MOLECULAR CLONING AND CHROMOSOMAL LOCALIZATION OF HUMAN MEMBRANE COFACTOR PROTEIN (MCP)
Evidence for Inclusion in the Multigene Family of Complement-Regulatory Proteins

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Control of the complement system is essential to prevent damage to autologous tissue. Multiple proteins have been described that serve this regulatory function. One group is focused at the C3 convertase stage of the complement cascade, providing critical control of the deposition of C3 that can lead to clearance or lysis of the marked particle or cell. This group includes serum (C4-binding protein [C4bp] and factor H [H]) and membrane proteins (C3b receptor [CR1], C3d/Epstein-Barr virus receptor [CR2], decay-accelerating factor [DAF], and membrane cofactor protein [MCP]). Each of these proteins binds to fragments of C3 or C4, and may block formation of the C3 convertase or serve as a cofactor for its proteolytic inactivation by serum factor I (reviewed in references 1, 2). This large number of proteins highlights the importance of regulating complement activity. Furthermore, it has been suggested that this might also be serving as a primitive self/non-self immunologic recognition system (3).

cDNAs for all of these proteins except MCP have been cloned and sequenced (4–12). This has revealed a common structural motif; each of the proteins is composed of multiple repeats of an ~60-amino acid consensus unit composed of conserved cys, pro, gly, trp, leu/ile/val, and tyr/phe residues (reviewed in reference 13). The genes for these five proteins have been localized to the long arm of human chromosome 1, band q32 (14–17). Thus, these genes form a multigene family encoding complement-
regulatory or receptor proteins, which we have designated the regulator of complement activation (RCA) gene cluster. Their common structural elements and close genetic linkage suggest that they might all have arisen from an ancestral C3-binding protein.

MCP was first identified in this laboratory by iC3/C3b affinity chromatography on surface-labeled peripheral blood cells (and named gp 45-70 to describe its Mr heterogeneity on SDS-PAGE) (18). We subsequently purified the protein from human mononuclear cell lines and demonstrated that it possesses cofactor activity but no decay-accelerating function (19). We have found that MCP is present on human T and B lymphocytes, granulocytes, monocytes, platelets, endothelial cells, epithelial cells, and fibroblasts (18, 20-22). Additionally, MCP occurs as a characteristic doublet with Mr of 63 and 58 x 10^3 on most of these cells, with a polymorphism governing the expression of these two bands (23). C3b-binding molecules of similar Mr have been identified on rabbit alveolar macrophages (24) and on murine cells (25), but their relationship to MCP has not been established.

Because of its wide tissue distribution and cofactor activity, we have proposed that MCP is an important membrane protein for protecting host cells from damage by complement (3, 19, 22). Its C3b binding and cofactor activity suggest that it should belong to the complement-regulatory multigene family, but structural homology and genetic linkage are necessary to place it within that group. In this report, we present data to establish that position.

Materials and Methods

Protein Purification and NH₂-Terminal Sequencing. MCP protein was purified from the human T cell line HSB2 by our previously reported procedure (19) using NP-40 solubilization followed by sequential chromatography on chromatofocusing, hydroxylapatite, C3 (methylamine)-Sepharose, and Mono Q columns. Approximately 20 µg of protein was then run on a SDS-10% polyacrylamide gel, electroeluted, and electrodialyzed as described (26). This material was divided in half and subjected to automated Edman degradation on polybrene-coated glass filters in an Applied Biosystems, Inc. (Foster City, CA) model 470A sequencer with a model 120A PTH analyzer.

Construction and Screening of cDNA Library. RNA was isolated from the U937 cell line by the quanidinium isothiocyanate/CsCl method (27). Poly(A)⁺ RNA was purified by oligo(dT)-cellulose chromatography (28). A cDNA library was prepared from 5 µg poly(A)⁺ RNA by the method of Gubler and Hoffman (29). cDNA inserts (size-selected >1.0 kb by agarose gel electrophoresis) were ligated into Xgt10 arms, packaged, and plated on C600 hI A Escherichia coli. The library consisted of 2.0 x 10⁶ recombinants.

The oligonucleotide probe was synthesized on an Applied Biosystems, Inc. DNA synthesizer (model 380A) by the phosphoramidite method (30). Failure sequences were removed by PAGE and the probe was end-labeled with γ-{³²P}ATP. Duplicate plaque lifts on nitrocellulose filters were hybridized overnight at 37°C in 6 x SSC (1 x SSC = 0.15 M sodium chloride/0.015 M sodium citrate)/6 x Denhardt's solution (1 x Denhardt's solution = 0.02% BSA/0.02% Ficoll/0.02% polyvinylpyrrolidone)/0.05 M sodium phosphate, pH 6.8, containing 100 µg sonicated herring sperm DNA and 5 x 10⁷ cpm labeled probe per milliliter. The filters were washed twice for 30 min with 2 x SSC/0.1% SDS at room temperature. Autoradiographs were prepared at ~70°C on Kodak X-Omat XAR film with Cronex intensification screens (Dupont Co., Wilmington, DE). Plaques yielding positive signals in duplicate were plaque purified by standard techniques (31).

DNA Sequence Determination. Phage DNA was prepared and the cDNA inserts were subcloned into the Eco RI site of pUC-19 by standard techniques (31). DNA sequencing was performed by dideoxy-chain termination (32) using alkaline-denatured, double-stranded DNA
templates (33) and T7 polymerase (34; U.S. Biochemical Corp., Cleveland, OH). Sequencing primers (used at ~15:1 molar ratio to DNA templates) included forward and reverse M13 primers (New England Biolabs, Beverly, MA), as well as synthesized oligonucleotides corresponding to already sequenced regions of the cDNA. Both strands were sequenced in their entirety.

**Northern Blot.** Total RNA, prepared as described above, was separated on formaldehyde/1% agarose gels and transferred to nylon filters as described (31). These were hybridized overnight at 42°C in 6x SSC/1x Denhardt’s solution/12.5% dextran sulfate/0.1% SDS/10 mM Tris, pH 7.6/50% formamide containing 100 μg sonicated herring sperm DNA and 106 cpm labeled probe per milliliter. cDNA probes were labeled with α-[32P]dCTP by random hexanucleotide priming (35). Hybridized filters were washed twice for 30 min at 56°C in 0.2x SSC/0.1% SDS and autoradiographs were prepared.

**Somatic Cell Hybrids.** Somatic cell hybrids were generated by polyethylene glycol 1000-mediated fusion of human VA2 or IMR90 fibroblasts to Chinese E36 or Syrian BHK-B1 hamster cells that were mutant in their hypoxanthine-phosphoribosyltransferase or thymidine kinase genes, respectively, permitting hybrid cell selection with HAT. A panel for mapping studies was selected from a series of hybrids that contain the entire rodent genome but that have selectively lost different combinations of human chromosomes. The human chromosomal composition of the hybrid clones was determined by screening for up to 34 gene-enzyme systems (36), and in selected cases by complete cytogenetic analyses using trypsin-Giemsa banding (37). High molecular weight DNA for Southern blots and cell homogenates for isozymes were prepared from the same passages of cells. DNA from the hybrid clones was digested to completion with Eco RI or Hind III restriction endonucleases (New England Biolabs), fractionated by electrophoresis through an agarose gel, transferred by Southern blotting to nylon membranes, and hybridized with a 32P-labeled MCP cDNA probe prepared by the random hexanucleotide priming method to a specific activity of 1-3 x 108 dpm/μg. Hybridized filters were washed twice for 30 min at 56°C in 0.2x SSC/0.1% SDS and autoradiographs were prepared.

**Results**

**cDNA Cloning.** MCP protein was purified from the HSB2 T cell line as described in Materials and Methods and subjected to automated Edman sequencing, yielding the NH2-terminal sequence through position 24 XEEPPTFAMELICPKPYYXIXE (standard single-letter codes; X, undetermined). A 64-fold degenerate 17-mer antisense oligonucleotide probe based on amino acid residues 7-12 was used to screen 1.3 x 105 recombinant plaques from the U937 cDNA library. One clone giving a positive hybridization signal in duplicate was plaque purified, and the 1.5kb insert was subcloned into the Eco RI site of pUC-19.

**DNA Sequence.** The DNA sequence of this cDNA clone was determined by dideoxy chain termination sequencing of both strands and contains a long open reading frame encoding 384 amino acids beginning with an initiation methionine codon (Fig. 1). The first 34 amino acids show the typical structure for a signal peptide (39) and would predict the signal peptide cleavage at the residue indicated in Fig. 1. The succeeding 24 amino acids exactly match the NH2-terminal protein sequence as determined above for MCP, confirming the identification of this clone. This cDNA encodes a polypeptide (without signal peptide) of 39 kD, which agrees with the size of the MCP precursor detected in biosynthetic studies (40). Furthermore, there are
three potential N-linked glycosylation sites, and multiple potential O-linked glycosylation sites in a ser/thr-rich region (12/25 residues) between amino acids 253-277; this is consistent with the oligosaccharide structure of MCP, which contains 2-3 N-linked oligosaccharide units and multiple O-linked units (40). Amino acids 278-294 represent a short region of unknown function. Hydrophobicity analysis (41) demonstrates a 23-amino acid region typical for a transmembrane hydrophobic domain at amino acids 295-317, followed by a 33-amino acid region corresponding to a cytoplasmic tail. The 335-bp 3'-untranslated region ends in a 16-bp poly(A) track,
Figure 2. Consensus repeat units from MCP. The four contiguous NH$_2$-terminal domains in MCP are shown in single-letter amino acid code, with the amino acid numbers listed to the right. Spaces (-) have been introduced to maximize the alignment. The consensus sequence of the 60-amino acid repeats from the other complement-regulatory proteins is listed below for comparison. Conserved amino acids or conservative substitutions present in at least three of the four repeat units are boxed.
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FIGURE 3. Northern blot analysis of MCP RNA. RNA was isolated from cell lines, and 10 μg was separated by electrophoresis, transferred to nylon filters, and hybridized with 32P-labeled MCP cDNA. The autoradiograph shows RNA from Raji (lane 1), U937 (lane 2), and HeLa (lane 3) cells. Migration of the 28S and 18S bands is indicated. In addition to the major MCP species at 4.2 kb, a minor band at 4.8 kb is also present.

preceded 17 and 29 bp upstream by the sequences AATGAA and AATATA, respectively, variants on the consensus polyadenylation signal AATAAA (42).

The majority of the encoded MCP protein consists of four contiguous domains of ~60 amino acids, starting at the NH2 terminus, which match the consensus sequence found in the multigene family of complement-regulatory proteins (Fig. 2). These four domains show 18–35% amino acid homology to each other (29–44% allowing conservative amino acid substitutions), similar to the degree of homology found between these repeat units in other proteins of this family (13). Northern Blot Analysis. To determine the size (and number) of mRNA species encoding MCP, Northern blots of RNA from Raji, U937, and HeLa cell lines were hybridized with the 32P-labeled MCP cDNA probe. The autoradiograph (Fig. 3) shows the main MCP mRNA at 4.2 kb in the latter two cells, which express MCP protein. Raji cells, which possess no detectable MCP protein, do not show a band on the Northern blot. This 1,546-bp cDNA clone contains 5'-untranslated sequence and ends in a poly(A) track. The main 4.2-kb MCP message either possesses a very long 5'-untranslated region, or, alternatively and more likely (see Discussion), this

FIGURE 4. Hybridization of MCP-specific probe to Eco RI-digested DNA from hamster × human somatic cell hybrid clones. Hamster and human DNAs are in lanes H and I, respectively. DNA from hybrid clones containing chromosome 1 are in lanes B, D, and F. DNA from hybrid clones lacking chromosome 1 are in lanes A, C, E, and G. Hybridization and Southern blotting were performed as described in Materials and Methods. Sizes are shown in kilobases.
cDNA and the 4.2-kb mRNA species represent products of differential polyadenylation or splicing.

**Chromosomal Localization.** Having found structural homology to the functionally similar complement-regulatory proteins, we next wished to ascertain if there was also a genetic linkage to this group. Therefore, we screened hamster–human somatic cell hybrids for concordance of the MCP gene and specific human chromosomes.

Southern blots of Eco RI and Hind III digests of DNA from hamster–human somatic cell hybrids were hybridized with the $^{32}$P-labeled MCP cDNA (a representative blot is shown in Fig. 4). This identified several Eco RI and Hind III fragments from human DNA. The full panel of somatic cell hybrids was analyzed for discordance of the MCP gene and specific human chromosomes, i.e., percent asynteny (Table I). The MCP gene is located on chromosome 1; all other possible chromosome localizations were associated with a minimum of six discordant clones.

To confirm the association of MCP with chromosome 1 (using an independent technique) and to sublocalize the MCP gene on chromosome 1, in situ hybridizations to normal human metaphase chromosomes were performed using two different
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Figure 5. Distribution of labeled sites on chromosome 1 in 100 normal metaphase cells from PHA-stimulated peripheral blood lymphocytes that were hybridized with the full-length MCP cDNA (A) or MCP8SCR cDNA (B) probe. The MCP8SCR probe is a 650-bp fragment of the full-length MCP cDNA that lacks any of the sequence encoding the 60-amino acid short consensus repeat units. The labeled sites observed in these hybridizations were clustered at q31-41; the largest cluster of grains was located at q32.

MCP probes. These hybridizations resulted in specific labeling of a single human chromosome, namely chromosome 1. All hybridizations were repeated twice and they all gave similar results. In hybridizations performed with the full-length MCP cDNA probe, we observed specific labeling of the distal long arm of chromosome 1. Of 100 metaphase cells examined from this hybridization, 37 (37%) were labeled on region q3 or q4, bands q31-41, of one or both chromosome 1 homologs ($p < 0.0005$). The distribution of labeled sites on chromosome 1 is illustrated in Fig. 5 A. A total of 71 grains were observed on this chromosome; of these, 51 (72%) were clustered at bands q31-41 and represented 20.6% (51/248) of all labeled sites. The largest cluster of grains was observed at q32.

The MCP clone contains four copies of the homologous repeat unit characteristic of the C3b-binding multigene family. Thus, this probe may crosshybridize to the genes encoding other C3b-binding proteins, which have also been localized to this region of chromosome 1. To eliminate the possibility that the specific labeling of q31-41 observed in this hybridization was due to crosshybridization to homologous repeat units of related genes, we hybridized the MCP8SCR probe to normal metaphase cells. In this case, also, we noted specific labeling only of chromosome 1. We examined 100 metaphase cells, and the distribution of labeled sites on this chromosome is illustrated in Fig. 5 B. Of these, 25 cells were labeled on region q3 or q4 (bands q31-41) of one or both chromosome 1 homologs. A total of 41 grains was observed on this chromosome; of these, 29 (71%) were clustered at bands q31-41 and represented 17.1% (29/170) of all labeled sites ($p < 0.0005$). The largest cluster of grains was observed at q32. Thus, the MCP gene is localized to human chromosome 1, at bands q31-41.

Discussion

Previous work from this laboratory had shown that purified MCP possesses cofactor activity for inactivation of C3b or C4b by serum factor I (19). This would allow MCP to regulate the complement system at the level of the C3 convertases, a role shared by other members of the family of complement-regulatory proteins: CR1, CR2, DAF, C4bp, and factor H. The present report of the complete primary structure and chromosomal localization of human MCP establishes that it is indeed a
member of this multigene family, sharing structural homology and genetic linkage as well as functional similarity to the other proteins.

The oligonucleotide probe used to identify the MCP cDNA clone was based on amino acid residues 7–12 of purified MCP. The identification of the clone was confirmed with 15 other amino acids surrounding this region. In addition, the polypeptide encoded by this cDNA clone matches several other properties for MCP: (a) typical structure for a membrane protein, with a signal peptide at the NH2 terminus, and a region of hydrophobic amino acids near the COOH terminus to serve as the transmembrane domain; (b) overall size of 39 kD, in agreement with the precursors (identified by anti-MCP antibody) seen in biosynthetic labeling experiments (40); (c) three sites for N-linked glycosylation and multiple sites for O-linked glycosylation, consistent with the oligosaccharide structure of MCP (40); and (d) tandemly arranged NH2-terminal homologous consensus repeat units characteristic of other C3b/C4b-binding proteins. Taken together, these data indicate that this cDNA clone encodes the full-length MCP polypeptide.

The most striking structural feature of the derived protein sequence for MCP is the presence at the NH2 terminus of four contiguous repeats of a 60-amino acid consensus sequence that has been found in other members of the complement-regulatory multigene family as well as in other complement and noncomplement proteins. This is the first structural data that links MCP to the other functionally related complement-regulatory proteins. However, knowledge of the primary structure of this whole group of proteins has not shed light on the ligand binding site or the functional domain for cofactor or decay-accelerating activity. Interestingly, comparison of the different repeat units of MCP to the other proteins shows that the highest homology match of repeat units two and three of MCP is to these same repeat units in CR1, C4bp, and H, as well as in C2 (43) and factor B (44, 45) of the major histocompatibility complex, but to repeat units three and four of DAF. Overall, this would support speculation that these genes evolved from an ancestral C3-binding protein that already possessed at least two repeat units. Genomic cloning and sequence analysis will shed further light on this question in terms of the intron/exon structure of the repeat units and a comparison of the intron sequences.

Northern blot analysis of RNA from U937, HeLa, and Raji cells shows a major species of 4.2 kb in the first two cell lines, but no MCP mRNA apparent in the latter. This correlates with the expression of MCP protein in these cells. The discrepancy between the size of the mRNA and the cDNA clone must reflect additional untranslated sequence at either the 5' or 3' end, or both. The difference of 2 kb would be unusually large for a 5'-untranslated sequence, suggesting that this clone uses an alternative, and earlier, polyadenylation signal than the major mRNA species. 17 and 29 nucleotides upstream of the poly(A) track are sequences (AATGAA and AATATA, respectively) that support polyadenylation at levels <10% of wild type (AATAAA) (42). Furthermore, the construction of the library with an oligo (dT) primer and screening with a probe corresponding to the NH2 terminus of the protein would bias selection of clones toward an earlier, minor polyadenylation signal. This does not exclude the additional possibility of alternative RNA splicing, perhaps leading to differences both in parts of the coding region as well as in the 3'-untranslated region. Indeed, two forms of MCP are found in most cells (18, 23, 40). Analysis of additional cDNA clones will be needed to elucidate this matter.
The multigene family of complement-regulatory proteins was first established by our family studies that, by following protein polymorphisms, showed close genetic linkage of CR1, C4bp, and H (14). Subsequently, another group used in situ chromosomal hybridization with a CR1 cDNA probe to map CR1 (and hence the linkage group of CR1/C4bp/H) to human chromosome 1, band q32 (15). These investigators also mapped CR2 to this location (15), and we and others have mapped the DAF gene to this same location (16, 17). In the present study, we have used somatic cell hybrid and in situ hybridization techniques to map MCP to human chromosome 1, band q32. Because of the possibility of crossreaction of the MCP probe to homologous sequences in the other genes, the in situ hybridizations were repeated with a probe that encodes none of the consensus repeat sequences; the chromosomal localization was confirmed. Thus, the multigene family of complement-regulatory proteins has been enlarged, now consisting of six members: CR1, CR2, DAF, MCP, C4bp, and H.

The large number of related proteins in this multigene family might reflect specialization of these regulatory functions: membrane vs. serum proteins, classical vs. alternative pathway control, and immune complex processing vs. protection of host cells from autologous complement damage. In this regard, MCP and DAF seem to form a pair in serving the latter protective function. MCP and DAF show a very similar structure (Fig. 6): membrane proteins with four NH2-terminal consensus repeat units, followed by a ser/thr-rich region (probable site of heavy O-linked glycosylation), and then a hydrophobic domain. This latter domain is replaced by a glycosylphosphatidyl membrane anchor in DAF (46, 47), but forms a transmembrane polypeptide anchor followed by a cytoplasmic domain in MCP. DAF functions intrinsically to protect host cells from amplification of the complement cascade on their surfaces (48, 49); the similar size, structure, and properties of MCP suggest that it might also function in this role (3). Indeed, DAF and MCP have complementary functions for preventing complement amplification, the former possessing decay-accelerating properties and the latter cofactor activity. Expression of either MCP, DAF, or both in transfected cells will allow the role of these proteins to be addressed.

Summary

Membrane cofactor protein (MCP), a regulatory molecule of the complement system with cofactor activity for the factor I-mediated inactivation of C3b and C4b, is widely distributed, being present on leukocytes, platelets, endothelial cells, epi-
thelial cells, and fibroblasts. MCP was purified from a human T cell line (HSB2) and the NH$_2$-terminal 24-amino acid sequence obtained by Edman degradation. An oligonucleotide probe based on this sequence was used to identify a clone from a human mononcytic (U937) cDNA library. Nucleotide sequencing showed a 43-bp 5'-untranslated region, an open reading frame of 1,152 bp, and a 335-bp 3'-untranslated region followed by a 16-bp poly(A) track. The deduced full-length MCP protein consists of a 34-amino acid signal peptide and a 350-amino acid mature protein. The protein has, beginning at the NH$_2$ terminus, four ~60-amino acid repeat units that match the consensus sequence found in a multigene family of complement regulatory proteins (C3b-receptor or CR1, C3d-receptor or CR2, decay-accelerating factor, C4-binding protein, and factor H), as well as several other complement and non-complement proteins. The remainder of the MCP protein consists of 25 amino acids that are rich in serine and threonine (probable site of heavy O-linked glycosylation of MCP), 17 amino acids of unknown significance, and a 23-amino acid transmembrane hydrophobic region followed by a 33-amino acid cytoplasmic tail. The MCP gene was localized to human chromosome 1, bands lg31-41, by analysis of human x rodent somatic cell hybrid clones and by in situ hybridization. This same genetic region contains the multigene family of complement-regulatory proteins, which is thereby enlarged to include the functionally and structurally related MCP.

We thank Drs. V. Michael Holers and Laura Ballard for useful discussions, Joseph Leykam for NH$_2$-terminal sequence analysis, and Avtar Khalsa for expert secretarial assistance.

Received for publication 1 March 1988.

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