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Characterization of two mannose-binding protein cDNAs from rhesus monkey (*Macaca mulatta*): structure and evolutionary implications

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Mannose-binding proteins (MBPs), members of the collectin family, have been implicated as lectin opsonins for various viruses and bacteria. Two distinct but related MBPs, MBP-A and MBP-C, with ~55% identity at the amino acid level, have been previously characterized from rodents. In humans, however, only one form of MBP has been characterized. In this paper we report studies elucidating the evolution of primate MBPs. ELISA and Western blot analyses indicated that rhesus and cynomolgus monkeys have two forms of MBP in their sera, while chimpanzees have only one form, similar to humans. Two distinct MBP cDNA clones were isolated and characterized from a rhesus monkey liver cDNA library. Rhesus MBP-A is closely related to the mouse and rat MBP-A, showing 77% and 75% identity at the amino acid level, respectively. Rhesus MBP-A also has three cysteines at the N-terminus, similar to mouse and rat MBP-A and human MBP. Rhesus MBP-C shares 90% identity with the human MBP at the amino acid level and has three cysteines at the N-terminus, in contrast to two cysteine residues found in rodent MBP-C. A stretch of nine amino acids close to the N-terminus, absent in both mouse and rat MBP-A, but present in rodent MBP-C, chicken and human MBPs, is also found in the rhesus MBP-A. The phylogenetic analysis of rhesus and other mammalian MBPs, coupled with the serological data suggest that at least two distinct MBP genes existed prior to mammalian radiation and the hominoid ancestor apparently lost one of these genes or failed to express it.

Key words: collectin/rhesus monkey/mannose-binding protein/MBP cDNA/mannan-binding protein

Introduction

Mannose-binding proteins (MBPs) are multimeric calcium-dependent (C-type) lectins that bind terminal mannose, fucose and *N*-acetylglucosamine (Kawasaki *et al.*, 1978). MBP belongs to a family of structurally and functionally related proteins termed collectins (collagenous lectins) (Sastry and Ezekowitz, 1993). The collectin family includes the two lung surfactant proteins, SP-A and SP-D (Lu *et al.*, 1992; Holmskov *et al.*, 1994), and two serum lectins, conglutinin (Lee *et al.*, 1991) and collagenous lectin-43 (CL-43, Lim *et al.*, 1994). Conglutinin and CL-43 have been isolated only from members of the bovidae family (Lim *et al.*, 1994). All members of the collectin family have collagenous tails attached to a globular carbohydrate recognition domain (CRD) that binds a range of sugars in a calcium-dependent manner (Drickamer and Taylor, 1993). These proteins appear to play a role in first line host defense as opsonins of various microorganisms and activators of the complement (Hoppe and Reid, 1994).

The high affinity of MBP for mannose/*N*-acetylglucosamine found on the surfaces of several viral and bacterial pathogens and the regulation of its synthesis as an acute phase reactant suggest that MBP plays a role in natural immunity (Ezekowitz *et al.*, 1988; Sastry *et al.*, 1991; Arai *et al.*, 1993; Tabona *et al.*, 1995). MBP after binding to carbohydrates triggers both the classical and also an unique complement pathway (Ikeda *et al.*, 1987; Matsushita and Fujita, 1992; Holt *et al.*, 1994). The collagenous domain of MBP interacts with the C1q receptor present on phagocytic cells to carry out its effector function (Malhotra *et al.*, 1993). MBP also directly acts as an opsonin to enhance oxidative killing of influenza virus by neutrophils (Hartshorn *et al.*, 1993; Anders *et al.*, 1994). Interaction of MBP with the human immunodeficiency virus (HIV) envelope glycoproteins inhibits *in vitro* infection of cells, and activates complement (Ezekowitz *et al.*, 1989; Haurum *et al.*, 1993). An opsonic defect in humans has been found to be associated with low serum levels of MBP (Super *et al.*, 1989). Decreased serum levels of MBP result from point mutations in the collagen domain (Sumiya *et al.*, 1991; Summerfield *et al.*, 1995), and polymorphisms in the promoter region of the MBP gene (Madsen *et al.*, 1995). Recent studies show that ~8% of the patients with immunodeficiencies are homozygous for alleles of the MBP gene which have collagen domain mutations (Garred *et al.*, 1995).

MBPs are synthesized in hepatocytes and are found in serum (Maynard and Baenziger, 1982). MBPs have been isolated from serum of chicken (Sugi and Hirota, 1994; Laursen *et al.*, 1995), mice (Holt *et al.*, 1994), rabbit (Kozutsumi *et al.*, 1980), cow (Holmskov *et al.*, 1993), serum and liver of humans (Kawasaki *et al.*, 1983; Wild *et al.*, 1983) and rat liver (Drickamer *et al.*, 1986). A single MBP polypeptide (~28–32 kDa) has four domains (Drickamer *et al.*, 1986). The N-terminal domain of ~18–21 amino acids contains cysteine residues that form interchain disulfide bonds as MBP polypeptide units assemble into oligomers (Kawasaki *et al.*, 1983). The collagenous domain, 53–59 amino acids long, has a stretch of 18–20 Gly-X-Y repeats that assemble into a collagen-like triple helix, contributing to the formation of the trimeric structural unit of MBP, as observed for other collectins. The short region of 23–25 amino acids that follows the collagen domain has α-helical coiled-coil conformation and forms the 'neck' or trimerization domain of the MBP molecules from which the globular carbohydrate recognition domains (CRDs) spread out (Hoppe and Reid, 1994). The CRD of 124 amino acids cross-linked by four invariant cysteines, is found at the C-terminus (Drickamer *et al.*, 1986).

Rodents have two forms of MBP (Drickamer 1988; Sastry *et al.*, 1991). The predominant rodent serum MBP (designated

MBP-A) is a glycoprotein with an apparent molecular mass of ~650–750 kDa, whereas the major liver form (MBP-C) has a molecular mass of ~200 kDa (Oka *et al.*, 1988). Rodent MBP-C is also found in serum (Oka *et al.*, 1988). Rodent MBP-A, but not MBP-C, has been shown to activate the complement pathway after binding to ligand (Ikeda *et al.*, 1987; Holt *et al.*, 1994). Humans have only one form of MBP, which has the ability to activate the complement pathway after binding to its carbohydrate ligand (Schweinle *et al.*, 1989; Lu *et al.*, 1990).

The two mouse and rat MBPs have ~55% identity at the amino acid level and are most likely, products of gene duplication (Drickamer and McCreary, 1987). Studies by Kurata and colleagues (Kurata *et al.*, 1994) have shown that both the liver and serum forms of the human MBP have identical amino acid sequences, and the differences in oligomerization between the two forms appear to be acquired by post-translational modifications. Human MBP has some features of both rodent MBP-A and MBP-C (Sastry *et al.*, 1991). The high oligomeric forms of human MBP, and rat and mouse MBP-A, composed of 18 identical polypeptide chains, are similar to C1q in overall organization (Lu *et al.*, 1993). This property has been attributed to the presence of three inter-chain cross-linking cysteines at the N-terminus, as compared to only two cysteines found in the MBP-C of the rat and the mouse (Hoppe and Reid, 1994; Kurata *et al.*, 1994). In common with the rodent MBP-C isoforms, human MBP has an additional nine amino acid stretch at the N-terminus and 23 amino acids (unlike MBP-A which has 25 amino acids) in the 'neck' domain (Ezekowitz *et al.*, 1988).

The collectins are believed to have arisen by an exon shuffling event that brought together the collagenous domain with the calcium-dependent globular CRD (Drickamer and McCreary, 1987; Drickamer, 1993). Subsequent gene duplication and divergence in various domains, especially the CRD, may have contributed to the diversity of these molecules. In bovidae, for example, an ancestral SP-D-like gene appears to have duplicated to give rise to the present day SP-D and conglutinin genes (Liou *et al.*, 1994). Conglutinin gene probably duplicated further and evolved by loss of a collagen domain exon giving rise to CL-43 (Lim *et al.*, 1994). Conglutinin and CL-43 share 81% homology in the CRD region (Lim *et al.*, 1994). In humans, all genes of the collectin family reside in a cluster on the long arm of chromosome 10, suggestive of an evolutionary relationship among this family of proteins (Kolble and Reid, 1993). The mouse MBP-A gene localizes to chromosome 14, in a region syntenic to human chromosome 10 (White *et al.*, 1994). However, the mouse MBP-C gene localizes to chromosome 19 (White *et al.*, 1994). Whether the duplication of MBP genes into the A and the C forms is an evolutionary event, unique to the rodent lineage, is unknown. This study of primate MBPs was undertaken to shed light on the evolutionary events that led to the single MBP gene status in humans. Serologic data on MBP from three non-human primate species and characterization of two distinct rhesus monkey MBP cDNAs is reported.

Results

Detection of primate serum MBPs

Chimpanzee (*Pan troglodytes*), rhesus monkey (*Macaca mulatta*) and cynomolgus monkey (*Macaca fascicularis*) sera were examined by ELISA (on mannose-BSA coated wells) and Western blot techniques. Mannose-binding proteins in primate sera were detected by employing polyclonal rabbit antisera raised against rat MBP-A and MBP-C, and a mouse monoclonal antibody (Mab#6) raised against human recombinant MBP. The polyclonal rabbit antisera against rat MBP-A and MBP-C are capable of distinguishing the two MBP isoforms with very low cross-reactivity by ELISA (Figure 1A). Rhesus and cynomolgus monkey sera appeared to have significant levels of both MBP-A and MBP-C as determined by ELISA using polyclonal rabbit antibodies to rat MBPs (Figure 1B). In contrast, sera from both monkey species did not show any reactivity with monoclonal human MBP antibody. Chimpanzee serum, on the other hand, reacted only with the antibodies raised against rat MBP-C and the recombinant human MBP, but not with anti-rat MBP-A antibody. These results suggest that two distinct forms of MBP occur in old world monkeys, while chimpanzees may have only one form of MBP, closely resembling the human MBP and the rodent MBP-C.

Western blot analysis was carried out to confirm the ELISA experiments. Monkey sera were resolved by SDS–PAGE and transferred onto a nitrocellulose membrane and their reactivity was examined with either rat MBP-A or MBP-C antibodies. Under denaturing conditions, serum MBP has been shown to resolve with a molecular weight between 28 and 32 kDa (Drickamer *et al.*, 1986). In agreement with the ELISA observations, sera from rhesus and cynomolgus monkeys reacted with both rat MBP-A and MBP-C antibodies (Figure 2). The molecular weights of reactive bands also corresponded to the recombinant human MBP used as a control.

Isolation of rhesus monkey MBP cDNA clones and their sequence analysis

Rhesus monkey liver cDNA library was screened under moderate stringency using mouse MBP-A and MBP-C cDNA probes. Two positive clones were detected out of 900,000 plaques screened. These clones were named MBP-A and MBP-C due to their hybridization with the mouse MBP-A and MBP-C probes, respectively. Analysis of the nucleotide and the deduced amino acid sequences reveal that rhesus MBPs share features common to the rodent MBPs and the human MBP (Figure 3). The deduced amino acid sequence of rhesus MBP-A cDNA has 77% and 75% identity to the mouse MBP-A (Sastry *et al.*, 1991) and to the rat MBP-A (Drickamer *et al.*, 1986), respectively (Table I). Rhesus monkey MBP-C, on the other hand, has greater identity to the human MBP, having 90% identity at the amino acid level. Rhesus MBP-A and MBP-C share 62% identity with each other at the amino acid level.

Rhesus MBPs, similar to the rodent MBPs and the human MBP, are organized into four domains. The N-terminal domain of rhesus MBP-A contains three cysteines which suggests the possibility of a higher oligomeric structure for this molecule, similar to the MBP-A forms of rodents and the human MBP. Rhesus MBP-A has an additional nine amino acids at the N-terminus domain, the absence of which is a characteristic feature of the rodent MBP-A forms (Drickamer *et al.*, 1986; Sastry *et al.*, 1991). The rhesus MBP-C closely resembles the human MBP, possessing three cysteine residues at the amino terminus, unlike the MBP-C of the rat and the mouse, which have only two cysteine residues in this domain (Drickamer *et al.*, 1986; Sastry *et al.*, 1991).

A stretch of 18 and 19 Gly-X-Y repeats are found in the

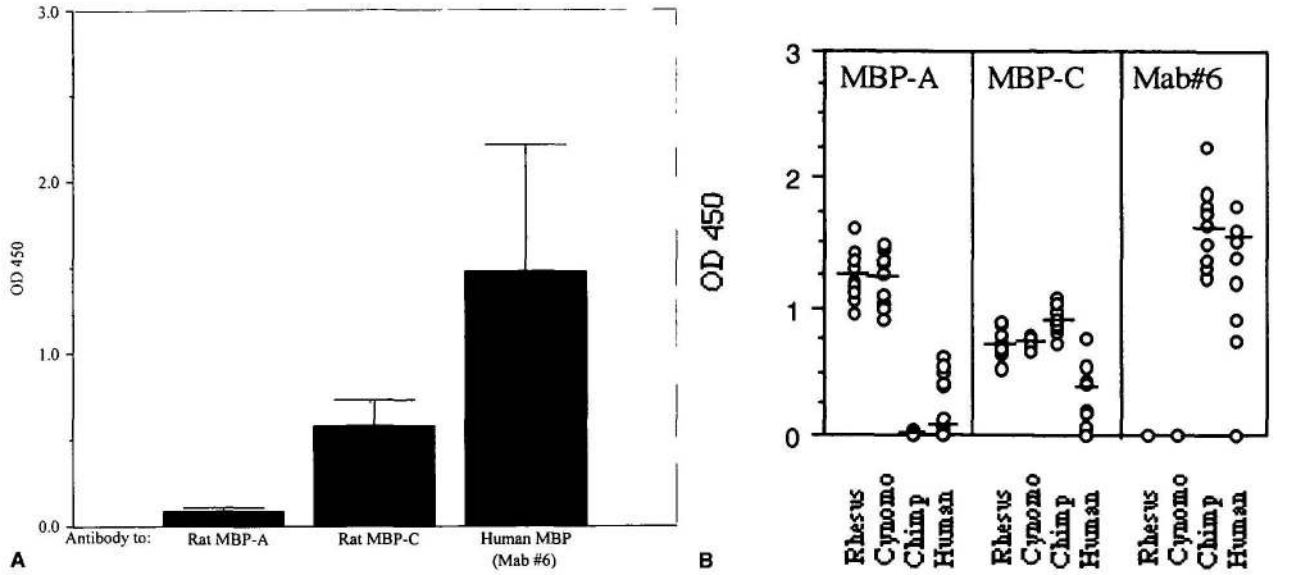

Fig. 1. (**A**) The reactivity of antibodies to MBP with human recombinant MBP as measured by ELISA. Recombinant human MBP (50 ng) was coated onto microtitre plates and reacted with the respective antibodies followed by anti-immunoglobulin horse radish peroxidase (HRP) conjugate. Results are expressed by reading OD at 450 nm and values indicated are averages of triplicates. Bars in the figure represent standard deviation. (**B**) A scattergram of individual OD values (shown as open circles) determined for sera from monkeys (rhesus and cynomolgus) as well as chimpanzees and humans. For detection of primate MBP, serum samples (1:10 dilution) were coated onto microtitre wells and reacted with the respective antibodies (MBP-A and MBP-C are rabbit anti-rat MBP-A and MBP-C antibodies respectively; Mab#6 is a mouse monoclonal antibody against human rMBP) followed by an anti-immunoglobulin HRP conjugate. Results are expressed as OD at 450 nm. Mean values are shown as dashed lines.

collagen domain of rhesus MBP-A and rhesus MBP-C, respectively. A contiguous stretch of 20 amino acids in the collagen domain, completely conserved in all other MBPs (Sastry and Ezekowitz, 1993), is also conserved in these newly characterized rhesus MBPs (Figure 3). The Gly-Gln-Gly interruption in the collagen repeats, observed in all MBPs, occurs at the seventh repeat in rhesus MBP-A (Hoppe and Reid, 1994). This interruption is observed at the eighth repeat in rhesus MBP-C, similar to the human MBP and mouse MBP-C. Following the collagen domain is the 'neck' region, which is 25 amino acids long in rhesus MBP-A, similar to the MBP-A of the rat and the mouse. This region is only 23 amino acids long in rhesus MBP-C due to a gap of two amino acids, in common with all other characterized MBP-C isoforms (Sastry *et al.*, 1991). The neck domain appears to be the least conserved region in terms of identity, although there is conservation of hydrophobic amino acids with a potential to form triple helical coiled coil as shown for SP-D (Hoppe *et al.*, 1994). The neck region of bovine SP-D expressed in *E. coli*, forms a trimer in solution owing to very strong hydrophobic forces, and is stable against denaturation by heat, acidic or alkaline environments (Hoppe *et al.*, 1994). It is of interest to note that rhesus MBP-C and human MBP share identity of 92% in the neck region while the identity drops to 18% for rodent MBP-C isoforms (Table I). The next stretch of 124 amino acids found towards the C-terminus comprise the calcium-dependent CRD of the rhesus MBPs. Rhesus MBP-A CRD is 82% and 81% identical to the mouse and rat MBP-A CRD, respectively. Rhesus MBP-C, on the other hand, shares 88% identity with the human MBP CRD. In both rhesus MBP-A and MBP-C CRD, the 14 completely conserved and 18 invariant residues, found in all C-type lectin CRDs, are conserved (Drickamer, 1993) (Figure 3).

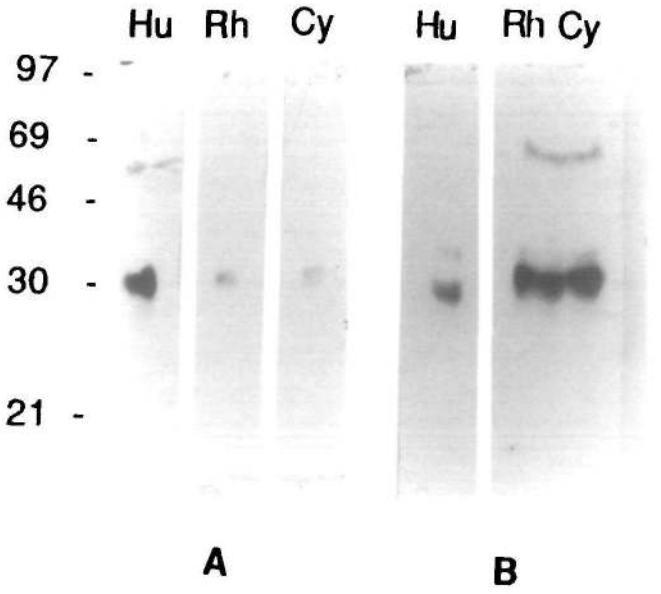

Fig. 2. Western blot analysis of rhesus and cynomolgus monkey serum. *Diluted sera (10 μg total protein) from each species were separated on 12%* SDS–PAGE gels, transferred to nitrocellulose membrane and analyzed by immunoblotting with rabbit anti-rat MBP-A (**A**) and MBP-C (**B**) antibodies. Human rMBP was used as control protein. Hu, human; Rh, rhesus; Cy, cynomolgus. The additional high molecular band observed in 'Rh' and 'Cy' lanes may be a doublet of MBP polypeptide unit due to unbroken disulfide bonds.

Evolutionary analysis of sequences

In order to investigate major evolutionary factors acting on MBP genes, the pattern of nucleotide substitution was examined by estimating the number of synonymous (*ds*) and non-synonymous (*dn*) substitutions per site (Nei and Gojobori, 1986). The analysis would reveal if the genes have evolved under positive Darwinian selection ($ds < dn$) as in the case of major histocompatibility complex genes (Hughes and Nei,

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                                        *       *       *
RhesusA   -15  ----PSFPVLLLSVVTASC|SETKACEDAQKTCS-VITCGIPVTNGTPGRDGRDGPKGEKGEPGQGLRGLQGPPGKSGPPGNTG  63
MouseA    -19  MLLL.LL..-..C..SV.S|.GSQT...TL....-..A.---------.........................L....SV.  54
RatA      -18  MLLL.LL-.-..C..SV.S|.GSQT..ETL....-..A.---------.........................L....SV.  54
Human     -20  MSLF..L.L....M.A..YS|..VT........PA..A.SS.GI..F..K.....T...................L.....P.  63
RhesusC   -20  MSLF..LTL......AT.YS|..VT...S..I.PA..A.NS.GI..F..K.....T...................L.....P.  63
RatC      -18  MSLF-T-SF...C.L..VYA|..LT-.G..SS.P-..A.SS.GL..F..K..H..A...................V..A.PP.  61
MouseC    -18  MSLF-T-SF...C....VYA|..LT-.GV.NS.P-.V..SS.GL..F..K.....A...................V..T.PP.  61

                                                                                          *
RhesusA         APGIPGPRGQKGDHGDNSVAEAKLANLERQLQSLRSELDHMKKLQAFSLGKMSGKKLFVTNGERMPFSEVKALCAGLQATVAA  146
MouseA          S..S...K.........RAI.E....M.AEIRI.K.K.QLTN..H...M..K.........H.K....K..S..TE..G...I  137
RatA            ...SQ..K.....R..SRAI.V....M.AEINT.K.K.ELTN..H...M..K....F....H......K.....SE.RG...I  137
Human           PS.S...K.....P.KSPDGDSS..AS..--KA.QT.MARI..WLT.....QV.N.F.L....I.T.EK.....VKF..S..T  144
RhesusC         SS.S...K.....P.ESPDC.SS..AS..--KA.QT.MARI..WLT....RQV.N.F.L....M.T.DK......RF..S..T  144
RatC            N..SK.AT.P...R.ESVEFDTT--.IDLEIAA.....RA.R.WVLL.MSENV...Y.MSSVR...LNRA....SE..G...T  142
MouseC          N..LK.AV.P...R..RAEFDTS--EIDSEIAA.....RALRNWVL...SEKV...Y..SSVKK.SLDR.....SEF.GS..T  142

Conser                                                  S     G K F      M     K LC      VA

                                                                        *           *       *
RhesusA         PKNAEENKAIQDVAKDTAFLGITDEATEGQFMYVTGGRLTYSNWKKDEPNDHGSGEDCVILLSNGLWNDISCTASYIAVCEFPA  230
MouseA          .R.........E..TGI.................................N.........I.D.........Q..FK.......  221
RatA            .R.........E...TS.........V.................................TIVD.........Q..HT.......  221
Human           .R..A..G...NLI.EE........K.....VDL..N....T..NEG...NA..D....L..K..Q...VP.ST.HL......I  228
RhesusC         .R..A..R...NLI.EE........N...E.VDL..NK...T..NDG...NA..N....L..K..K....P.SS.HL.L....I  228
RatC            .R.....R...N....V.......QR..NV.EDL..N.VR.T..NEG...NV....N..VL.T..K...VP.SD.FLV....SD  226
MouseC          .R.....S...K....I.Y.....VRV..S.EDL..N.VR.T..NDG...NT.D.....VI.G..K...VP.SD.FL.I...SD  226

Conser          P NA EN AIQ       A LGITD   E  F   TG    Y NW   EPN  G  E CV    NG WND  C  S    CEF
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Fig. 3. Alignment of the deduced amino acid sequences of rhesus MBP-A (RhesusA), mouse MBP-A (MouseA) (Sastry *et al.*, 1991), rat MBP-A (RatA) (Drickamer *et al.*, 1986), rhesus MBP-C (RhesusC), human MBP (Human) (Sastry *et al.*, 1989), rat MBP-C (RatC) (Drickamer *et al.*, 1986), and mouse MBP-C (MouseC) (Sastry *et al.*, 1991). Arrow denotes the beginning of N terminus. A period (.) indicates identity with the rhesus MBP-A amino acid sequence. A dash (-) indicates a gap. Conserved cysteine residues at the N-terminus and the CRD region are indicated with an asterisk. Conserved amino acids of the CRD region are also shown on a separate line below the amino acid sequences (Conser). Rhesus MBP-A and MBP-C cDNA sequences have been deposited in Genbank: Accession numbers L43912 and L43911, respectively.

1989). The results, shown in Table II, indicate that in all but one case, *ds* > *dn* for rodent and primate MBPs. This is consistent with the neutral theory of molecular evolution and indicates that the MBP gene has mainly been subjected to purifying selection but not to positive selection. However, it remains unclear if positive selection operates on few sites or regulatory elements of the gene (see Discussion). The analysis also shows that both *ds* and *dn* are generally larger for the rodent MBP-Cs than those for the rodent MBP-As. Especially, *dn* for the rodent MBP-As (0.06) is significantly different from one of rodent MBP-Cs (0.12), despite that no significant difference is observed for *ds* for the same comparison (Table II). This is likely attributable to distinct selectional constraints on these two MBPs.

To understand the evolutionary relationships of mammalian MBPs, a phylogenetic tree has been constructed (Saitou and Nei, 1987) using amino acid sequences of the CRD region, with human SP-D and bovine collectins, CL-43 and conglutinin, as outgroup genes (Figure 4). The result suggests that divergence of MBP-A and MBP-C preceded the rodent-primate separation. However, the clustering of rodent MBP-Cs with the cluster of human MBP and rhesus MBP-C is not well resolved. It is possible that the group of the human MBP and rhesus MBP-C separated from the MBP-A cluster, indicating

Table I. Comparison of Rhesus MBP nucleotide and deduced amino acid sequence identity (%) with human, mouse and rat MBPs

	Nucleotide	Amino acid				
		Total	N-terminus	Collagen	Neck	CRD
RhesusA &						
MouseA	75	77	61	89	39	82
RatA	74	75	56	85	39	81
Human	73	63	65	79	33	62
RhesusC	73	62	58	79	38	61
RatC	64	56	52	70	33	55
MouseC	70	54	48	74	21	53
RhesusC &						
Human	94	90	89	96	92	88
RatC	72	61	72	77	18	59
MouseC	72	61	64	74	18	62
MouseA	68	61	59	85	25	57
RatA	69	60	53	83	25	58

Note. i) Nucleotide sequence identity was determined only for the translated region. ii) Identity was determined by pairwise alignment of sequences and all gaps have been excluded during comparison.

Table II. The number of synonymous (*ds*) and nonsynonymous (*dn*) substitutions per site for mammalian mannose binding proteins (MBPs)

	Entire coding region	Exon coding S/N/C[1]	Exon coding collagen domain	Exon coding trimerization domain	Exon coding CRD[2]
Human MBP vs Rhesus MBP-C					
n	247	61	38	22	123
ds	0.12 (0.03)	0.07 (0.04)	0.14 (0.07)	0.15 (0.11)	0.14 (0.04)
dn	0.05 (0.01)	0.05 (0.02)	0.02 (0.02)	0.04 (0.03)	0.05 (0.01)
Mouse MBP-A vs Rat MBP-A					
n	237	49	38	24	123
ds	0.22 (0.04)	0.27 (0.10)	0.20 (0.09)	0.07 (0.07)	0.25 (0.06)
dn	0.06 (0.01)	0.01 (0.01)	0.06 (0.03)	0.10 (0.04)	0.07 (0.02)
Mouse MBP-C vs Rat MBP-C					
n	243	57	38	22	123
ds	0.32 (0.05)	0.43 (0.13)	0.22 (0.09)	0.21 (0.12)	0.33 (0.08)
dn	0.12 (0.02)	0.09 (0.03)	0.09 (0.04)	0.13 (0.06)	0.14 (0.03)

Note: i) n: the number of codons compared, ii) Numbers in parentheses are standard errors, iii) Initiation codons (ATG) were not included in the analyses.
[1]Signal peptide/N-terminus/collagen domain
[2]Carbohydrate recognition domain.

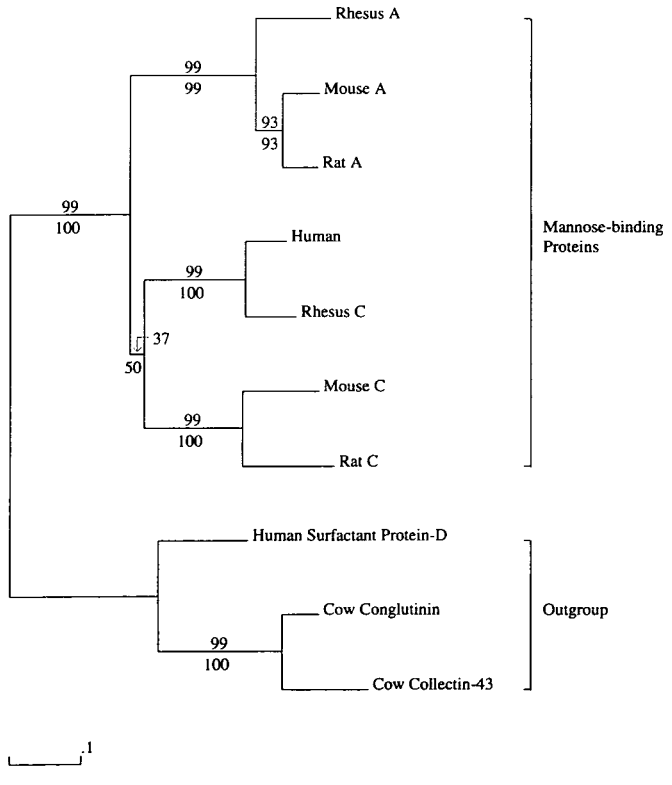

Fig. 4. Phylogenetic relationship of seven mammalian MBP CRDs as estimated by the neighbor joining method of Saitou and Nei (1987). In order to obtain root for the tree, sequences from cow collectin-43 (CL-43) (accession no. X75912), cow conglutinin (accession no. L18871), and human surfactant protein D (accession no. X65018) were selected as outgroup based on their sequence similarity with the mammalian MBP sequences. Amino acids numbered 106, 107, 229 and 230 in rhesus MBP-A CRD and other MBPs aligned to this region (Figure 3) were excluded from analysis due to ambiguity of alignment with the collectin genes included as outgroups. The scale bar represents 0.1 unit of genetic distance. The numbers above and below the lines are confidence probabilities and bootstrap numbers (results of 2000 replicates), respectively.

three distinct ancestral MBP genes prior to mammalian radiation. If this were the case, a new nomenclature should be created for human MBP and rhesus MBP-C genes.

Discussion

Analysis of MBPs isolated from a rhesus monkey liver cDNA library was undertaken and their relationships to the previously characterized MBPs was determined. The two rhesus monkey cDNAs isolated have been designated MBP-A and MBP-C based on their hybridization pattern and homology to rodent MBP-A and MBP-C and the human MBP amino acid sequences (Figure 3). Notable features of the rhesus MBP clones are the presence of an additional nine amino acid stretch at the N-terminus of rhesus MBP-A compared to rodent MBP-A, and the conservation of three cysteine residues at the N-terminus of rhesus MBP-C, in common with the structure of human MBP. In mice and rats, the absence of the nine amino acid stretch at the N-terminus characterizes MBP-A forms, whereas its presence together with the loss of a cysteine residue in this region are the identifying features of MBP-C (Sastry and Ezekowitz, 1993). Studies with a mutant form of human MBP lacking this nine amino acid stretch, synthesized in Cos-1 cells, shows an impairment in its ability to activate the classical pathway of complement, indicating an important structural role for this region (Kurata *et al.*, 1994).

Rhesus and cynomolgus monkeys have two forms of MBPs as shown by ELISA experiments (Figure 1B). Western blot analysis using the same rat MBP antibodies showed reactive bands of ~30 kDa in support of the ELISA results (Figure 2). Rhesus MBP-C did not react with the mouse monoclonal antibody against recombinant human MBP, although a high degree of homology is observed between rhesus MBP-C and human MBP (90% identity at the amino acid level, Table I). This could be due to loss of the reactive epitope in the rhesus monkey MBP. In contrast, chimpanzee sera reacted with antibodies to both human MBP and rat MBP-C, but not with rat MBP-A antibody, suggesting that chimpanzee serum has one form of MBP, similar to humans. These results suggest that a loss or a failure of expression of one MBP gene, similar to MBP-A, occurred after the divergence of monkeys, during hominoid evolution.

Although one MBP gene appears to be lost in humans, four

allelic forms of the human MBP gene are known. The predominant wild-type allele is designated A and the other three alleles B, C, and D, differ from each other due to point mutations in the collagen domain of the MBP gene (Sumiya *et al.*, 1991; Lipscombe *et al.*, 1992; Madsen *et al.*, 1994). Individuals with the wild type MBP allele have an average of 1.6 μg/ml of MBP in the serum, which circulates as mixtures of polymers of apparent molecular weights of 200–700 kDa (Lipscombe *et al.*, 1995). Individuals homozygous to alleles B and C, however, have very low levels of MBP in the serum (<10 ng/ml) mainly comprising material of an apparent molecular weight of 120–130 kDa (Lipscombe *et al.*, 1995). When expressed *in vitro,* mutant MBP alleles retained opsonic functions but failed to activate complement pathway (Super *et al.*, 1992; Kurata *et al.*, 1993; Matsushita *et al.*, 1995). The B, C and D alleles occur at high frequency in humans world wide, and because of their persistence in population, have been proposed to confer some selective advantage to the host, similar to the sickle cell and TNF-α promoter allele traits, which are also believed to provide protection to individuals exposed repeatedly to infectious agents (Garred *et al.*, 1994). High levels of serum MBP prior to infection may promote certain infections by allowing entry of opsonized pathogens (either directly or following complement activation) into cells (Fischer *et al.*, 1994). Thus loss of an MBP gene or having low levels of circulating MBP may be advantageous to the host by decreasing susceptibility to infections.

The presence of two distinct MBP forms in rhesus monkey strongly suggests that the duplication of MBP genes occurred prior to mammalian radiation. However, the loss of the nine amino acid stretch from the N-terminal domain of the rat and mouse MBP-A, and the missing cysteine residue from the N-terminus of MBP-C of rodents, appear to be evolutionary events that occurred after the divergence of rodents from primates 80 million years ago (Li and Graur, 1991).

The N-terminus sequence of the chicken MBP, determined recently, has the highest identity (43%) to the human MBP and revealed the presence of three cysteine residues and nine amino acids characteristic of complement activating MBP isoforms (Laursen *et al.*, 1995). These observations and the presence of the nine amino acid stretch in the N-terminus of rhesus MBP-A and rodent MBP-Cs suggest that the ancestral MBP gene also possessed this nine amino acid stretch. Chromosomal localization of the two rhesus MBP genes would be of interest to determine if they are localized on a single chromosome, syntenic to human chromosome 10.

Recent studies on the isolation of human MBP from serum, employing carbohydrates for elution from a mannan column (instead of EDTA), have shown that it is associated, in a calcium-dependent manner, with a novel serine protease called MASP (MBP associated serine protease, Matsushita and Fujita, 1992). MASP is related to C1r and C1s subcomponents of the first complement component- C1 (Matsushita and Fujita, 1992; Sato *et al.*, 1994). When MBP binds to carbohydrate ligands, it can activate C4 and C2 components of complement through the activation of MASP (Matsushita and Fujita, 1992). Previous structural studies on oligomers of human MBP isolated from serum has shown that it is only the pentamer and hexamer (15 to 18-chain, i.e., C1q-like) forms of MBP that have the ability to also directly activate $C1r_2/C1s_2$ components of the classical pathway of complement after binding zymosan (Lu *et al.*, 1990; Yokota *et al.*, 1995). Since both the rhesus monkey MBPs have three cysteines near the N-terminus and the nine amino acid stretch, perhaps both rhesus MBP-A and MBP-C, may be able to form pentamers and hexamers, and thus have the potential to activate the complement pathway independently. The knowledge of whether monkey MBP-A and MBP-C are associated with serine protease(s) similar to MASP in humans will provide valuable information on the functional and evolutionary aspects of these proteins.

Methods

Primate sera and other reagents

Chimpanzee (*Pan troglodytes*), rhesus monkey (*Macaca mulatta*), and cynomolgus monkey (*Macaca fascicularis*) serum samples were obtained from White Sands Research Center (Alamagodro, NM), with the approval of the Biosafety Committee of Boston University. Test antibodies included i) mouse monoclonal antibody (Mab#6) raised against human recombinant MBP (rMBP), generously provided by Dr. Alan Ezekowitz (Children's Hospital, Boston, MA); ii) polyclonal rabbit antibodies to both rat MBP-A and MBP-C, kindly provided by Dr. Kurt Drickamer (Glycobiology Institute, Oxford, UK). Secondary antibodies, horseradish peroxidase (HRP) conjugated anti-mouse and anti-rabbit IgG were obtained from SIGMA (St. Louis, MO). 3,3',5,5'-tetramethyl benzidine (TMB) was purchased from Bio-Rad (Hercules, CA). The Enhanced Chemiluminescence (ECL) kit was obtained from Amersham (Arlington Heights, IL). Rhesus monkey liver lambda gt10 cDNA library was purchased from Clontech (Palo Alto, CA). Reagents for polymerase chain reaction (PCR) were purchased from Perkin Elmer (Roche Molecular Systems, Branchburg, NJ).

Enzyme linked immunosorbent assays

To detect MBP in sera by ELISA, mannose-BSA or BSA (SIGMA, St. Louis, MO) was adsorbed to the wells of microtitre plates (10 μg/well) for 1 h at 37°C. Wells were washed with Tris buffered saline (TBS: 0.15 M NaCl; 50 mM Tris–HCl, pH 7.5) supplemented with 2 mM $CaCl_2$ and 0.1% Tween-20 and then blocked with 0.25% BSA. Sera were diluted to 10% in TBS with 2 mM $CaCl_2$ prior to addition to mannose-BSA or BSA coated wells (50 μl/well) and incubated overnight at 4°C. Bound MBP was then detected by sequential incubation with primary antibodies (1:500 dilution of mouse anti-human rMBP, rabbit anti-rat MBP-A or rat MBP-C) and then with 1:2000 dilution of HRP-labelled secondary antibody for 1 h at 37°C followed by incubation with TMB. The reaction was stopped with 1N H_2SO_4 and absorbance was quantified at 450 nm.

SDS–PAGE and Western blot analysis

Sera (1:10 dilution) and purified human RMBP were resolved by SDS–PAGE in a homogeneous 12% polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes. Western blot technique was carried out following protocols provided by the manufacturer (Amersham, Arlington Heights, IL) with modifications. To detect MBP, nitrocellulose membranes were incubated overnight at 4°C with rabbit anti-rat MBP-A and MBP-C antibodies (1:500 dilution) and after washes with HRP-conjugated secondary antibodies (1:2000 dilution) for 45 min at room temperature. Following extensive washing with TBS supplemented with 2 mM $CaCl_2$ and 0.3% Tween-20, the nitrocellulose membranes were treated with ECL reagent and exposed to Kodak X-ray films (Eastman, Rochester, NY) to detect reactive bands.

Screening of rhesus monkey cDNA library

Random and oligo dT primed 5'-stretch rhesus monkey (*Macaca mulatta*) liver cDNA library was screened to isolate MBP cDNA clones. Approximately 900,000 plaques were screened with (^{32}P)-dCTP random labeled mouse MBP-A and MBP-C cDNA probes. The mouse cDNA probes were ~700 bp in size and contained both the CRD and the collagen domain sequences (Sastry *et al.*, 1991). Prehybridization was carried out at 42°C for 4 h (in 5X SSC, 5X Denhardt's solution, 0.5% SDS and 100 μg denatured salmon sperm DNA/ml). Hybridization was performed at 42°C in the same solution with the addition of 1×10^6 CPM/ml of labeled probe for at least 16 h. Washing the membranes in 2× SSC/0.1% SDS for 15 min twice at room temperature and then at 55°C in 1× SSC/0.1% SDS for 30 min was found to be optimal. The membranes were then exposed to Kodak X-ray film at −70°C with intensifying screens to identify positive clones.

Amplification of the first exon of the rhesus monkey MBP-C gene by PCR

The rhesus MBP-C cDNA clone isolated from the liver cDNA library did not contain the most 5′ end of the protein coding region. The first 70 base pair sequence at the 5′ end of this clone contained a ribosomal sequence that may have been introduced during library construction. The sequence corresponding to the first exon of rhesus MBP-C gene was therefore determined from the genomic DNA by polymerase chain reaction (PCR). To amplify the first exon of rhesus MBP-C sense primer 5′-CCA AAT CCC CAG CTA GAG GC-3′ and antisense primer 5′-GAC CTT CCA TTT CTT AAC GTC T-3′ derived from noncoding sequences that flank the first exon of the human MBP gene were used. The PCR mixture (50 μl) contained 5 μl of 10× PCR buffer, 1 mM $MgCl_2$, 200 μM dNTPs, 0.25 μM sense primer, 0.25 μM antisense primer, 1 μg rhesus monkey genomic DNA, and 2.5 units of Taq DNA polymerase. The amplification protocol used was one cycle of 7 min at 95°C, 2 min at 55°C, and 1 min at 72°C followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C with a final extension step for 10 min at 72°C. The gel purified PCR products were cloned into pT7blue-T-vector (Novagen, Madison, WI) and sequenced as described below.

Subcloning and sequencing

Phage DNA of positive clones was amplified by PCR using lambda gt10 reverse and forward primers. The PCR products of MBP cDNA clones were digested with *Eco*RI (MBP-A) or *Eco*RI and *Pst*I (MBP-C) and ligated to appropriately digested pUC18 vectors. Sequencing reaction was performed on double stranded DNA template of these pUC18 MBP subclones utilizing Sequenase Version 2.0 kit (United States Biochemicals, Cleveland, OH) following the Sanger dideoxy chain termination method (Sanger *et al.*, 1977). The DNA was sequenced on both strands with the use of vector primers or rhesus MBP specific oligonucleotide primers synthesized at Life Technologies, Inc. (Grand Island, NY).

Sequence analysis of rhesus MBP cDNA clones

Nucleotide sequence data were analyzed with the use of the MacVector Sequence Analysis software (International Biotechnologies, New Haven, CT). Comparison of the rhesus MBP sequences with Genbank sequences was done using the BLASTN program at NCBI (Altschul *et al.*, 1990) and deduced amino acid sequences compared by protein scoring matrix PAM 120. Evolutionary analyses of rhesus MBP sequences with the human and rodent MBPs were carried out following the methods described by Nei and Gojobori (1986) and a phylogenetic tree was constructed by the neighbor-joining method of Saitou and Nei with the pairwise genetic distances obtained by poisson correction (Saitou and Nei, 1987). Confidence probabilities and bootstrap numbers were generated by utilizing MEGA (Kumar *et al.*, 1994) and METREE (Rzhesky and Nei, 1992) computer programs.

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