# Molecular cloning of membrane cofactor protein (MCP; CD46) on B95a cell, an Epstein–Barr virus-transformed marmoset B cell line: B95a-MCP is susceptible to infection by the CAM, but not the Nagahata strain of the measles virus

Yusuke MURAKAMI*, Tsukasa SEYA†‡, Mitsue KURITA†, Aya FUKUI*, Shigeharu UEDA§ and Shigeharu NAGASAWA*[1]

*Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-ku Sapporo 060, Japan, †Department of Immunology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Higashinari-ku, Osaka 537, Japan, ‡PROBRAIN, Tokyo 105, Japan, and §Department of Neurovirology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan

Measles virus (MV) infects not only human beings but also some simian species. The MV receptor on Vero cells (a cell line established from African Green monkey kidney cells) and human cells has been shown to be the membrane cofactor protein MCP/CD46, which is an inhibitor of autologous complement (C) activation. B95a, an Epstein–Barr virus (EBV)-transformed marmoset B cell line, is a simian cell line used for MV selection and is much more susceptible to MV than Vero cells. In the present study, we isolated cDNAs encoding MCP homologues from B95a cDNA library and assessed whether B95a-MCP is responsible for the high susceptibility of B95a to MV. The deduced amino acid sequence of the cDNA of B95a-MCP was 76% identical to that of human-MCP, and the recombinant B95a-MCP exerts C inhibitor activity. Although CAM, a vaccine strain of MV, infected Chinese hamster ovary (CHO) cells expressing B95a-MCP, Nagahata strain, a wild type of MV, failed to infect the CHO transfectants, suggesting that additional membrane molecules of B95a are responsible for the high susceptibility of B95a to the Nagahata strain.

## INTRODUCTION

Measles virus (MV) is an enveloped RNA virus that is responsible for a highly contagious infection during childhood. MV contains two glycoproteins in the viral envelope: the haemagglutinin (H) protein, which mediates virus attachment to susceptible cells, and the fusion (F) protein, which, together with H, is responsible for fusion with the cell membrane and virus entry into the host cell. Interaction between these viral proteins with cellular proteins also induces fusion between infected and uninfected cells to form multinucleated giant cells (syncytia) [1].

Recent studies have shown that the MV receptor on human cells is the membrane cofactor protein (MCP; CD46) [2,3]. MCP is a member of the regulators of complement (C) activation (RCA) gene family [4,5]. It acts as a cofactor in the factor I-catalysed cleavage of C3b/C4b deposited on the same cell membrane, thus protecting host cells from autologous C attack [6–10].

Human-MCP is composed of three extracellular domains, one transmembrane (TM) domain, and a cytoplasmic tail (CYT). The extracellular domains are composed of four tandem repeats of a domain named short consensus repeat (SCR) consisting of about 60 amino acids, a serine/threonine (ST)-rich domain, and a 13-amino acid sequence of unknown significance (UK) [4,11]. SCR2, SCR3 and SCR4 are required for the cofactor activity, while SCR1 and SCR2 are essential for MV infection [12,13]. In addition, the ST domain, which is rich in *O*-glycosylated residues was shown to modulate MV infectivity [14–16]. Alternative splicing of the mRNA of MCP can produce many MCP isoforms, which differ in ST-rich regions ($ST^A$, $ST^B$ and $ST^C$) and cytoplasmic tails (CYT1 and CYT2) [4,17–19].

Although MV exists as a single dominant immunotype, a number of MV strains, which differ in nucleotide sequence and biological properties have been established from exudates of measles patients by using monkey cell lines [20]. Vero cells (an African green monkey kidney cell line) and B95a cells (an EBV [Epstein–Barr virus]-transformed marmoset B cell line) [21] are two commonly used cell lines. However, there is increasing evidence that Vero and B95a cells differ in their sensitivity to MV; B95a cells are 10000-fold more sensitive to MV present in clinical specimens than are Vero cells, and the MV strains isolated by passage in B95a cells are more likely to resemble those of the circulating wild type strains than those obtained by passage in Vero cells [21]. In the previous paper, we reported Vero-MCP is 86% identical to human-MCP and shows similar MV receptor activity as human-MCP [22]. Thus, it is of interest whether high MV susceptibility can be reproduced by B95a-MCP alone or requires additional molecules. Here, we cloned the cDNA of the MCP homologue from B95a cells. Expression analysis revealed that although CAM, a vaccine strain [23–25], can infect CHO (Chinese hamster ovary) cells expressing B95a-MCP, Nagahata, a wild MV strain [24,26], fails to infect the CHO cells expressing B95a-MCP. These results suggest that additional membrane molecules are necessary for the infection of B95a cells with the Nagahata strain.

Abbreviations used: C, complement; CHO, Chinese hamster ovary; CYT, cytoplasmic tail; DAF, decay-accelerating factor; DMEM, Dulbecco's modified Eagle's medium; EBV, Epstein–Barr virus; F, fusion; FCS, fetal calf serum; H, haemagglutinin; mAb, monoclonal antibody; MCP, membrane cofactor protein (CD46); MFI, mean fluorescence intensity; MV, measles virus; NP-40, Nonidet P-40; PFU, plaque-forming unit; RCA, regulators of C activation; RT, reverse transcriptase; SCR, short consensus repeat; ST, a serine/threonine (-rich domain); TM, transmembrane; UK, unknown significance.

[1] To whom correspondence should be addressed.

## MATERIALS AND METHODS

### Cell, virus, antibodies, proteins, and reagents

Wild type CHO cells were obtained from the American Type Culture Collection (Bethesda, MD, U.S.A.), B95a and Vero cells from the Research Institute for Microbial Diseases (Osaka University, Japan). CHO cells were cultured in Ham's F12 (Sigma, St. Louis, MO, U.S.A.) supplemented with 10 % (v/v) fetal calf serum (FCS), 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin (Wako, Osaka, Japan). B95a cells were grown in RPMI 1640 (Sigma) supplemented with 10 % (v/v) FCS, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin. Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 5 % (v/v) FCS. Cultures were kept in a 5 % $CO_2$, 95 % air atmosphere at 37 °C. PBS containing 0.02 % (w/v) EDTA and 0.05 % (w/v) trypsin was used to detach cells.

The MV strains CAM (Hiraoka) [23–25] and Nagahata (HB) [24,26] were obtained form the Research Institute for Microbial Diseases (Osaka University, Japan). MV stocks were propagated in 70 % confluent Vero cells. After 2 or 3 days, the cells and culture supernatants were collected and lysed by a freeze and thaw cycle. MV-containing medium was centrifuged to remove cell debris, and titres were determined by plaque assay with Vero cell monolayers [27].

Monoclonal antibodies (mAbs) against human-MCP (M75, M160 and M177) were produced and purified in our laboratory as previously described [28]. mAbs MH61 [29] and E4.3 [30] were gifts from Drs. M. Okabe (Osaka University, Japan) and B. Loveland (Austin Institute, Australia), respectively. J48, S-19S, 3-81SA and 4-23SB were from Dr. J. M. Pesando (Onco membrane Inc., Seattle, WA, U.S.A.) [31]. H316 mAb was a gift from Dr. P. M. Johnson [32]. A mAb against MV-H protein was provided by Dr. Ueda (Osaka University, Japan). FITC-labelled goat anti-mouse IgG was a product of TAGO (Burlingame, CA, U.S.A.).

Complement C3 and factor I were purified from human plasma, and C3b was prepared from C3 as previously described [33–35]. Neuraminidase was obtained from Sigma, and *O*-Glycanase from Genzyme, Cambridge, MA, U.S.A. Restriction enzymes were purchased from Takara Biomedicals (Kyoto, Japan).

### cDNA cloning

Poly(A) mRNA was purified from B95a cells by a commercial oligo(dT) selection method (FastTrack; Invitrogen, San Diego, CA, U.S.A.). A cDNA library was constructed from 1 $\mu$g mRNA according to manufacturer's instructions (ZAP Express cDNA Synthesis Kit; Stratagene, La Jolla, CA, U.S.A.). The cDNA of human-MCP ($ST^c$/CYT2) [11] was labelled with [$\alpha$-$^{32}$P]dCTP and used to screen $1 \times 10^6$ plaques of the library. Hybridization was carried out at 40 °C for 14 h in a solution of 7 % (w/v) polyethylene glycol 6000, 10 % SDS, and 100 $\mu$g/ml yeast tRNA, and washed with $2 \times$ SSC ($1 \times$ SSC = 15 mM sodium citrate and 150 mM NaCl, pH 7.0) containing 0.1 % SDS at 50 °C [36]. After a third screening of plaques, 89 positive plaques were cloned and subjected to PCR by using T3 and T7 primers to determine inserted cDNA sizes. According to their cDNA sizes, the plaques were divided into three groups (1.3, 1.7 and 3.0 kbp) and converted into pBK-CMV phagemid clones (named pBKB3, pBKB1 and pBKB2, respectively). The three clones were sequenced from both directions by sequence-primer walking (ABI 373A Applied Biosystems, Foster City, CA, U.S.A.). The nucleotide and the deduced amino acid sequences of the clones were aligned and analysed using GENETYX-MAC software (Software Development Co., Tokyo, Japan).

### Reverse transcriptase (RT)-PCR

The SCR1 region of B95a-MCP was reverse-transcribed and amplified using a Geneamp RNA PCR kit (Perkin–Elmer Cetus, Norwalk, CT, U.S.A.). The sequence of the 5′ non-coding region-specific primer was CTTTGTGAGTTTGGGGATTGTTG and that of the 3′ SCR2 primer was ACTCCAAACTGCGTCTGATCCTT. mRNA (0.1 $\mu$g) from B95a cells was used for RT-PCR. PCR (30 cycles) was performed with denaturation at 94 °C for 1 min, annealing at 67 °C for 2 min, and extension at 72 °C for 3 min. The PCR products were electrophoresed on a 1.5 % agarose gel, subcloned into the pCRII vector (Invitrogen), and sequenced. ST-CYT regions were amplified by RT-PCR using the following primers: 5′-GACACAATTGTCTGTAACAGTAAC-3′ in the SCR4 region (sense strand); 5′-AAAAGATGAACTGCCAAACCAAG-3′ in the 3′ non-coding region (antisense). PCR was performed as above, except the annealing step was at 65 °C for 2 min.

### Expression of MCP homologue on CHO cells

pBKB2 and pBKB3 were digested with *Pst*I and *Sal*I/*Xho*I, respectively. The fragments were blunt-ended (Takara Biomedicals Blunting Kit) and ligated into pCXN2, a high expression vector with a neomycin-resistance gene [37]. The subcloned vector was transfected into CHO cells by calcium phosphate precipitation [38]. Transfected CHO cells were maintained in Ham's F12 medium supplemented with 10 % FCS in a humidified 5 % $CO_2$ atmosphere at 37 °C. The cells were transferred to identical medium containing 0.5 mg/ml of G418 (Gibco-BRL, Grand Island, NY, U.S.A.) for selection. G418-resistant colonies were isolated with cloning cylinders and grown on tissue culture plates. MCP homologue expression was confirmed by flow cytometry using M160 mAb as described below. CHO cell lines expressing the human-MCP $ST^c$/CYT2 isoform (nos. 2 and 8) [39] and decay-accelerating factor (DAF; no. 6) [40] were previously established.

### Flow cytometry

Transfected cells ($5 \times 10^5$) were incubated with 1 $\mu$g of murine anti-human-MCP mAb for 1 h at 4 °C. The cells were washed and treated with FITC-labelled secondary antibody. The stained cells were analysed on an Epics Profile II (Coulter, Hialeah, FL, U.S.A.) or a FACSort (Becton Dickinson, Mountain View, CA, U.S.A.). Mean fluorescence intensity (MFI) was evaluated on the attached computer.

### Immunoblotting

Preparation of 1 % Nonidet P-40 (NP-40)-solubilized MCP [6], and SDS/PAGE [41] followed by immunoblotting [42] were performed as reported previously [12]. MCP was detected with M160 mAb, peroxidase-conjugated secondary antibody, and chemiluminescence (ECL system, Amersham Life Science, U.K.).

### Glycosidase treatment

Solubilized MCP solution (50–100 $\mu$l) was mixed with an equal volume of 40 mM Tris-maleate containing 0.2 % NP-40 (pH 6.0), and incubated with 100 microunits of neuraminidase for 1 h at 37 °C. The samples were then incubated with 3 milliunits of *O*-

Glycanase for 16 h at 37 °C [14]. Samples were resolved by SDS/PAGE (10 % acrylamide) and Western blotted with M160 mAb. Control (designated as 'untreated') samples were subjected to neither neuraminidase nor *O*-Glycanase.

### Factor I-cofactor activity

Cofactor activity was determined by immunoblotting of factor I-treated C3b [22]. Human C3b (0.4 $\mu$g) and factor I (0.04 $\mu$g) were incubated in a solution of 20 mM phosphate buffer (pH 6.0) for 3 h at 37 °C with various amounts of MCP solubilized from CHO transfectants expressing B95a- and human-MCP. The cofactor activity of 1 $\mu$l of lysate obtained from CHO clone no. 2 expressing human-MCP was defined as one unit. Half the samples were resolved by SDS/PAGE (7.5 % acrylamide), blotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, U.S.A.), and detected with rabbit anti-C3d polyclonal antibody and peroxidase-labelled secondary antibody.

### Determination of MV infectivity

CHO transfectants ($5 \times 10^5$) were infected with the MV strains (CAM and Nagahata) at 1 plaque-forming unit (PFU) per cell in a 6-well plate (Becton Dickinson Labware, NJ, U.S.A.). After infection for 63 h at 37 °C, the cells were fixed with 10 % formalin and stained with 0.1 % neutral red. The syncytia formed were photographed using a Nikon inverted microscope. The CHO transfectants expressing DAF [40] were used as the control.

Another set of plaque-forming assays were performed under different conditions [12]. Briefly, monolayers of CHO cell clones were cultured at 37 °C in 24-well plates (Corning, New York, NY, U.S.A.) with Ham's F12 supplemented with 10 % FCS for 12 h and infected with the MV strains at $1–5 \times 10^4$ PFU/well. After incubation for 1 h at 37 °C, infected cells were overlaid with 0.5 % agarose (SeaKem LE; FMC BioProducts, Rockland, ME, U.S.A.) in basic culture medium. The cells were cultured at 37 °C for 3–5 days. The syncytia formed were counted under a microscope. These experiments were performed in duplicate and a minimum of two times.

### Nucleotide sequences

The nucleotide sequences for cDNA of B1, B2, B3, B2C, a SCR1 region, and a CYT1 region in this paper have been deposited in the DDBJ/EMBL/GenBank databases with the accession numbers D82076, D85750, D63848, D89756, D78369 and AB001991, respectively.

## RESULTS

### cDNA cloning and sequence

A full-length cDNA of human-MCP ($ST^C$/CYT2 isoform) [11] was used as a probe for screening $1 \times 10^6$ plaques from the cDNA library of B95a cells, 89 positive clones were obtained after the third screening. Positives were divided into three nucleotide lengths of about 1.3, 1.7 and 3.0 kbp. Most positives belonged to the 1.3 and 1.7 kbp groups. Each of the 1.3, 1.7 and 3.0 kbp groups were excised from the phage in the form of phagemids, termed B3, B1 and B2, respectively. At first, we attempted to sequence the B1 and B3 clones, since the mRNA corresponding to B1 and B3 were judged to represent the major MCP molecules of B95a. Both of the cDNAs contained a single open reading frame but were devoid of the SCR1 domain (Figure 3B). Since SCR1 together with SCR2 was shown previously to be essential for MV infectivity [12], we next attempted to sequence the remaining B2 clone. It contained a cDNA of 2745-bp which encoded the entire MCP molecule with an open reading frame of 370 or 378 amino acids including a putative signal peptide of 34- or 42-amino acids (Figure 1). Comparison of the deduced amino acid sequence of B95a-MCP with that of human-MCP suggested that the translation of the cDNAs began at the second ATG codon to produce a MCP homologue composed of 370 amino acids, including a signal peptide of 34 amino acids (Figure 2A). The molecular mass of the predicted mature protein was estimated to be 37265 Da. There were three potential *N*-glycosylation sites (-Asn-X-Ser/Thr; $Asn^{49}$, $Asn^{80}$ and $Asn^{239}$) in the SCR domains, and the ST domain contained eight Ser/Thr residues and resembled the $ST^B$ isoform. Hydropathy analysis revealed that the amino acid residues 282–302 constitute a TM domain, suggesting that the carboxyl terminal cytoplasmic tail (CYT) is composed of 33 amino acids, which resembles the longer CYT2. Thus, B95a-MCP from the B2 clone consists of four typical domains resembling SCRs, $ST^B$, TM, and CYT2, which are 77, 60, 58 and 78 % identical to the corresponding domains in human-MCP (Figure 2A). The homologies in translated SCR1, SCR2, SCR3, and SCR4 regions between B95a- and human-MCP are 67, 70, 88 and 85 %, respectively. Overall homology between B95a- and Vero-MCP is 76 %.

The SCR1-deficient B1 and B3 clones were different from each other in the ST region; the shorter B3 clone contained three tandem repeats of $ST^A$, $ST^B$ and $ST^C$ ($ST^{ABC}$), while the longer B1 clone contained only a single $ST^C$ (Figure 2B).

To determine the relative amounts of SCR1-deficient and SCR1-containing MCP homologues in B95a cell mRNA, the 5′ and 3′ sides of the SCR1 region in B95a mRNA were amplified by RT-PCR. Products at 370- and 580-bp were detected by agarose gel electrophoresis (Figure 3A). The relative ratio of the two bands was estimated to be approx. 9:1. The minor 580-bp product showed the same nucleotide sequence as the SCR1 portion of the B2 clone, while the major 370-bp product showed the same sequence as the corresponding region in SCR1-deficient B1 and B3 clones (Figure 3B). Thus, the SCR1-deficient MCP homologue was the major form in B95a cells.

Northern blot analysis of RNA from B95a cells using the [$\alpha$-$^{32}$P]dCTP-labelled B3 clone as a probe, showed two main mRNA at 1.2–1.7 and 4.0 kb (data not shown). These results suggested that B2 clone originated in the longer mRNA of B95a-MCP, while B1 and B3 clones originated in the shorter mRNA species.

### Analysis of ST and CYT-variants

To assess the existence of ST- and CYT-variants, these regions in B95a cell mRNA were amplified by RT-PCR. The products were extracted and subcloned into pCRII vector. Twenty clones were isolated and sequenced. We found at least seven isoforms which were five ST-variables with CYT1 or CYT2 such as $ST^C$/CYT2 (1 clone), $ST^B$/CYT1 (1) or CYT2 (1), $ST^{AB}$/CYT1 (6) or CYT2 (9), $ST^{BC}$/CYT2 (1), and $ST^{ABC}$/CYT2 (1). The nucleotide and deduced amino acid sequences of a CYT1 region of B95a-MCP are given in Figure 2C. These results showed the polymorphic properties of human-MCP are conserved in B95a-MCP.

### Expression of B95a-MCP on CHO cells

CHO cells were transfected with the cDNA of B95a-MCP (B2 clone; $ST^B$/CYT2 isoform) and assessed for reactivity to anti-human-MCP mAbs by flow cytometry. Almost all mAbs, except for M160, hardly bound with parent B95a cells, suggesting that

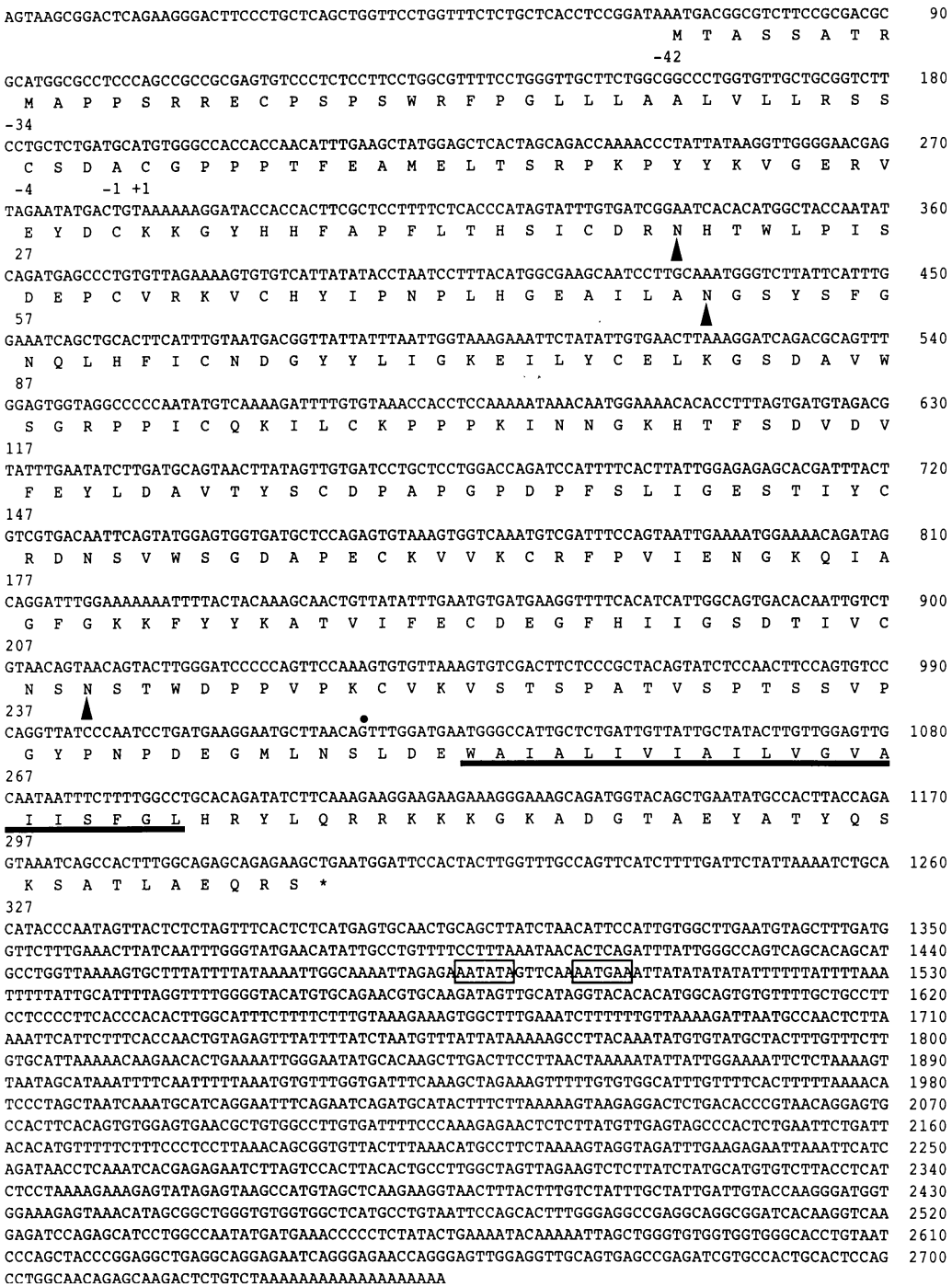


**Figure 1 The nucleotide and deduced amino acid sequences of the B2 clone**

The numbers on the right indicate nucleotide position, amino acids are numbered from the first residue of the mature protein and are shown under the sequence. The hydrophobic TM domain is indicated by a bold underline. The potential *N*-glycosylation sites are marked with arrowheads. The difference between B2 and B2C nucleotide sequences in the UK region is dotted (at nucleotide 1027 of B2). The asterisk denotes the stop codon, and the polyadenylation signals are boxed.

the number of MCP molecules expressed on parent B95a cells was very low. Among 10 mAbs tested, only H316 and M160, which bind to SCR1 and SCR3 respectively, bound to the CHO transfectants expressing B95a-MCP (Table 1). The SCR2-reactive mAbs M75 and M177, which inhibit MV- and C3b-binding to human-MCP [10], did not bind to the CHO transfectants expressing B95a-MCP. Next, the membrane fractions of the CHO transfectants expressing B95a-MCP and the parent B95a cells were subjected to SDS/PAGE, followed by immunoblotting with M160 mAb, revealed a single band at 54 kDa from both the CHO transfectants expressing the B95a-MCP $ST^C$/CYT isoform and the parent B95a cells, while a single 59 kDa band was detected from the B95a-MCP $ST^B$/CYT2 isoform (Figure 4). Immunoblotting profiles of *O*-Glycanase-treated and untreated

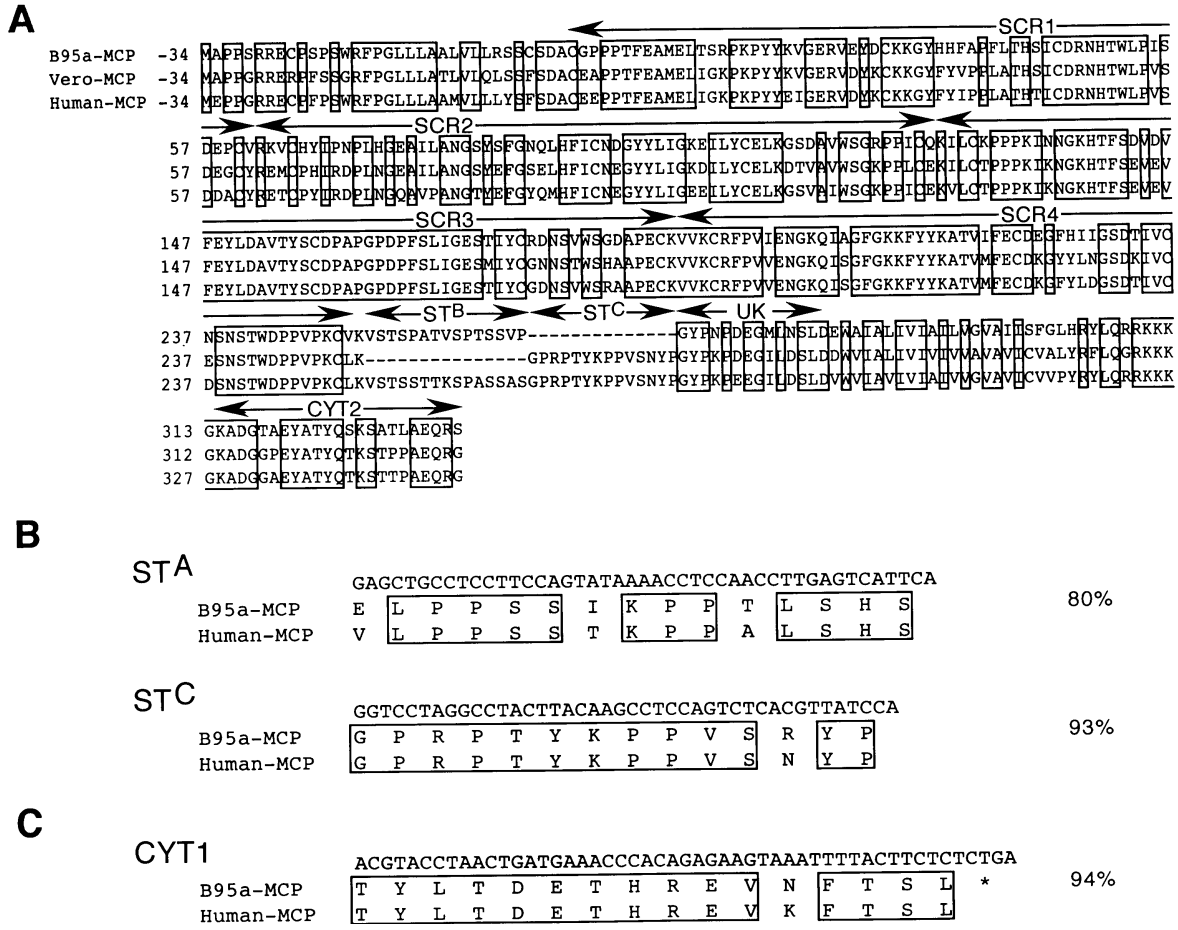


**Figure 2 Alignment of the deduced amino acid sequences of B95a-, Vero- [22], and human-MCP [11]**

(**A**) Amino acids numbered from the first residue of the predicted mature proteins are on the left. The amino acid identity among three primate MCPs is boxed. Based on human-MCP, SCR, ST, UK and CYT domain boundaries are shown by arrows. Dashed lines within B95a- and Vero-MCP sequences represent gaps. (**B**) The nucleotide and deduced amino acid sequences of $ST^A$ and $ST^C$ in B3 clone ($ST^{ABC}$) are shown. The amino acid residues (upper in each set) which are identical to $ST^A$ or $ST^C$ domains in human-MCP (lower) [4] are boxed. The percentage of amino acid identity are indicated on the right. (**C**) CYT regions in B95a mRNA were amplified by RT-PCR and the nucleotide sequences analysed. The amino acid sequences (upper) corresponding to the CYT1 domain in human-MCP (lower) [4] are shown. The asterisk denotes the stop codon.

membrane fractions from the CHO transfectants expressing the $ST^B$ and $ST^C$ isoforms of B95a-MCP revealed that the apparent molecular sizes of *O*-linked sugars in the $ST^B$ and $ST^C$ regions of B95a-MCP were 10 and 5 kDa, respectively.

### Cofactor activity of B95a-MCP

We assessed whether B95a-MCP showed the cofactor activity in factor I-catalysed cleavage of human C3b. Human C3b and factor I were incubated with or without the membrane fractions of CHO transfectants expressing B95a-MCP ($ST^B$/CYT2 or $ST^C$/CYT2 isoform) or the $ST^{ABC}$/CYT2 isoform of SCR1-deficient B95a-MCP. Control CHO cells and CHO transfectants expressing human-MCP were also tested. The degree of cleavage of C3b was determined by SDS/PAGE, followed by Western blotting with anti-C3d polyclonal antibodies. The membrane fraction of the CHO transfectants expressing B95a-MCP but not the control CHO cells allowed the factor I-catalysed cleavage of the $\alpha'$ chain of human C3b (105 kDa) producing the $\alpha 1'$ (68 kDa) fragment. The SCR1-deficient B95a-MCP also showed the cofactor activity. Thus, both isoforms of the SCR1-containing and the SCR1-deficient B95a-MCP retain cofactor cleavage activity (Figure 5).

### MV infection of CHO cells expressing B95a-MCP

To assess whether B95a-MCP is responsible for the high susceptibility of B95a cells to MV, CHO cells expressing B95a- or human-MCP were incubated with two MV strains, Nagahata and CAM. The CHO cells expressing human-MCP formed many syncytia with small amounts (100–500 PFU) of both MV strains (data not shown). The CHO cells expressing B95a-MCP ($ST^B$/CYT2 isoform) formed a few syncytia only when they were infected with extraordinary large amounts ($5 \times 10^5$ PFU) of the CAM strain (Figure 6). No syncytia were formed with the Nagahata strain (data not shown). In addition, flow cytometric analysis with anti-H protein of MV showed that the Nagahata strains did not bind to the CHO transfectants (data not shown).

The inability of MV to infect CHO transfectants expressing B95a-MCP ($ST^B$/CYT2 isoform) seemed to be due to the ST, but not the SCR domains, since we reported the size of the ST also

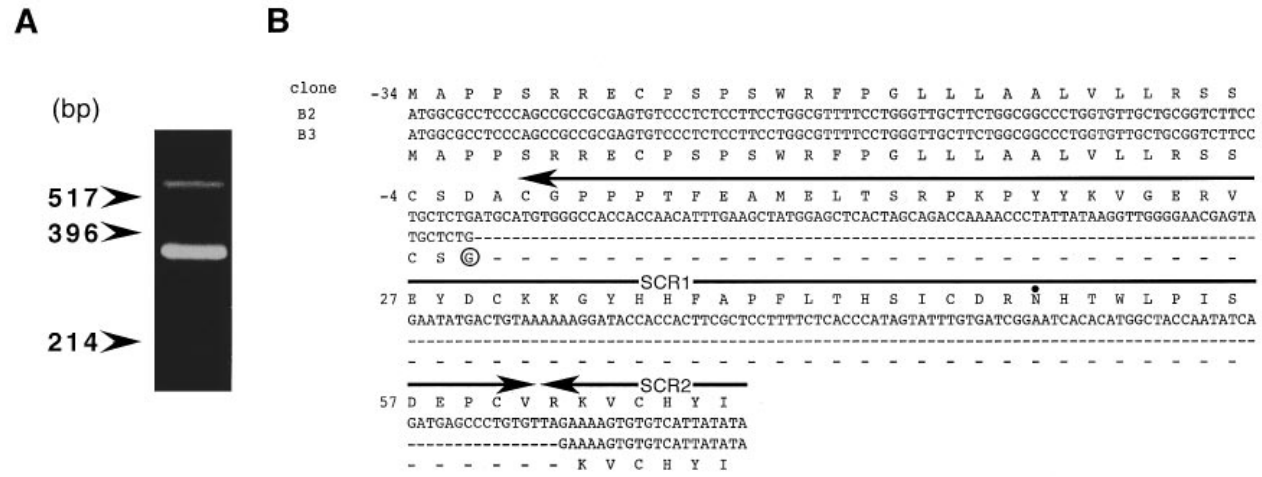


**Figure 3 Analysis of the SCR1 region of B95a-MCP**

(**A**) Poly(A) mRNA was isolated from B95a cells. RT-PCR was performed using specific-primers. Half of the PCR products were subjected to 1.5% agarose gel electrophoresis. Marker sizes are shown by arrow heads. The sequence analysis of two amplified products revealed a 580-bp product containing the SCR1 region and a 370-bp product lacking SCR1. (**B**) The numbers on the left correspond to amino acids from the *N*-terminus of the mature protein encoded by the B2 clone. The partial deduced amino acids sequences of B2 and B3 clones are shown at the top and bottom in each sequence set, respectively. SCR1 and SCR2 boundaries are shown by arrows. An amino acid substitution in a coding region of the B3 clone, which is presumed to be caused by alternative splicing of mRNA, is circled. An *N*-linked glycosylation site is dotted. Dashed lines indicate gaps.

**Table 1 Reactivities of anti-human-MCP mAbs with B95a cells and CHO cell clones expressing B95a-MCP**

The results were shown as MFI.

| | | | CHO cells expressing | |
|---|---|---|---|---|
| mAb | Epitope | B95a cells | B95a-MCP | Human-MCP (clone no. 8) |
| Mouse IgG | — | 1.73 | 2.32 | 2.70 |
| E4.3 | SCR1 | 1.83 | 2.46 | 738.86 |
| S-19S | SCR1 | 1.86 | 2.44 | 787.31 |
| 3-81SA | SCR1 | 1.87 | 2.52 | 459.94 |
| 4-23SB | SCR1 | 1.80 | 2.49 | 682.51 |
| J48 | SCR1 | 1.85 | 2.33 | 730.87 |
| H316 | SCR1 | 1.98 | 356.87 | 736.82 |
| M75 | SCR2 | 1.98 | 2.46 | 945.62 |
| M177 | SCR2 | 1.86 | 2.26 | 940.06 |
| M160 | SCR3 | 3.26 | 182.14 | 414.52 |
| MH61 | SCR3 | 2.55 | 2.67 | 794.97 |

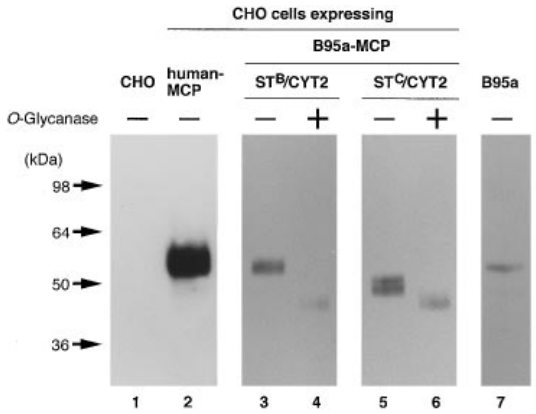


**Figure 4 Western blots of B95a-MCP on CHO transfectants and B95a cells**

CHO cell clones expressing the human-MCP ST$^C$/CYT2 isoform [39] (lane 2), the B95a-MCP ST$^B$/CYT2 isoform (lanes 3 and 4), the B95a-MCP ST$^C$/CYT2 isoform (lanes 5 and 6), and parent B95a cells (lane 7) were solubilized and resolved by SDS/PAGE (10% acrylamide) under non-reducing conditions. CHO cells having no MCP were used as the control (lane 1). Total proteins from solubilized cells were incubated with (+) or without (−) *O*-Glycanase. Immunoblotting was performed using M160 mAb as the primary antibody. Molecular mass markers are indicated by arrows.

affected MV infectivity [12]. ST$^B$ is probably more bulky than ST$^C$, since ST$^B$ is rich in serine/threonine and supposedly heavily glycosylated. We attempted to sequence another cDNA clone of the ST$^C$ isoform and obtained a 2743-bp cDNA (B2C) which included an open reading frame encoding the B95a-MCP ST$^C$/CYT2 isoform (data not shown). A single mutation in the UK region of the B2C clone was detected, a G at nucleotide 1027 of B2 in Figure 1 was substituted for a T. The mutation resulted in the change from a Ser (AGT) to Ile (ATT). However, the nucleotide sequence in the UK region of all clones was identical to that of the B2 clone. In addition, the presence of the mutation in the UK region was not detected by RT-PCR, suggesting an artificial mutation that happened during the preparation of the cDNA library. We then constructed a modified cDNA encoding ST$^C$/CYT2 isoform with the 'right' UK region by using B2 and B2C clones. The CHO transfectants expressing the B95a-MCP ST$^C$/CYT2 isoform of the modified B2C clone showed the same MV susceptibility to the CAM strain (Figure 6), but not to the Nagahata strain (data not shown). Thus, it appears likely that the SCR1 and SCR2 of B95a-MCP itself are effective as the MV receptor to the CAM strain, but not to the Nagahata strain. Further, the extremely low MV infectivity of the B95a-MCP ST$^B$ isoform was due to the ST$^B$ domain.

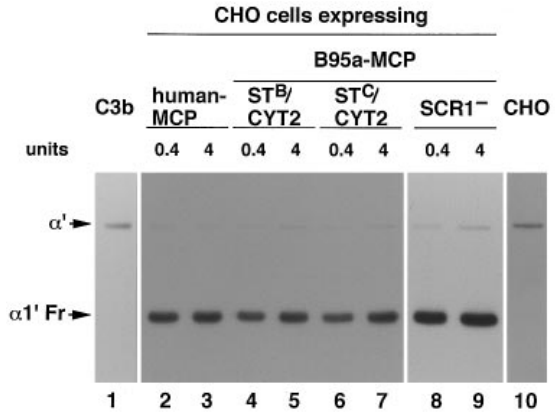


**Figure 5 Human complement regulatory activity of B95a-MCP**

Human complement regulatory activity of B95a-MCP was determined by immunoblotting factor I-mediated C3b fragments. Each transfectant was solubilized in 1% NP-40 and resolved by SDS/PAGE (7.5% acrylamide) under reducing conditions. CHO cells expressing no MCP (lane 10) and C3b incubated with only factor I (lane 1) were used as controls. The expression level of MCP was evaluated by flow cytometry using M160 mAb. The cofactor activity equivalent to one microlitre of cell lysate from CHO cells expressing human-MCP (clone no. 2) [39] was defined as one unit. The samples were blotted onto a membrane and detected with anti-C3d polyclonal antibody, peroxidase-conjugated secondary antibody, and chemiluminescence. Lanes 2 and 3, the human-MCP $ST^C$/CYT2 isoform; lanes 4 and 5, the B95a-MCP $ST^B$/CYT2 isoform; lanes 6 and 7, the B95a-MCP $ST^C$/CYT2 isoform; lanes 8 and 9, the SCR1-deficient B95a-MCP $ST^{ABC}$/CYT2 isoform ($SCR1^-$). The $\alpha'$ chains of C3b (105 kDa) and the $\alpha 1'$ fragment of iC3b (68 kDa, designed as $\alpha 1'$ Fr) are marked.

## DISCUSSION

We cloned different cDNAs from the B95a homologue of human-MCP, including a cDNA clone (B2) consisting of 2745-bp with an open reading frame encoding 370 amino acids. The cDNA had a long 3′ untranslated region which included the same sequences (AATGAA and AATATA) as the human-MCP [11] variant on the poly(A) signal [43]. The overall homology in the amino acid sequence of B95a-MCP to human-MCP was 76%, which was lower than that between Vero- and human-MCP (86%) [22]. The B95a-MCP corresponded to the human-MCP $ST^B$/CYT2 isoform.

The molecular mass of B95a-MCP was estimated to be 37 kDa from its amino acid sequence and 54–59 kDa by western blotting. The differences in the molecular masses were probably due to *N*- and *O*-glycosylation of B95a-MCP, since there are three potential *N*-glycosylation sites in the SCR domains and several putative *O*-glycosylation sites in the $ST^B$ and $ST^C$ domains. The apparent molecular mass of B95a-MCP was similar to that of human- and Vero-MCP [22]. RT-PCR revealed the presence of a smaller CYT1 phenotype in B95a mRNA, which was also observed in human [4] and Vero cells [22]. There are RRKKK [44] and FTSL [45] sequences in the CYT domain of human-MCP. RRKKK is a targeting sequence for the endoplasmic reticulum [46] and a moesin-binding site [44]. FTSL may serve as a retention signal in the cytoplasm [45]. These sequences were conserved in B95a-MCP.

Recombinant B95a-MCP was found to act as the cofactor in factor I-catalysed cleavage of human C3b. Evidence that mAbs M75 and M177, which bind to SCR2 and inhibit the cofactor activity of human-MCP [12], did not bind to B95a-MCP suggests that the epitopes for M75 and M177 in human-MCP are not directly responsible for the cofactor activity. It seems possible that the epitopes for these mAbs are located in the vicinity of the binding site for C3b in SCR2, and the binding of these mAbs sterically interferes with C3b binding to MCP.

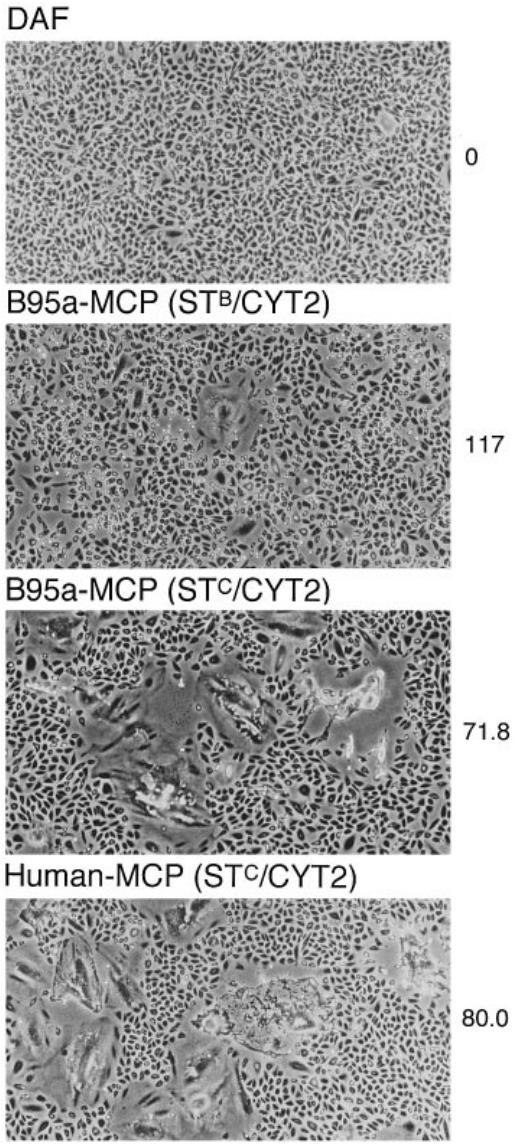


**Figure 6 Syncytium formation of CHO cells expressing primate MCP induced by the MV CAM strain**

CHO cell clones expressing DAF [40] and the human-MCP $ST^C$/CYT2 isoform (clone no. 8) [39], were used as controls. B95a-MCP CHO transfectants were inoculated with MV (1 PFU/cell) and viewed by phase-contrast microscopy (magnification × 10) after 63 h. Very few syncytia were formed in monolayers of CHO transfectants expressing the B95a-MCP $ST^B$/CYT2 isoform under the conditions employed. The CHO cells expressing the B95a-MCP $ST^C$/CYT2 isoform formed many syncytia similar to those formed in human-MCP. The experiment was performed twice in triplicate, typical pictures are shown. The MCP expression levels were simultaneously evaluated by flow cytometry using M160 mAb, shown by MFI on the right.

Although the CHO transfectant expressing B95a-MCP was stained with two (H316 and M160, which recognize SCR1 and SCR3, respectively) of the 10 anti-human-MCP mAbs, parent B95a cells were ambiguously stained with only M160 mAb. SDS/PAGE of the membrane fractions prepared from CHO transfectants and parent B95a cells followed by Western blotting with anti-MCP mAb M160 detected faint bands of MCP

homologues in B95a cells. Flow cytometric analysis also revealed the number of MCP molecules expressed on B95a cells was far less than those on Vero cells (data not shown).

The CHO transfectants expressing the $ST^B$ isoform did not form any syncytium upon treatment with extremely high concentrations of the CAM strain. The CAM preparation used in the present experiment formed syncytia at low MV titre with CHO transfectants expressing the $ST^C$ isoforms of B95a-, Vero-, and human-MCP (data not shown). Iwata et al. showed that CHO cells expressing the human-MCP $ST^{BC}$ isoform induce less syncytia than those expressing the $ST^C$ isoform [14]. Our present observations, taken together with those of Iwata et al., strongly suggest that the bulkiness of *O*-linked sugars in the ST region adjacent to the target cell membranes render the cell resistant to MV infection. This is further supported by reports that MV infectivity becomes weaker as the distance from the MV binding domain (SCR1 and SCR2) to the membrane anchoring unit becomes longer [47].

Neither the B95a-MCP $ST^B$ isoform nor the $ST^C$ isoform can act as a MV receptor for the Nagahata strain. The Nagahata strain tested could infect parent B95a cells (data not shown), excluding the possibility that the MV strain lacked the ability to infect B95a cells. The B95a-MCP homologue is not sufficient for the entry of the Nagahata strain. Some molecules present on B95a but not CHO cells may associate with B95a-MCP and facilitate MV binding to SCR1 and SCR2. There are two cDNAs encoding B95a-MCP isoforms which are lacking in the SCR1 and differ from each other in composition of the ST region. RT-PCR analysis suggests that the SCR1-deficient MCP is the major form of B95a-MCP. mRNA encoding SCR1-deficient MCP was not detected in human [11] and Vero cells [22]. The SCR1-deficient B95a-MCP homologue still retained the cofactor activity. Thus, B95a-MCP serves as a C3b inactivating factor, even if it lacks SCR1, suggesting the essential domains for cofactor activity in simian MCP are SCR2, SCR3 and SCR4. The inability of SCR1-reactive H316 to bind parent B95a cells suggests that the SCR1-deficient MCP isoform may be the major form expressed on parent B95a cells. If this is the case, CAM MV uses an alternative receptor for binding to B95a cells, since both SCR1 and SCR2 of MCP are essential for MV binding to MCP [12]. Indeed, the SCR1-deficient B95a-MCP ($ST^{ABC}$/CYT2 isoform) is not susceptible to infection by the MV strains CAM and Nagahata (data not shown).

Another possibility is that MV-H could not bind to the SCR1 and SCR2 of B95a-MCP. Indeed, M177 and M75, which are capable of blocking human-MCP-mediated binding and MV infection [12], fail to recognize B95a-MCP. Additionally the homologies of SCR1 and SCR2 between B95a-MCP and human-MCP are only 67 and 70 %, respectively. Recently, Buchholz et al. determined the epitopes recognized by anti-human-MCP mAbs to search the primary binding site of MV to human-MCP [48]. It was revealed that $Arg^{69}$ was the most critical amino acid residue for binding of both mAbs M75 and M177 and $Asp^{70}$/$Glu^{103}$ had some effect on binding of M177. The residue $Asp^{70}$, which is close to $Arg^{69}$, was essential for H and MV binding. Interestingly, both residues $Arg^{69}$ and $Asp^{70}$ in human-MCP were substituted for Pro and Asn in B95a-MCP, respectively. These changes may reduce binding of the Nagahata strain MV-H to B95a-MCP. MV-H primary sequences were reported to be slightly different between wild and vaccine strains of MV [25]. There are 14 amino acid variations between the MV-H primary sequences of the MV strains Nagahata (HB; DDBJ/EMBL/GenBank accession number D63924) and CAM (Hiraoka; unpublished data). These amino acid variations in the Nagahata strain MV-H may possibly affect the binding of MV to B95a-MCP expressed on CHO cells. The B95a-MCP homologues used in this study are functionally active as a cofactor but all of them do not cause high susceptibility to MV strains.

B95a cells were established from marmoset B lymphocytes transformed with EBV [21]. An alternative explanation is that B95a-specific host factors are responsible for MV entry into B95a cells and high susceptibility to MV may be amplified by gene products derived from EBV. Horvat et al. proposed the usage of an alternative receptor for entry of MV into activated murine B cells [49], and Yanagi et al. have reported the entry of MV into mouse fibroblast cell lines, which are lacking in MCP [50]. However, the molecular nature of these alternative MV receptors are not characterized. Whether B95a cells may express alternative MV receptor must await further investigation.

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