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Measles virus and C3 binding sites are distinct on membrane cofactor protein (CD46)

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ABSTRACT **The human complement regulatory protein membrane cofactor protein (CD46) is the cellular receptor for measles virus (MV), whereas decay accelerating factor (DAF; CD55), a structurally similar complement regulatory protein, does not bind MV. To characterize the interaction between MV and CD46, mutants of the CD46 protein and hybrid molecules between CD46 and DAF were tested for their ability to act as MV receptors. The transmembrane domain and cytoplasmic tail of CD46 were not required for receptor function as cells expressing the CD46 extracellular domain linked to the glycosyl-phosphatidylinositol tail of DAF were rendered susceptible to MV infection. Chimeric proteins exchanging the four extracellular short consensus repeat (SCR) domains between CD46 and DAF indicated that only molecules with both SCR1 and SCR2 from CD46 allowed a productive MV infection. Further, monoclonal antibodies (mAbs) against SCR1 or SCR2 of CD46 blocked MV infection, whereas a mAb against SCR3 and SCR4 did not. The latter mAb blocks C3b/C4b binding (which maps to SCR3 and SCR4) whereas the former mAbs do not. Thus, our data indicate that both SCR1 and SCR2 make up the MV receptor determinant in CD46. These results also suggest avenues for development of therapeutic agents to inhibit MV binding and thus infection and disease.**

Measles virus (MV) is among the most contagious infections of humans. Although an effective MV vaccine has been in use since the 1960s, >40 million infections and ≈2 million deaths occur yearly from measles (1). Vaccine failure is primarily due to interference by maternal antibodies that prevents vaccination in young children up to 15 months of age (2). High-dose vaccination in children of this age has not been effective and in fact has increased morbidity and mortality after MV infection (3, 4). Thus, a window of time exists when susceptibility to MV in young children is high and vaccination is not effective. Anti-MV therapeutics that could inhibit the spread or severity of MV infection may be useful during this period.

Understanding the interaction between the virus and host cell opens avenues for the design of effective antiviral agents. We (5) and others (6, 7) have identified the MV receptor as CD46 (membrane cofactor protein). CD46 is a member of the regulators of complement activation (RCA) family located on human chromosome 1 (8, 9). Proteins in this family protect the host from attack by its own complement system by regulating the activation state and deposition on host cells of complement proteins. CD46 binds complement components C3b and C4b and acts as a cofactor for their cleavage by serine protease factor I (8). The structural hallmark of proteins in the RCA family is the short consensus repeat (SCR) domain, an ≈60-amino acid domain with four conserved cysteines forming two disulfide bonds (10, 11). Members of the RCA family vary widely in the numbers of SCRs they possess, from 4 [CD46 and decay accelerating factor (DAF/CD55) to as many as 30 (CR1/CD35) (8). CD46 and DAF are unique in that they contain extracellular Ser-Thr-Pro-rich (STP) regions near the membrane. CD46, in particular, is expressed as a series of isoforms that arise by alternative splicing of the STP regions and cytoplasmic domain (8). Each of the isoforms consists of the four SCRs, followed by variable STP domains, a small region of unknown significance, a transmembrane domain, and one of two cytoplasmic tails. All of the four primary isoforms expressed in human cells and tissues can function as MV receptors (5).

To determine the region(s) of CD46 that interact with MV, chimeras between CD46 and DAF and deletion mutants of CD46 were constructed. Although it does not function as a MV receptor, DAF has a structure very similar to CD46, with four SCRs and a STP domain. Instead of a transmembrane domain, however, DAF has a glycosyl-phosphatidylinositol (GPI) linkage to the cell membrane. We investigated which combinations of SCRs, STP, and membrane anchor domains from CD46 and DAF are required to produce a functional MV receptor. We observed that the presence of both of the two outermost SCRs of CD46 (SCR1 and SCR2) are required for MV infection. Further, monoclonal antibodies (mAbs) against either SCR1 or SCR2 of CD46 could block MV infection, whereas a mAb that blocks C3b/C4b binding in SCR3 and SCR4 did not block infection. The cytoplasmic tail of CD46 was not required for MV receptor function.

MATERIALS AND METHODS

Cells and Virus. MV (Edmonston strain) was prepared as described (5). CHO cells were maintained in Ham's F-12 medium supplemented with 10% (vol/vol) fetal calf serum and G418 (Geneticin; Life Technologies, Grand Island, NY) as indicated.

CD46–DAF Chimeras and CD46 Deletion Mutants. The CD46–GPI chimera has been described (12). Chimeras of DAF and CD46 were generated by using PCR to substitute various domains of CD46 into DAF in the plasmid vector Bluescript KS+ (Stratagene). The CD46 domain was amplified from the CD46 cDNA by using phosphorylated primers, and the DAF cDNA lacking the corresponding domain was

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Abbreviations: MV, measles virus; DAF, decay accelerating factor; SCR, short consensus repeat; mAb, monoclonal antibody; RCA, regulators of complement activation; STP, Ser-Thr-Pro; GPI, glycosyl-phosphatidylinositol; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; moi, multiplicity of infection; EBV-Epstein–Barr virus.

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amplified by inverse PCR from the DAF cDNA, followed by gel purification of the two products and ligation. The chimeras were verified by DNA sequence analysis and cloned into the expression vector PCRα, which consists of the vector pcDNA3 (Invitrogen) with the R-U5′ segment from human T-cell leukemia virus I inserted downstream from the promoter. Plasmids were introduced into CHO cells by LipofectAmine reagent (Life Technologies) followed by selection in G418 (0.2–0.5 mg/ml). Colonies were pooled and cell populations were subjected to multiple rounds of fluorescence-activated cell sorting (FACS) with mAbs corresponding to SCR regions in CD46 (see below) or with polyclonal rabbit anti-DAF antiserum (13). CD46 mutants with deletions of either SCR1 or SCR2 were modified from soluble CD46 mutants (14) by adding a transmembrane and cytoplasmic domain of CD46. These were introduced into the plasmid pSFFV.neo (15). Plasmids were transfected into CHO cells and G418-resistant colonies were pooled and screened by FACS as described above with rabbit anti-membrane cofactor protein (a gift of Cytomed, Cambridge, MA). Sorted cells were stained with mAbs to SCR1 (E4.3, gift of D. Purcell, Melbourne, and TRA-2-10, gift of P. Andrews, Philadelphia; refs. 14, 16, and 17), to SCR2 (M75, gift of T. Seya, Osaka; ref. 18), and to SCR3/4 (GB24, gift of B.-L. Hsi, Nice, France; ref. 19), followed by goat anti-mouse IgG antibody conjugated to fluorescein isothiocyanate (FITC), and quantitated by FACS. Receptor expression was again confirmed by FACS analysis at the time of MV infection.

MV Binding Assay. To prepare sucrose-gradient-purified MV stocks, Vero cells were infected at a multiplicity of infection (moi) of 0.3. After 48 h, cells and supernatants were pooled and cells were lysed by freezing/thawing. Cellular debris was cleared from the supernatants by centrifugation at 1500 × *g* for 10 min. MV was concentrated from the cleared supernatants by centrifugation at 100,000 × *g* for 1 h. Virus pellets were resuspended in 10 mM Tris·HCl, pH 8.0/1 mM EDTA (TE) and layered on a 20–60% discontinuous sucrose gradient. Gradients were centrifuged at 250,000 × *g* for 1 h. Virus bands were removed from the interface, layered on a continuous 20–60% sucrose gradient, and centrifuged at 200,000 × *g* for 3 h. Virus bands were collected and centrifuged once more at 125,000 × *g* for 30 min. Virus pellets were resuspended in TE and stored at −70°C. For binding assays, purified MV was added to CHO, CHO-BC1, and CHO-DAF cells at a moi of 1 for 30 min on ice. Cells were washed four times in washing buffer [ice-cold PBS containing 3% (wt/vol) bovine serum albumin and 0.1% sodium azide]. Cells were incubated with human anti-MV antiserum for 1 h on ice, followed by goat anti-human FITC-conjugated antibody. Cells were washed again and fixed in ice-cold 1% formaldehyde in PBS. MV binding was analyzed by FACS.

Infections and Immunofluorescence Assays. Receptor-expressing cell lines were plated on glass coverslips and infected with MV at a moi of 3. At 48 h after infection, cells were fixed and stained for expression of MV proteins by using an immunofluorescent assay as described (5). Individual cells were counted by using an Olympus fluorescence microscope at ×200. Alternatively, cells were infected at a moi of 3 in flasks. At 48 h, cells were resuspended in medium and stained with a human antiserum to MV followed by a FITC-coupled anti-human antibody. Cells were analyzed by FACS and MV-positive cells were quantitated. Infectious center assays were performed as reported (5).

Blocking of MV Infection with mAbs to CD46. The mAbs E4.3 (recognizing SCR1; refs. 16 and 17), TRA-2-10 (SCR1; refs. 14, 16, and 20), M75 (SCR2; ref. 18), and GB24 (SCR3/4; refs. 14 and 19) were used. In addition, ascites fluid containing a mAb against influenza hemagglutinin (Berkeley Antibody, Richmond, CA) was used as a control. Serial 1:5 dilutions of mAbs were incubated with CHO cells expressing the BC1 isoform of CD46. After 30 min, MV was added at a moi of 3 and incubated at 37°C for 1 h. Cells were plated on glass coverslips, allowed to incubate overnight, and then fixed and stained for immunofluorescence as described (5). Fluorescence-positive cells were quantitated by using an Olympus fluorescence microscope at ×200. At least three fields were quantitated per sample and are expressed as the mean ± SEM.

RESULTS

The Cytoplasmic Tail of CD46 Is Not Required for MV Receptor Function. CD46 and DAF are both members of the RCA family; among the members, they are structurally the most similar to one other. Both proteins have four SCRs followed by a STP domain, a region unique to these two proteins within the RCA family. DAF, however, is linked to the cell membrane via a GPI linkage rather than the peptide transmembrane domain found in CD46 (Fig. 1). To determine whether DAF could be used as a receptor for MV, DAF was compared to CD46 for its ability to bind MV. CHO cells expressing CD46 or DAF were compared to control CHO cells in a MV binding assay. Purified MV was added to cells in suspension at a moi of 1. Virus was bound to the cells for 30 min at 4°C, followed by extensive washing. Cells were incubated with a human anti-MV antiserum, followed by a goat anti-human antibody conjugated to FITC. MV binding was analyzed by FACS (Fig. 2). CD46 cells showed MV binding as an increase in fluorescence intensity compared to control cells.

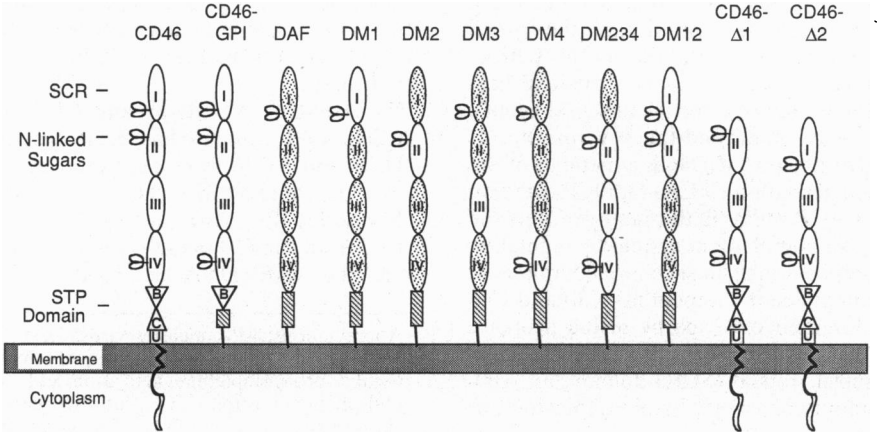

FIG. 1. Chimeras of CD46 and DAF. Chimeras were generated by substituting regions of DAF with the corresponding single or multiple SCRs of CD46. The BC1 isoform of CD46 is depicted. CD46 SCRs are open, and SCRs of DAF are stippled. Regions of N-linked glycosylation on the respective SCRs are indicated. The STP domain is a site for extensive O-linked glycosylation.

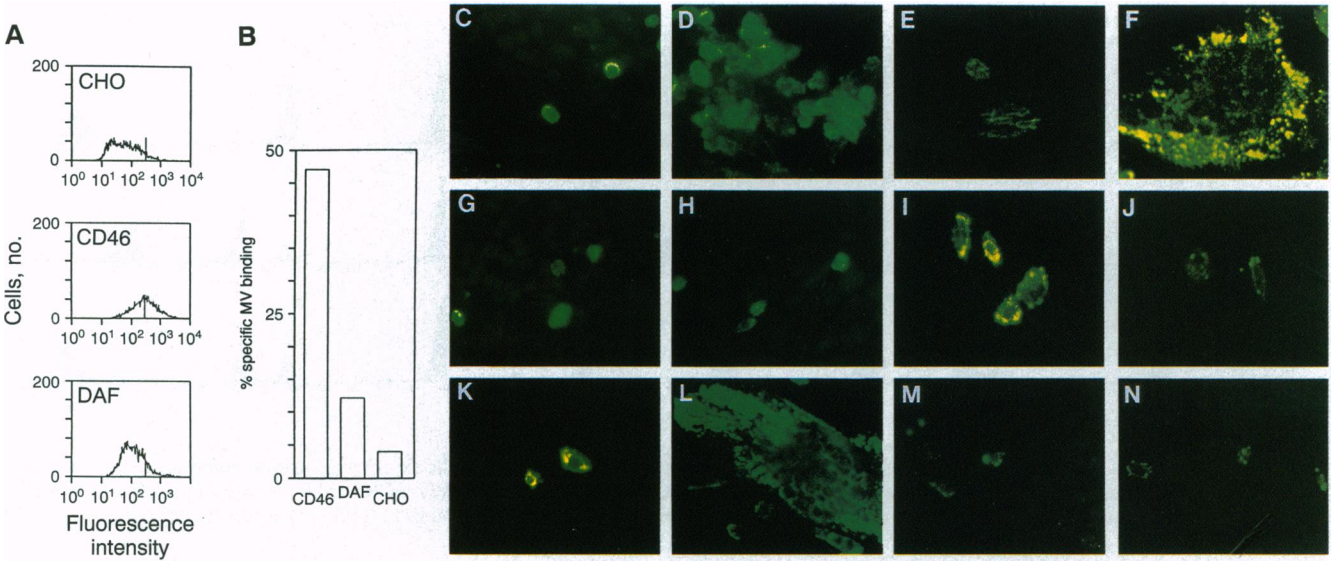

FIG. 2. (*A* and *B*) Binding of MV to cells expressing CD46 but not DAF. CHO cells expressing CD46 or DAF or control CHO cells were incubated with sucrose-gradient-purified MV. After washing, cells were stained with a human antiserum to MV and a FITC-conjugated goat anti-human antibody. Cells were analyzed by FACS (*A*) and binding was quantitated (*B*). (*A*) (*Top*) CHO cells. (*Middle*) CD46-expressing cells. (*Bottom*) DAF-expressing cells. (*B*) Percentage of total cells showing increased fluorescence intensity relative to the vertical marker in *A*. (*C–N*) Immunofluorescence staining of MV-infected cells expressing CD46–DAF chimeras. Cells were plated on glass coverslips and infected with MV. At 48 h, cells were fixed and stained with a human anti-MV antibody, followed by FITC-conjugated goat anti-human antibody. Cells were photographed by using an Olympus fluorescence microscope. (*C*) Control CHO cells. (*D*) CHO cells expressing CD46-BC1 isoform. (*E*) CHO cells expressing DAF. (*F*) CHO cells expressing CD46-GPI. (*G–L*) CHO cells expressing chimeras DM1 (*G*), DM2 (*H*), DM3 (*I*), DM4 (*J*), DM234 (*K*), and DM12 (*L*) and CD46 SCR deletion mutants CD46Δ1 (*M*) and CD46Δ2 (*N*). (×200.)

MV binding to DAF-expressing cells was not significantly higher than control cells (Fig. 2).

To further define the regions of CD46 that are responsible for recognition by MV, chimeras between CD46 and DAF were studied (12). First, CHO cells expressing CD46 (BC1 isoform), DAF, or a GPI-linked form of CD46 (CD46–GPI) (Fig. 1) were infected with MV and stained for the presence of MV antigens (Fig. 2). Expression of receptor proteins on the cell surface at similar levels to control CD46-expressing cells was confirmed by FACS analysis using mAbs against CD46 (17) or a polyclonal antibody against DAF (data not shown; ref. 13). Cells expressing DAF and the negative control CHO cells showed similar numbers of fluorescence-positive cells (Fig. 2 *C* and *E*). Cells expressing a chimera of CD46 and DAF, whereby the four SCRs of CD46 were coupled to the STP domain and GPI linkage of DAF, were infectable with MV (Figs. 1 and 2*F*). Infected cells showed the characteristic MV-associated syncytium formation and similar numbers of fluorescence-positive cells compared to positive-control BC1 cells. These data indicate that the cytoplasmic domain of CD46 is not required for receptor function, MV infection, or membrane fusion as shown by syncytium formation. In addition, the STP region of CD46 is not specifically required as part of the STP region of DAF may be substituted in a functional MV receptor.

Infection of Cells Expressing Combinations of SCRs from CD46 and DAF. Since MV receptor function mapped to the SCRs of CD46, additional chimeras between CD46 and DAF were constructed that consisted of substitutions of SCR domains from CD46 on a DAF background (Fig. 1). All of the chimeras and CD46 deletion mutants tested were stained with mAbs against each of the SCR domains to confirm that they both expressed the proper SCR of CD46 and that expression was at similar levels (Fig. 3). None of the anti-CD46 mAbs showed significant binding to DAF (Fig. 2*B*, compare DAF to CHO cells). Individual substitution of each of the CD46 SCR domains on the DAF backbone did not allow MV infection (chimeras DM1–DM4; Fig. 2 *G–J*). Similarly, a receptor consisting of SCR1 of DAF and SCR2 to SCR4 of CD46 (DM234; Fig. 2*K*) did not allow MV infection. Only a chimera consisting of SCR1 and SCR2 of CD46 and SCR3 and SCR4 of DAF (DM12; Fig. 2*L*) showed fluorescence-positive cells and MV-associated syncytium formation. CD46 deletion mutants that had either SCR1 or SCR2 deleted were not infectable with MV compared to controls (CD46Δ1 and CD46Δ2; Fig. 2 *M* and *N*). Similar results were seen when infected cells were analyzed for surface expression of MV antigens by FACS (data not shown). Thus, these results indicate that both SCR domains 1 and 2 are recognized by MV and are required for receptor function.

Cells Expressing CD46 SCR1 and SCR2 Substituted in DAF Are Productively Infected with MV. DM12 chimeras were compared to singly SCR-substituted receptors DM1 and DM2 in an infectious center assay to determine whether the cells were productively infected. CHO cells expressing DM1, DM2, DM12, or wild-type CD46 and CHO and Vero cells were infected with MV at a moi of 1. At 48 h, cells were resuspended and serial 1:10 dilutions were plated on MV-susceptible Vero cell monolayers. After 6 days, infectious centers were counted (Table 1). Cells expressing either wild-type CD46 or the DM12 chimera gave rise to comparable numbers of infectious centers, while cells expressing either SCR1 or SCR2 alone produced at least 10-fold fewer infectious centers, similar to control CHO cells. Control CHO cells have been previously shown to be infectable by MV and to produce infectious centers at 0.5–5% (5). These data confirm that both SCR1 and SCR2 are required for productive infection with MV.

mAbs Against SCR1 or SCR2 Block MV Infection. To further map the regions of CD46 that are involved in MV infection, antibodies that recognize different SCRs of CD46 were used to block MV infection. mAbs against SCR1 (E4.3 and TRA-2-10; refs. 14, 16, 17, and 20), SCR2 (M75; R.K. and D.M.L., unpublished data, and ref. 18), or SCR3/4 (GB24; refs. 14, 16, and 19) were tested. Serial dilutions of antibodies were incubated with CD46-BC1 cells for 30 min after which MV was added at a moi of 1. After 24 h, MV-positive cells expressing MV proteins were quantitated (Fig. 4). Specific anti-CD46 binding activity in the ascites fluid samples was

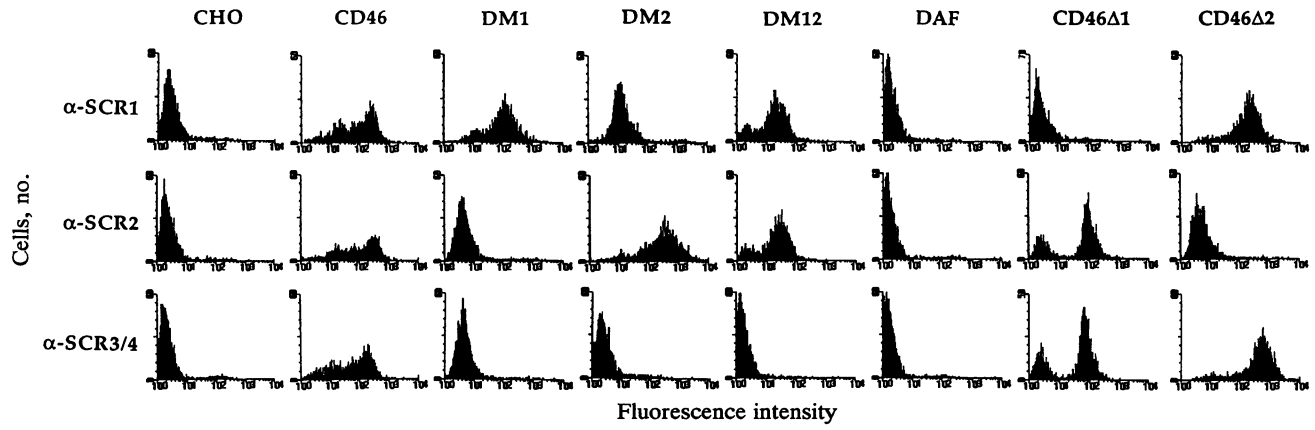

FIG. 3. Expression of CD46–DAF chimeras detected by mAbs against SCRs from CD46. Cells expressing various chimeras were resuspended in minimum essential medium. Cells were stained with mAbs against SCR1 (TRA-2-10; α-SCR1), SCR2 (M75; α-SCR2), and SCR3/4 (GB24; α-SCR3/4). Cells were analyzed by FACS. All chimeras listed in Fig. 1 were tested by this assay and were found to express the proper SCR of CD46 and at similar levels.

quantitated for each antibody; E4.3 was found to be 1000-fold more reactive than GB24, whereas TRA-2-10 was 45-fold more reactive than GB24. After correcting for these differences in concentration, antibodies against SCR1 or SCR2 were still 500-fold (TRA-2-10) to 10,000-fold (E4.3) more effective in blocking MV infection than was the GB24 antibody against SCR3/4. A control antibody raised against a noncrossreacting viral protein, influenza hemagglutinin, showed nonspecific MV-blocking activity only at high concentrations, similar to the SCR3/4 antibody (data not shown). Thus, by this assay both SCR1 and SCR2 are also shown to be involved in MV recognition.

DISCUSSION

CD46 deletion mutants and chimeras between CD46 and the related complement regulatory protein DAF were used to determine the regions of CD46 that are important for MV receptor function. The results are summarized in Table 2. Data from the mutant receptors and from mAb blocking experiments indicate that both of the two outermost SCRs of CD46 (SCR1 and SCR2) must be present on the cell surface for MV to infect cells at levels comparable to levels in cells expressing wild-type CD46. CHO cells expressing SCR1 and SCR2 of CD46 on a DAF backbone were infected with MV, while cells expressing other combinations of CD46 and DAF SCRs were not infectable. Similarly, CD46 proteins with deletions of either SCR1 or SCR2 were not functional MV receptors. Further, neither the cytoplasmic tail nor the STP region of CD46 was required for MV receptor function. These observations confirm and extend our earlier studies showing that different isoforms of CD46, which have the same composition of SCR but differ in their STP regions and cytoplasmic tail domains, are equally functional receptors for MV (5).

The SCR is the hallmark structural motif in the RCA family of proteins. The SCR is conserved among all the members of the family, with at least 30% amino acid similarity; four cysteines in the module are completely conserved and form two disulfide bonds, the first and third forming one disulfide and the second and fourth forming the other (10, 11). The solution structure of an SCR from factor H shows that the module is composed of a hydrophobic core wrapped in β-sheet, with a number of exposed variable loops (11, 21). It appears that, although multiple SCRs are found in all of the RCA proteins, only a small subset of modules are actually involved in ligand binding. For example, in the complement receptor CR2, the complement binding regions consist of 2 SCRs out of 16,

Table 1. Infectious centers on Vero cell monolayers

Cell line/receptor	No. infectious centers formed/no. cells plated				
	10^6	10^5	10^4	10^3	10^2
Vero	TM	TM	TM	TM	36
CHO-CD46 BC1	TM	TM	TM	9	0
CHO-DM12	TM	TM	TM	9	0
CHO-DM1	TM	TM	12	0	0
CHO-DM2	TM	TM	5	0	0
CHO-DAF	TM	TM	11	0	0
CHO	TM	TM	5	0	0

Infectious center assays were performed by infecting CHO cells expressing CD46 receptors and DAF chimeras with MV. After 48 h, cells were washed and serial 1:10 dilutions of infected cells (10^2 to 10^6 cells) were plated onto susceptible Vero cell monolayers. After 6 days, infectious centers were counted. TM, too many to count (>100 plaque-forming units per sample).

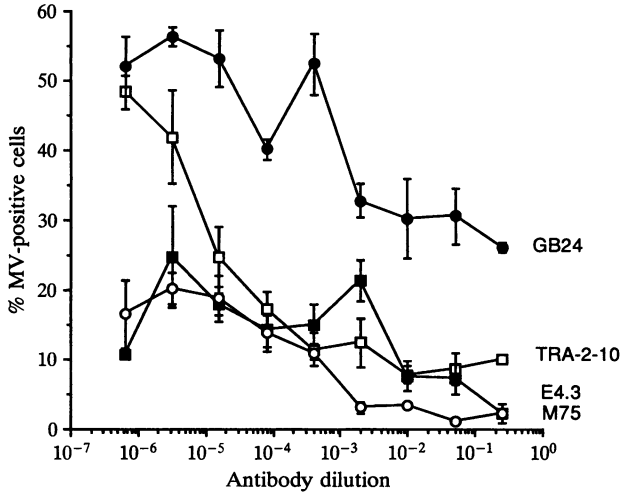

FIG. 4. mAb blocking of MV infection. mAbs E4.3 and TRA-2-10 (both recognizing SCR1; solid and open squares, respectively), M75 (recognizing SCR2; open circles), and GB24 (recognizing SCR3/4; solid circles) were used. Concentration of specific anti-CD46 binding activity in ascites fluid was measured with a quantitative ELISA assay (14). Serial 1:5 dilutions of ascites fluid were incubated with CHO cells expressing the BC1 isoform of CD46 and then infected with MV at a moi of 1. After 24 h, cells were stained for immunofluorescence with an antiserum to MV. MV-positive cells were counted; three fields were quantitated and are expressed as the mean ± SEM.

Table 2. Summary of infection by MV

Cell line	Infection by MV
CHO	−
CD46-BC1	+
DAF	−
CD46-GPI	+
DM1	−
DM2	−
DM3	−
DM4	−
DM234	−
DM12	+
CD46-Δ1	−
CD46-Δ2	−

whereas in CR1 only 12 of the 30 SCRs can bind complement ligands (22, 23). In CD46, the C3b and C4b binding and cofactor activity is found primarily in SCR3 and SCR4, with cofactor and some binding activity being contributed by SCR2 (14). Thus it is thought that the SCR modules form a framework with certain units playing primarily structural or communicative roles and others having specific ligand-recognition functions (11). SCR1, SCR2, and SCR4 of CD46 also contain sites for N-glycosylation (ref. 8 and Fig. 2), and Maisner *et al.* (24) has shown that both N-glycans and disulfide bonds are important for the MV receptor function of CD46. The presence of N-glycans attached to SCR1 and SCR2 may influence MV binding by affecting the conformation of the SCR or by forming part of the receptor determinant.

Interestingly, mAbs that bind SCR1 and block MV infection (Fig. 4) do not impair the complement regulatory functions of CD46 (14). Similarly, CD46 proteins deleted in SCR1 retain C3b and C4b binding activity in culture (14) although they are not functional MV receptors (Figs. 1 and 2). Since the MV and C3/C4 binding regions of CD46 appear to be discrete, it seems reasonable that deletion of the MV binding regions of the molecule would confer a selective advantage by avoiding MV infection without compromising protective complement binding and cofactor activities. Our data indicate that reagents that specifically block the MV–receptor interaction might be designed so as not to perturb the complement regulatory functions of CD46. This should reduce the likelihood of generating side effects from anti-MV drugs designed to target the MV–CD46 interaction.

Most MV infections currently occur in the time window between waning of maternal antibodies and the age at which the individual is vaccinated. Therapeutic strategies for controlling MV infection during this period might reduce the significant morbidity and mortality associated with measles. The related complement regulatory protein CR2 (CD21) is the receptor for Epstein–Barr virus (EBV) (25). Interestingly, EBV also binds to the two outermost SCRs of CR2. In this case, C3d binding and EBV binding regions overlap. Soluble SCR1 and SCR2 of CR2 or peptides corresponding to portions of these SCR have shown promise for blocking EBV replication *in vitro* and *in vivo* (26). Peptides that block EBV infection correspond to an exposed loop region of the SCR model (11, 21, 26). Similarly, by comparing the primary sequences between DAF and CD46 and the consensus model of the SCR structure, it should be possible to identify peptides that are unique to CD46 and that could block the MV–CD46 interaction.

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