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The N-Glycan of the SCR 2 Region Is Essential for Membrane Cofactor Protein (CD46) To Function as a Measles Virus Receptor

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Membrane cofactor protein (MCP) (CD46), a complement-regulatory protein, serves as a cellular receptor for measles virus. Its amino-terminal portion is composed of four short consensus repeats (SCR), three of which (SCR1, SCR2, and SCR4) carry an N-linked oligosaccharide. In order to determine the importance of the three N-glycans for the function of MCP as a measles virus receptor, we established Chinese hamster ovary (CHO) cell lines that stably express mutant MCPs lacking one of the three motifs for N glycosylation (NQ1, NQ2, and NQ4). In an additional mutant (NQ1-2), two glycosylation motifs were altered, allowing the addition of an N-linked oligosaccharide only in SCR4. The abilities of the mutant MCPs to function as measles virus receptors were analyzed with three different assays: (i) binding of measles virus hemagglutinin to MCP immobilized on nitrocellulose; (ii) binding of measles virus to CHO cells expressing wild-type or mutant MCP; and (iii) infection of the transfected CHO cells by measles virus. In all three assays, the abilities of the NQ2 and NQ1-2 mutants to serve as measles virus receptors were drastically impaired. The NQ1 and NQ4 mutants were recognized by measles virus almost as efficiently as the wild-type protein. These results indicate that the N-glycan attached to SCR2 is essential for MCP to serve as a measles virus receptor, while the oligosaccharides attached to SCR1 and SCR4 are of only minor importance.

Measles virus (MV) is a highly contagious agent causing an acute respiratory childhood disease. A transient cellular immunosuppression, which increases the risk of opportunistic secondary infections, is responsible for the high infant mortality rates associated with MV infections in developing countries. In rare instances, persistent infection can lead to lethal neurological disorders, subacute sclerosing panencephalitis, or measles inclusion body encephalitis. MV is a negative-strand single-stranded RNA virus belonging to the genus *Morbillivirus* within the family *Paramyxoviridae*. It is an enveloped virus containing two membrane glycoproteins, the hemagglutinin (H) and the fusion protein (F). Infection of cells is initiated by attachment of the H protein to the host cell via a specific cellular receptor and a subsequent fusion event mediated by the F protein. Conclusive evidence that membrane cofactor protein (MCP) (CD46) is the cellular counterpart of H in the binding of MV to the cell surface has been presented (4, 17). CD46 is expressed on all nucleated human cells, being a suitable receptor for a virus that infects a wide variety of cell types in primate hosts, including lymphocytes, monocytes, macrophages, and glia cells as well as epithelial and endothelial cells of lung, skin, kidney, liver, placenta, and prostate tissues (18). CD46 is a type I membrane glycoprotein and functions normally as a cofactor for the plasma serine protease factor I (11). CD46 binds complement factors C3b and C4b, promoting their proteolytic degradation, and thus protects the cell from complement-mediated damage. CD46 consists primarily of an extracellular portion of four short consensus repeats (SCRs), a combination of serine-, threonine-, and proline-rich (STP-rich) regions and either of two distinct cytoplasmic tails. By alternative splicing multiple CD46 isoforms that differ in both amino acid sequence (in the STP region and the cytoplasmic tail) and O glycosylation arise. It has been shown that different isoforms can serve as MV receptors (6, 14, 15). Binding of MV to CD46 was shown to be independent of the O-glycans which are linked to the STP region of MCP. In contrast, enzymatic release of the three N-glycans attached to SCRs 1, 2, and 4 abolished MV binding (14). Both CD46 with mannose-rich and CD46 with complex N-glycans were recognized by H protein in a binding assay (13). Cells expressing CD46 with different types of N-glycans could be infected by MV (13). These findings indicate that MV binds to a receptor determinant on CD46 which requires the presence of N-glycans. In order to study the importance of each of the three N-linked oligosaccharides, the glycosylation motifs in SCRs 1, 2, and 4 of CD46 were altered by site-directed mutagenesis. The resulting CD46 mutants were stably expressed in CHO cells and analyzed for their ability to function as MV receptors. CD46 lacking the N-glycan in SCR 2 showed strongly reduced MV binding and could mediate only a weak MV infection. In contrast, N-linked oligosaccharides attached to SCRs 1 and 4 were found to be of minor importance for the function of CD46 as an MV receptor.

MATERIALS AND METHODS

Cell lines. Chinese hamster ovary (CHO) cells (American Type Culture Collection) were maintained in Ham's F-12 medium supplemented with 2 mM glutamine and 5% fetal calf serum (FCS). Geneticin (Sigma) at a concentration of 0.5 mg/ml was added to the medium to select for neomycin resistance.

Construction of cDNA mutants and expression in CHO cells. Four modified constructs of the BC1 isoform of CD46 were used in this study (Fig. 1). The N-glycosylation site in SCR 1 (NHT) was mutated to QHT for the NQ1 mutant, the site in SCR 2 (NGT) was changed to QGT for the NQ2 mutant, and both sites were modified for the NQ1-2 construct. The NQ4 mutant was obtained by mutating the N-glycosylation site in SCR 4 (NST) to QST. Mutagenesis was

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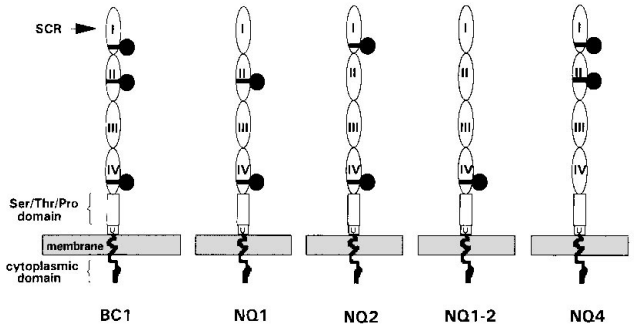

FIG. 1. Schematic drawing of CD46 N-glycosylation mutants. The NH_2 terminus of the CD46 isoform BC1 is composed of four SCRs. Three N-linked oligosaccharides are linked to SCRs 1, 2, and 4. The mutants differ only in the number of N-glycans. By site-directed mutagenesis the glycosylation motifs were altered, creating CD46 molecules lacking the N-glycan in SCR1 (NQ1), in SCR2 (NQ2), in SCR4 (NQ4). The mutant NQ1-2 has retained only the oligosaccharide in SCR4.

performed by using PCR methodology as described by Liszewski et al. (12). The cDNAs for wild-type BC1 and mutant CD46 were cloned into the *Eco*RI site of the expression plasmid pCR3 (Invitrogen).

CHO cells were transfected by using the Lipofectin reagent (Life Technologies) and selected for neomycin resistance. Cell sorting was performed on a FACStar cell sorter (Becton Dickinson). Stable cell lines containing 25 to 92% CD46-positive cells, as determined by fluorescence-activated cell sorter (FACS) analysis, were established.

Surface biotinylation and isolation of CD46. Cell surface proteins were labeled with sulfosuccinimidobiotin (S-NHS-biotin; Pierce). Immunoprecipitation of CD46 from unlabeled or surface-biotinylated cells was carried out with a monoclonal antibody directed against CD46 (J4/48; Dianova) as described previously (14). Immunoprecipitated CD46 was separated on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel under nonreducing conditions and blotted to nitrocellulose.

Detection of CD46 on nitrocellulose. The nitrocellulose was blocked in a solution of 10% nonfat dry milk in phosphate-buffered saline (PBS) for 45 min at 4°C. To detect surface-biotinylated CD46, the blot was incubated with streptavidin-biotinylated horseradish peroxidase (streptavidin/POX; Amersham) for 45 min at 4°C at a dilution of 1:500 in PBS. Bound peroxidase was visualized by the enhanced chemiluminescence system (ECL; Amersham).

In vitro binding assay. The glycoprotein overlay protein binding assay was performed as described previously (14). Briefly, nonlabeled immunoprecipitated CD46 immobilized on nitrocellulose was blocked and incubated with biotin-labeled MV H protein. The bound virus glycoprotein was detected with streptavidin/POX and ECL.

Flow cytometry. For detection of cell surface CD46, 3×10^5 cells were detached from culture dishes at 80% confluency by the addition of 2 ml of PBS containing 5 mM EDTA. After three washes in PBS containing 1% FCS, cells were resuspended in 100 μl of the same buffer containing 2 μg of the anti-CD46 monoclonal antibody (MAb) J4/48. Following an incubation for 1 h on ice, cells were washed twice in PBS–1% FCS and resuspended in 100 μl PBS-1% FCS containing 2.7 μg of fluorescein isothiocyanate-labeled rabbit anti-mouse immunoglobulin (Dako). After 30 min on ice and three washes, cells were fixed in 0.5% paraformaldehyde and flow-cytometric analyses were carried out on a FACScan (Becton Dickinson). For the detection of cell surface-bound MV, a polyclonal rabbit antiserum raised against MV glycoproteins was used as first antibody. Fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins (Dako) were applied as second antibody.

Virus binding assay. To compare MV binding to the CHO cell lines expressing different N-glycosylation mutants, the expressing cells were mixed with nonexpressing CHO cells in order to obtain a mixture of cells containing 25% CD46-positive cells. Cells were detached by PBS-EDTA, and the amount of CD46-expressing cells was determined by flow cytometry. Four hundred thousand cells were resuspended in 50 μl of Ham's F-12 containing 5% FCS and incubated for 2 h on ice with 0.2 μg of the partially purified Edmonston strain of MV (14). Further washes and antibody incubation steps were carried out in the presence of 0.1% NaN_3. Flow-cytometric analyses were performed as described above.

Determination of virus infectivity. As for the virus binding assay, CHO cell mixtures containing 25% CD46-expressing cells were used to determine virus infectivity. Cells at 80% confluency were incubated for 4 h at 37°C with MV at a multiplicity of infection of 5 (Edmonston strain). After three washes with PBS, cells were cultured in complete growth medium at 37°C. Two days postinfection no syncytium formation was observed because of the low percentage of CD46-expressing cells (25%). The MV-infected cell monolayers were surface biotinylated and lysed in RIPA buffer as described by Maisner et al. (14). Immunoprecipitation was carried out with MAb K83 directed to MV H protein (10). After an incubation for 2 h on ice, the mouse immunoglobulins were precipitated by addition of 50 μl of protein A-Sepharose (Sigma) preincubated with rabbit anti-mouse immunoglobulins. After three washes and boiling in sample buffer containing 5% mercaptoethanol, the precipitates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were blotted to nitrocellulose and biotinylated H protein was detected with streptavidin/POX and ECL as described for biotin-labeled CD46.

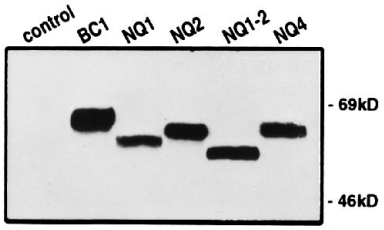

FIG. 2. Surface expression of wild-type and mutant CD46 in CHO cells. Nontransfected CHO cells (control) and CHO cells stably expressing wild-type CD46 (isoform BC1) or an N-glycosylation mutant of CD46 (NQ1, NQ2, NQ1-2, or NQ4) were surface biotinylated. After lysis of cells, immunoprecipitation was performed with J4/48. The precipitates were separated by SDS-PAGE under nonreducing conditions and blotted to nitrocellulose. Biotinylated proteins were detected by streptavidin/POX.

RESULTS

Cell surface expression of glycosylation mutants of CD46. To analyze the importance of the individual N-linked oligosaccharides for the receptor function of CD46, mutants that lacked one or two oligosaccharides were generated (Fig. 1). The motif for N-glycosylation (Asn-X-[Thr/Ser]) was altered by site-directed mutagenesis to prevent glycosylation in SCR1 (mutant NQ1), SCR2 (mutant NQ2), or SCR4 (mutant NQ4). Two glycosylation sites were altered in the NQ1-2 mutant, leaving only the glycosylation motif in SCR4 intact. To analyze the electrophoretic mobility of the wild-type and mutant CD46 proteins, CHO cells stably expressing wild-type CD46 (isoform BC1) or either of the N-glycosylation mutants NQ1, NQ2, NQ1-2, and NQ4 were surface biotinylated. CD46 was immunoprecipitated from cell lysates, separated by SDS-PAGE under nonreducing conditions, and blotted to nitrocellulose. On the blot stained with streptavidin/POX a protein band was detectable in all transfected cell lines but not in control CHO cells (Fig. 2). CD46 isoform BC1 has an estimated molecular mass of 67 kDa; NQ2 and NQ4 have faster electrophoretic mobilities (about 62 kDa), as expected for a CD46 molecule lacking one N-glycan. Though NQ1 also lacked only one oligosaccharide, it was found to have a faster electrophoretic mobility than NQ2 and NQ4. This difference was found only after analysis of the proteins under nonreducing conditions. Upon SDS-PAGE under reducing conditions, NQ1 had the same migration behavior as NQ2 and NQ4 (not shown). The differential electrophoretic mobility of the mutant under nonreducing conditions is probably a conformational effect. The absence of an oligosaccharide in SCR1 appears to have a different effect on the conformation of CD46 than the lack of the SCR2 or SCR4 oligosaccharide. NQ1-2 lacking two of the three N-glycans had the fastest electrophoretic mobility of all mutants, with an estimated molecular mass of about 55 kDa.

In vitro binding of MV H protein to the N-glycosylation mutants of CD46. In order to study the in vitro binding of isolated MV H protein to CD46 molecules immobilized on nitrocellulose, nonbiotinylated CHO cells expressing wild-type or mutant CD46 were used for immunoprecipitation. SDS-PAGE was performed under nonreducing conditions, because we have shown previously that reduced CD46 is not recognized

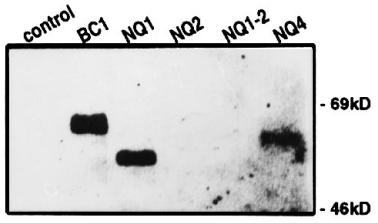

FIG. 3. Binding of biotinylated MV H protein to wild-type and mutant CD46. Lysates of nontransfected CHO cells (control) and CHO cells stably expressing CD46 (isoform BC1) or an N-glycosylation mutant of CD46 (NQ1, NQ2, NQ1-2, or NQ4) were immunoprecipitated with J4/48. The immunoprecipitates were analyzed by SDS-PAGE and blotted to nitrocellulose. The binding of biotinylated MV H protein was detected with streptavidin/POX.

by MV (14). After immobilization of the proteins on nitrocellulose, biotinylated MV H protein was used for a glycoprotein overlay protein binding assay. Bound viral glycoprotein was detected by streptavidin/POX. As shown in Fig. 3, only BC1, NQ1, and NQ4 were recognized by MV H protein. In contrast, NQ2 and NQ1-2 were not detectable in the overlay binding assay, even after longer exposure times (not shown). This finding indicates that the in vitro binding of MV to its receptor is dependent on the presence of the oligosaccharide in the SCR 2 region of CD46.

Binding of MV to CHO cells expressing N-glycosylation mutants of CD46. To analyze the importance of the three N-glycans of CD46 for the binding of MV to intact cells, an MV binding assay was performed by using flow cytometry. Cell lines containing 25% CD46-expressing CHO cells either in the wild-type form (BC1) or in a mutant form (NQ1, NQ2, NQ1-2, or NQ4) were incubated with virus, and the amount of virus-positive cells was determined by using a rabbit antiserum raised against MV glycoproteins (Fig. 4). Binding of MV to cells expressing BC1 was very efficient (20%). With cells carrying NQ1 and NQ4 the number of virus-positive cells was somewhat smaller (9 to 11%). Cells expressing NQ2 and NQ1-2 showed only background binding (<1.5%), similarly to nontransfected CHO cells. This result is in agreement with the

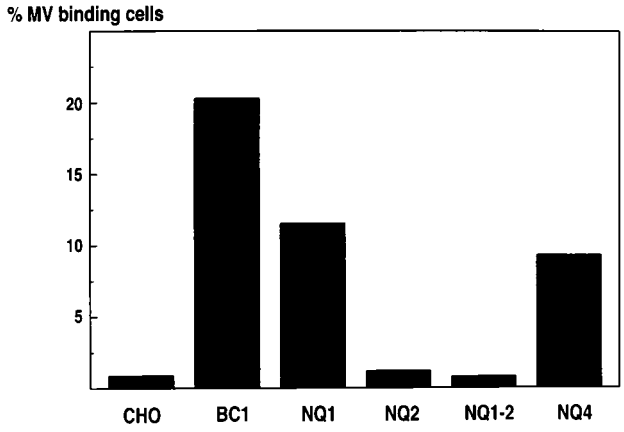

FIG. 4. MV binding to CD46-expressing CHO cells. CHO cells stably expressing wild-type CD46 (isoform BC1) or an N-glycosylation mutant of CD46 (NQ1, NQ2, NQ12, or NQ4) were mixed with nontransfected cells to get a ratio of 25% CD46-expressing cells. Nontransfected (CHO) and CD46-expressing cells were incubated with MV. Surface-bound virus was detected with a polyclonal anti-MV H rabbit antiserum and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins. By flow-cytometric analysis the percentage of MV-positive cells was determined. This experiment was performed three times with almost identical results.

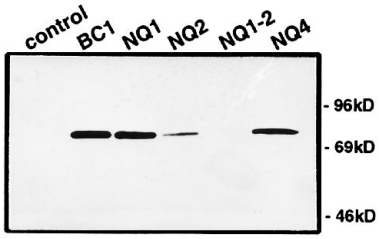

FIG. 5. Detection of MV H protein in MV-infected CD46-expressing CHO cells and control cells. CHO cells stably expressing the wild type (isoform BC1) or an N-glycosylation mutant of CD46 (NQ1, NQ2, NQ1-2, or NQ4) were mixed with nontransfected cells to get a ratio of 25% CD46-expressing cells. Nontransfected (CHO) and CD46-expressing cells were infected with MV at a multiplicity of infection of 5. Two days postinfection, infected cells were labeled by surface biotinylation and lysed. Immunoprecipitation was carried out with MAb K83, and samples were separated by SDS-PAGE under reducing conditions. After blotting to nitrocellulose, biotinylated MV H protein was detected with streptavidin/POX.

in vitro binding assay (Fig. 3) and confirms that the binding of MV to intact CD46 depends on the presence of the N-glycan in SCR 2.

MV infection of CHO cells expressing CD46 N-glycosylation mutants. The importance of the N-glycans of CD46 was further analyzed by an MV infection assay. Nontransfected CHO cells and cell mixtures containing 25% CD46-expressing cells (BC1, NQ1, NQ2, NQ1-2, or NQ4) were infected for 48 h with MV at a multiplicity of infection of 5. During this time no relevant virus release and reinfection of cells occurs, and because of the low percentage of receptor-positive cells no fusion of cells was observed. Therefore, the amount of newly synthesized viral proteins is proportional to the number of infected cells. As almost no virus was released during 48 h (determined by plaque assay of the cell supernatant), surface-expressed MV H protein was used to quantify virus replication. After surface biotinylation of the infected cells, MV H protein was immunoprecipitated, analyzed by SDS-PAGE, and blotted to nitrocellulose. The blot shown in Fig. 5 was stained with streptavidin/POX. Almost equal amounts of viral H protein were detected in CHO cells expressing wild-type CD46 (BC1), NQ1, or NQ4. In NQ2-expressing cells the amount of MV H protein was reduced by 80% (as determined from densitometric scanning). In nontransfected and NQ1-2-expressing CHO cells no viral H protein was visible. Only after extension of the exposure time from 10 to 50 s was faint staining observed (data not shown). This result confirms that the oligosaccharide in SCR2 is important for the function of CD46 as an MV receptor.

DISCUSSION

Having shown recently that N-glycans are important for the binding of MV to CD46 (14), we present evidence here that it is mainly the oligosaccharide in SCR2 that is required for the receptor function of CD46. Three different assays (binding of MV H protein to immobilized CD46, binding of MV to cells, and infection of cells by MV) indicated that MV binds efficiently not only to wild-type CD46 but also to the mutants NQ1 and NQ4. Recognition of NQ2, however, was drastically reduced or abolished. In the FACS analysis of virus binding to cells, the absence of the oligosaccharide in SCR1 or SCR4, respectively, appeared also to have a negative effect on virus binding. However, this decrease was minor compared with the reduction observed with the NQ2 mutant. The somewhat reduced MV binding to cells expressing NQ1 or NQ4 may at least in part be due to lower expression of CD46 in these cells.

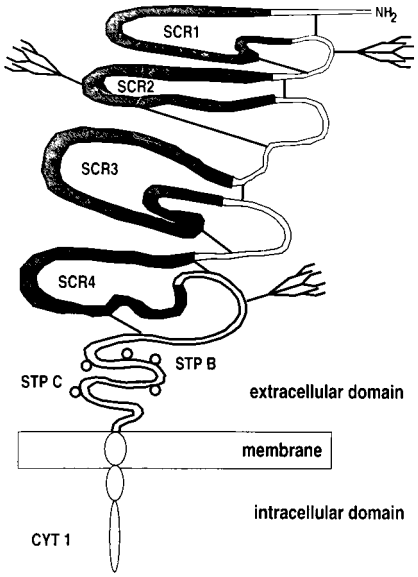

FIG. 6. Orientation of the N-glycans of CD46 isoform BC1 (according to reference 5). The proposed positions of the three N-linked carbohydrate side chains (tree-like structures) in SCRs 1, 2, and 4 are shown. The lines between the chains indicate disulfide bonds. Circles represent O-glycosylation sites in the STP regions.

The different assays were found to differ in the power of resolution. As indicated above, the FACS analysis resolved differences in the optimal and suboptimal ranges of virus binding. The virus infection assay indicated that the low level of receptor activity of the NQ2 mutant is further decreased when the oligosaccharide in SCR1 is also deleted.

Studies with glycosylation inhibitors have indicated that for virus binding it is not important whether the N-glycans are present as complex or mannose-rich oligosaccharides (13). This finding argues against a direct involvement of carbohydrates in the recognition of CD46 by MV. The oligosaccharides appear to be important for maintaining a conformation of CD46 that can be recognized by MV. Similarly, the disulfide bonds have been shown to be essential for preserving the recognition site on CD46 for MV (14). The domain of CD46 to which MV binds has been assigned to SCR1 and SCR2 (8, 16). The amino acid residues of MCP that interact with MV are not known but they appear to form a conformational site that requires disulfide bonds and the presence of the oligosaccharide in SCR2. It should be noted that, though both SCR1 and SCR2 contribute to the recognition site of CD46 for MV, only the oligosaccharide attached to SCR2 is of major importance for virus binding. The N-glycan attached to SCR1 had a minor effect on the binding of MV to CD46. This finding may be surprising, because the increased electrophoretic mobility of NQ1 compared with NQ2 and NQ4 suggested that the lack of the oligosaccharide in SCR1 had a more pronounced effect on the conformation of CD46 than the lack of N-glycans in SCR2 and SCR4, respectively. The oligosaccharides of SCR1 and SCR2 appear to affect different conformational sites of CD46, as illustrated in Fig. 6 (according to reference 4). Recent studies have shown that the endoplasmic reticulum contains a unique machinery for the folding and quality control of glycoproteins (2). N-linked glycans are important in this process for two reasons: (i) they increase the overall solubility of folding intermediates and lower the risk of aggregation (9), and (ii) they mediate the interaction with lectin-like chaperones, such as calnexin (7). In this respect each of the three N-linked oligosaccharides of MCP may contribute to the final conformation of this protein. The oligosaccharide of SCR2 is important for the MV binding site. The oligosaccharides of SCR1 and SCR4 affect the conformation of other parts of the molecule, as indicated by the differential electrophoretic mobility. It will be interesting in future studies to find out how the N-linked oligosaccharides affect the interaction of MCP with complement factors.

Another complement-regulatory protein, decay-accelerating factor (DAF), is the closest known relative of CD46. It also consists of four SCR domains and an STP region. However, DAF differs from MCP in the membrane anchor. DAF is inserted into the cell membrane via a glycosylphosphatidylinositol anchor, rather than by a transmembrane anchor as in the case of MCP. Recently, DAF has been shown to serve as receptor for several Echo viruses (1, 19). The interaction of these viruses with DAF is different from the interaction of MV with CD46. While MV requires SCR1 and SCR2 for binding to CD46, SCRs 2, 3, and 4 are indispensable for Echo viruses to recognize DAF as a receptor. Another difference is the dependence of virus binding on N-glycans. DAF contains only a single N-linked oligosaccharide that is located close to SCR1. Deletion of SCR1 together with the glycosylation site did not abrogate virus binding (3). Therefore, the single N-glycan of DAF is not required for the function of DAF as virus receptor.

In order to more completely understand how the oligosaccharide in SCR2 affects the conformation of CD46 and consequently the recognition site for MV, one has to await the elucidation of the three-dimensional structure of this complement-regulatory protein.

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