Mapping of the Primary Binding Site of Measles Virus to Its Receptor CD46*

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The measles virus (MV) hemagglutinin binds to the complement control protein (CCP) CD46 primarily through the two external modules, CCP-I and -II. To define the residues involved in binding, 40 amino acids predicted to be solvent-exposed on the CCP-I-II module surface were changed to either alanine or serine. Altered proteins were expressed on the cell surface, and their abilities to bind purified MV particles, a soluble form of hemagglutinin (sH) and nine CD46-specific antibodies competing to different levels with sH attachment, were measured. All proteins retained, at least in part, MV and sH binding, but some completely lost binding to certain antibodies. Amino acids essential for binding of antibodies weakly or moderately competing with sH attachment are situated in the membrane-distal tip of CCP-I, whereas residues involved in binding of strongly sH competing antibodies cluster in the center of CCP-I (Arg-25, Asp-27) or in CCP-II (Arg-69, Asp-70). Both clusters face the same side of CCP-I-II and map close to amino acid exchanges impairing sH binding (E11A, R29A, P39A, and D70A) or MV binding (D70A and E84A) and to a six-amino acid loop, previously shown to be necessary for sH binding.

Although numerous viral receptors and coreceptors have been identified, virus binding sites have been defined on the three-dimensional structure of only three proteins, all belonging to the immunoglobulin (Ig) family: CD4 (HIV1 receptor), poliovirus receptor (PVR), and ICAM-1 (rhinovirus receptor). These sites are located in the first (external) module of each of these type I transmembrane proteins composed of four (CD4), three (PVR), or five (ICAM-1) Ig-like modules (1). The three-dimensional structure of CD4 was determined by x-ray crystallography (2, 3), that of the other two proteins predicted by molecular modelling (4–7). For CD4, amino acids forming the virus binding site cluster mainly in a single loop (CDR2) (8), whereas they are more distributed over the first module in ICAM-1 and PVR (7, 9).

Members of the regulators of complement activation (RCA) protein family have been selected as receptors by different viruses: CD21 by Epstein-Barr virus (EBV), CD55 by several echoviruses, and CD46 by measles virus (MV) (10–13). RCA are type I transmembrane proteins composed of CCP modules, which contain two intramolecular disulfide bridges and have a size similar to Ig modules (14). In contrast to the Ig receptors, in CCP proteins a single module is not sufficient for virus binding. For example, CCP-II to -IV of CD55 are critical for binding of echovirus 7 (15).

Virus binding sites have not yet been mapped in molecular detail in CCP modules. In human CD21, short amino acid stretches of the EBV-binding CCP modules I and II were exchanged with homologue segments of the non-EBV binding murine CD21. These studies revealed that several amino acids are necessary to maintain a conformation allowing virus binding (16, 17) but did not identify residues directly involved in binding.

For the MV receptor CD46, a murine homologue is not available. CD46 homologues are known only in primates where they protect autologous tissue from complement mediated lysis (18, 19). The CD46 CCP-I and -II modules are sufficient for hemagglutinin (H) protein binding, but virus particle attachment is enhanced by CCP-III and -IV (20–23), suggesting secondary interactions with these modules. These secondary interactions, which may be mediated by H and/or by the viral fusion (F) protein (24), may trigger virus-cell and cell-cell fusion (25, 26).

We previously approached the definition of the primary MV binding site by H protein binding studies based on a three-dimensional model of CCP-I-II (27). The three-dimensional model was based on the NMR structure of the CCP modules from another member of the RCA protein family, the human complement factor H. Each of the two CD46 modules is predicted to be about 35 Å long and to form a five-stranded β-barrel structure (27). The two modules are tilted. N-glycosylation sites are situated in the CCP-I-II interface (Asn-80) and on top of CCP-I (Asn-49). We constructed proteins with intermodule exchanges of CCP-I and -II segments. Two of the few altered proteins that reached the cell surface efficiently, a six-amino acid CCP-I exchange mutant (P39-T44, the one letter amino acid code is used) and a 12-amino acid CCP-II exchange mutant (N94-L105), lost H protein binding (27). Partial (P39-T44) or total (N94-L105) loss of reactivity with conformation-depend-
ent antibodies indicated that both mutant proteins did not maintain the native conformation of the CCP modules. Nevertheless, it was noted that both exchanged segments reside on the concave side of the CCP-I-II module, near the N-glycosylation site (Asn-80) essential for the CD46/MV interaction (28).

Here we refine the localization of the MV binding site on CD46. To reduce conformational alterations leading to impaired folding and intracellular transport to a minimum, we based mutagenesis on semi-conservative modifications of single amino acids. We chose residues predicted to be on the CD46 CCP-I-II solvent exposed surface, avoiding alteration of amino acids predicted to constitute the hydrophobic protein core. The mutant proteins were analyzed for H protein binding, viral particle binding, and for binding to conformation-dependent anti-CD46 monoclonal antibodies that exhibited different degrees of interference with the CD46/H protein interaction.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Epitope Mapping**—mAb B97 was selected from hybridomas derived from BALB/c mice immunized with Vero cells by screening for supernatants that inhibited MV infection. mAb 10/88 was selected from hybridomas derived from BALB/c mice immunized with human U937 cells by the same method. All other mAbs have been described: M177, M75, and M160 were kindly provided by T. Seya (Centre for Adult Diseases, Osaka, Japan) (29), Tra-2.10 and GB24 by J. Atkinson (Washington University School of Medicine, St. Louis, MO) (30). The mAb MC120.6, which led to the identification of CD46 as MV receptor, was described by Naniche et al. (12), and 13/42 and 11/88 were described by J. Schneider-Schaulies et al. (31). mAbs E4.3 and J4/48 were obtained from Serotec (Oxford, United Kingdom).

Mouse mAbs from ascites or hybridoma supernatant were purified by affinity chromatography on protein G columns according to standard protocols. Avidities of the mAbs for human CD46 were estimated from Lineweaver-Burk plots of equilibrium binding measured by immunocytofluorometry after incubation of CHO-CD46 cells with serial dilutions of purified antibodies at 37 °C. For epitope mapping, cells transfected with plasmids coding for the altered CD46 proteins were incubated with mAbs at non-saturating concentrations, resulting in half-maximal fluorescent levels with the standard CD46 molecule.

**Generation of Plasmids Encoding Mutant CD46 Proteins**—Plasmids encoding mutant CD46 proteins were generated by site-directed mutagenesis (plasmid pCMV-CD46), which contains a cDNA coding for a C2 isorm of CD46 (clone 63 in Ref. 32). The site-directed mutagenesis system was based on double-stranded DNA (Chameleon kit, Stratagene) using a mutagenic primer and a selection primer (CAGATAT-ACGTATGACATTGT; the SmaI site substituting the unique MluI is in italics). Mutagenic primers were designed such that a restriction site was introduced together with the codon change to facilitate identification of the antibody Reaction with the mutagenic primer of the reactivity sites on the mutation. Further details on the mutagenesis procedure and sequences of most mutagenic primers were described by Koller (33). The following mutagenic primers were used in addition (mismatches in italics, introduced restriction sites are underlined): GCACCTATTGCCGAGAGGGTTA (N94A), GTAATGA-GGGGCTGACTTAAAAATGTT (Y97A), ATAGGGGATGACATATGGGAA (V98A), and GGTTATACGGCAAGCGTGG (L99S). All nucleotide changes were verified by sequencing.

**Inhibition of sH Binding and F/H-induced Syncytia Formation**—Binding of sH, derived from the MV Halle strain, to CD46 was measured by immunocytofluorometry analysis as described (24). All incubations were performed in 0.05% Na2HPO4 to prevent endocytosis. To measure the inhibitory capacity of mAbs, CHO-CD46 cells were incubated with serial dilutions of antibodies for 1 h at 37 °C. After washing the cells, sH was added and subsequently detected using the monkey polyclonal anti-MV antibody BSM94. Based on the median fluorescence values, the percentage of inhibition was calculated by the following formula: (% inhibition with mAbB - % inhibition with mAbA) / 100 × percentage of sH binding without mAb.

**Inhibition of syncytia formation** was assayed on 2 × 10⁵ HuLa cells/well in a 96-well plate. The day after seeding, cells were washed and medium added containing the antibody and 1.5 × 10⁵ pfu/well of a recombinant vaccinia virus coding for the MV F and H proteins (12). The day after infection, syncytia formation was observed under the microscope. The concentration of a mAb was scored as inhibitory if the number of syncytia was reduced to less than 10% of that formed without the mAb.

**TABLE I**

| mAb | Avidity for human CD46 | Binding to simian CD46 | Inhibition of sH binding |
|-----|------------------------|-----------------------|-------------------------|
|    | (nmol/mg)              | (nmol/mg)             | (nmol/mg)               |
| I.1.a (E4.3) | 0.4 | – | 40–50% at 10 μg/ml |
| I.2.a (MC120.6) | 0.3 | – | 75–85% at 11 μg/ml |
| I.2.b (J4/48) | n.d. | – | > 60–70% at 0.3 μg/ml |
| I.2.e (Tra-2.10) | 0.4 | – | 75–85% at 11 μg/ml |
| I.3.a (B97) | 0.1 | – | 100% at 10 μg/ml |
| I.3.b (11/88) | 7 | – | 100% at 15 μg/ml |
| I.3.c (13/42) | 14 | – | 100% at 30 μg/ml |
| II.3.a (M75) | 0.2 | 0 | 100% at 3 μg/ml |
| II.3.b (M77) | 0.4 | 0 | 100% at 10 μg/ml |
| III-IV.1.a (GB24) | 0.3 | 0 | 25–35% at 30 μg/ml |
| III-IV.2.a (10/88) | 0.3 | 0 | 75–85% at 11 μg/ml |

* Roman numbers indicate the CCP module containing the epitope of the mAb, and Arabic numbers indicate the efficiency of MV-inhibition (1 weak, 2 intermediate, 3 strong). mAbs are further distinguished by a small letter. Previous names are given in parentheses.

# RESULTS

## Efficiency of Different mAbs in Inhibiting sH Binding to CD46

In an indirect approach toward the identification of the MV H binding site on CCP-I and -II, we set up to determine the efficiency of different mAbs in inhibiting sH binding to CD46. First, we verified that the antibodies reacted with conformation-dependent epitopes. Indeed, all 11 mAbs lost more than 95% of their reactivity when bound to CD46 proteins separated under reducing conditions (data not shown).

Second, we verified the antibody specificity using hybrid CD46/CD4 proteins containing different combinations of CCP modules (22). mAb reactions were seven against CCP-I, two against CCP-II, and two against CCP-III or -IV. Based on this analysis, the antibodies were renamed with a two number, one letter code. The first (Roman) number defines the CCP module to which each mAb reacts, the second (Arabic) number corresponds to the magnitude of inhibition (1 weak, 2 intermediate, 3 strong).

### Expression and Functional Analysis of Mutant Proteins—Standard (C2 isorm) and mutant CD46 proteins were expressed transiently in mouse Ltk⁻ cells using the vaccinia virus T7 expression system (34). Details of the transfection procedure were as in Buchholz et al. (32).

Binding of mAbs, sH, and MV was performed 20 h after transfection using published incubation conditions, and immunocytofluorometry was used for detection of the bound ligands (24, 32).

Unspecific inhibition observed with this mAb was at or below 10% of the maximal inhibition yielding maximum inhibition is indicated.

### Efficiency of Different mAbs in Inhibiting sH Binding to CD46

Measles Virus Binding to CD46

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All anti-CD46 mAbs inhibited sH binding but at different levels. An example of a weakly inhibiting antibody was I.1.a (E4.3), of which 10 μg/ml inhibited to 40–50% sH binding (Table I, top line and Fig. 1, open circles). To indicate weak sH binding inhibition, we used the Arabic number 1 in the systematic name. Although mAb I.1.a. exhibited low sH inhibition, its CD46 avidity was in the same range as that of strongly inhibiting mAbs (Table I). Thus, low inhibition was not due to low avidity.

mAbs I.2.a, I.2.b, and I.2.c (Fig. 1, closed squares) inhibited sH binding between 60 and 85% (Table I). Interestingly, mAb III-IV.2.a, recognizing CCP-III or -IV, had similar effects on sH binding (Table I). Intermediary inhibition was indicated by the number 2 in the systematic name.

mAbs I.3.a (B97, Fig. 1, closed triangles), I.3.b, I.3.c, II.3.a, and II.3.b completely inhibited sH binding, as indicated by a 3 in the systematic name. Three of these antibodies are directed against CCP-I and two against CCP-II. It is noteworthy that even antibodies with low CD46 avidity exhibited strong sH binding inhibitory capacity. For example, the CD46 avidity of I.3.a was almost two orders of magnitude lower than the average avidity of the other mAbs.

Several of these antibodies had previously been tested for inhibition of MV plaque or syncytia formation (12, 13, 20, 21, 31). All antibodies were found to be inhibitory, with one exception: Iwata et al. (21) showed that I.1.a (E4.3) was not inhibitory when 25 μg/ml were added to CHO-CD46 cells, whereas Manchester et al. (20) reported 80–90% inhibition of MV plaque formation with 100-fold diluted I.1.a ascitic fluid. With purified I.1.a, we found inhibition of syncytia formation at concentrations of 10 μg/ml (data not shown), about 10-fold higher than required for complete inhibition with other antibodies of similar avidity assayed in parallel (not shown). Thus, in different assays, the inhibitory effect of I.1.a was considerably lower than that of other CCP-I mAbs.

We hypothesize that the strongly inhibiting mAbs (group 3) are directly competing for the MV binding site while the others (group 2 and 1 mAbs) are indirectly hindering the H/CD46 complex formation. The reactivities of the anti-CD46 mAbs with simian CD46 are in agreement with this hypothesis. Vero cell CD46 has eight amino acid differences in CCP-I and 14 in CCP-II, as compared with human CD46 (Fig. 2A), and retains MV receptor function (35). Thus, if group 3 mAbs would bind to the MV attachment site of CCP-I and -II, they should react with simian CD46. All group 3 mAbs, besides the CCP-III/IV mAbs, indeed bound Vero cell CD46 with a similar efficiency as the human homologue. However, no binding could be detected with the other CCP-I mAbs, including the low inhibiting mAb I.1.a (Table I). Evidently, one or several of the amino acid changes in the CCP-I module of simian CD46 interfered with binding of these mAbs (see below).

We can thus distinguish between strongly MV-inhibiting mAbs (group 3), which may recognize epitopes at or close to the MV attachment site, and moderately or low inhibiting mAbs (all on CCP-I), which may recognize epitopes located elsewhere. The latter CCP-I mAbs do not react against simian CD46.

**Fig. 1. Inhibition of hemagglutinin binding to CD46 by anti-CD46 antibodies.** CHO-CD46 cells were first incubated with different amounts of purified anti-CD46 mAbs and subsequently with sH. Bound sH was quantified by cytofluorometry, and the percentage of inhibition was calculated. Graphs are shown for the non-CD46 specific control mAb WM1 (closed circles), mAb III-IV.1.a (open squares), mAb I.1.a (open circles), mAb I.2.c (closed squares), and mAb I.3.a (closed triangles).

**Fig. 2. Predicted sequence and tertiary structure of CCP-I and -II.** A, predicted amino acid sequence of CCP-I and -II (residue numbers refer to the processed CD46 molecule). Amino acid stretches recently shown to be important for MV receptor function (27) are underlined. Variable residues in simian CD46 are indicated below the human sequence (35). B, front (left) and back (right) sides of the predicted tertiary structure of CCP-I and -II (27). Two flank views (left and right) of the solvent-accessible surface are shown. The asparagines to which glycans are linked are highlighted by enlarged black spheres centered in the 0°-positions of N49 (top) and N80 (center). Amino acids selected for mutagenesis are shown in blue (positively charged), red (negatively charged), cyan (polar), and yellow (nonpolar). Residues Y61 and E103 are located inside the molecule and therefore are not visible. The figure was prepared with the program MOLMOL (44).
native conformation of the CCP modules. On the basis of the three-dimensional CCP-I-II model, we selected residues with high solvent accessibility, aiming at covering homogeneously the CCP-I-II surface. We examined with more care the amino acid segments shown to directly or indirectly influence sH binding (27). In particular, three mutations in the amino acid 39–44 segment, including P39A (the only proline residue exchanged), and seven mutations in the residues 94–105 segment were introduced (Fig. 2A). Together, the 10 selected amino acids covered about 50% of the CCP-I-II solvent-accessible surface (Fig. 2B).

The CD46 mutant proteins were expressed transiently in the mouse fibroblast cell line Ltk-. Cell surface expression was determined by cytofluorometry using mAbs III-IV.1 and M160 reacting against the non-mutagenized modules CCP-III-IV (data not shown). Of the 40 mutant proteins, 38 were expressed at the cell surface at a similar efficiency as the standard CD46 molecule. This observation and the fact that none of the mutants lost reactivity against several mAbs (Fig. 3) indicate that the native conformation was maintained in these proteins. This confirms the expectations and validates the three-dimensional model, in particular for surface location of the polar and apolar residues L40, V55, V77, I92, Y97, Y98, I104, and Y106. Since L99S was not detected at the cell surface, probably due to misfolding, it is conceivable that the side chain of L99 does not point toward the solvent, but interacts with the hydrophobic core of CCP-II. Protein L99S was not included in further studies.

Surface expression of protein E2A was reduced to 20–30% of that of the standard, similar to that of the six-amino acid exchange mutant P39-T44 used as a control. E2A showed a reduced reactivity only against two of the CCP-I mAbs (Fig. 3A, top left row), suggesting a limited conformational change, while protein P39-T44 lost part of its reactivity against four (group 1 and 2 mAbs) and all reactivity against the other three CCP-I mAbs (group 3 mAbs) (Fig. 3A, bottom row), indicating a more relevant change in conformation. To gain insight into the effect of these different conformational changes on intracellular transport, we examined the composition of the oligosaccharides side chains of the two mutant proteins by endoglycosidase H and peptide:N-glycosidase F digestions. SDS-gel electrophoresis of the digested proteins revealed that formation of the complex oligosaccharides was severely impaired in the mutant proteins (data not shown). Moreover, after peptide:N-glycosidase F treatment, an increased mobility of the E2A protein on gels became apparent (data not shown). From this we conclude that conformational changes in CCP-I prevented efficient modification of proteins E2A and P39-T44 with complex oligosaccharides and thus reduced their surface expression.

Mapping of mAb Epitopes on CCP-I and CCP-II—Next we analyzed the 19 proteins mutated in CCP-I for binding to the seven CCP-I antibodies (Fig. 3A, left panel), the 17 proteins mutated in CCP-II for binding to the two CCP-II antibodies (Fig. 3B, left panel), and the three proteins mutated in the linker peptide for binding to all nine antibodies (Fig. 3C). Standard (C2 isoform) and mutant CD46 proteins were expressed transiently in mouse Ltk- cells using the vaccinia virus T7 expression system (32, 34). The transfected cells were incubated with mAbs at nonsaturating concentrations, and bound mAbs were detected by cytofluorometry. Median fluorescent values of binding were normalized for cell surface expression, which was monitored in parallel with CCP-III/IV mAbs used in saturating amounts. Amino acid exchanges resulting in impaired mAb binding were then localized on the CCP-I-II three-dimensional model. In describing our results, we name as front side the side of the molecule shown in the left panel of Fig. 2B and as back side that shown in the right panel.

In contrast to the P39-T44 control protein (Fig. 3A, bottom row), all mutant proteins completely retained binding to several mAbs (Fig. 3A, open rectangles), confirming that all these mutants maintained the original conformations of the CCP-I-II modules. Significantly, pronounced loss of binding of some mutants toward certain mAbs was monitored, in particular with mAbs I.1.a, I.2.c, I.3.a, and II.3.b.

The weakly sH-competing mAb I.1.a lost its reactivity against CD46 completely when residue E3 was altered. Since binding of all other mAbs was unaffected by this change (Fig. 3A, horizontal row E3A), we conclude that the conformation of CCP-I was maintained in this protein and that E3 is directly
recognized by I.1.a. Interestingly, the same amino acid exchange (E3A) is present in simian CD46 (Fig. 2A), explaining the inability of I.1.a to react against this antigen. mAb I.1.a bound with moderately reduced efficiency a protein in which the neighboring residue of E3, E2 was altered (Fig. 3A). This reduction in binding may be due either to a direct contact of E2 with I.1.a or to an indirect effect due to the local conformational change characterizing E2A (see above). The same holds true for the reduction in binding of mAb I.1.a to mutant P39-T44, which lost in part or completely binding to all seven CCP-I mAbs. Apart from these two, all other mutant proteins were recognized by I.1.a with standard efficiency. In conclusion, the I.1.a epitope likely includes the residues E2 and E3 that cluster on top of the CCP-I back side (Fig. 4, first panel, red and orange residues).

Of the moderately sH-competing mAbs, mAb I.2.c exhibited strongly reduced binding to protein K17A, moderately reduced binding to E2A, and weakly reduced binding to three other mutants (Fig. 3A, vertical row I.2.c). Interestingly, simian CD46 does not react with I.2.c although residues K17 and E2 are conserved (Fig. 2A). As mutant E3A bound I.2.c at standard level, an exchange different from E3A must be responsible for this effect. Apart from E3A, only the exchange E21K is severe and, indeed, situated close to K17 in the three-dimensional model (Figs. 2B and 4, second panel, arrowhead). Since the mutant protein E21A bound I.2.c with a similar efficiency as standard CD46 (Fig. 3A), we assume that E21 is not in direct contact with the antibody but that K21 in the simian CCP-I module alters the local conformation. This might also be the reason why another group 2 mAb lost reactivity against the simian CCP-I module, mAb I.2.a lost part of its reactivity when residue R48, which is situated close to E21, was changed to alanine (Figs. 2B and 3A). Thus, epitopes of two group 2 mAbs may include residues K17, R48, and E24, which all cluster on the top (upper third) of the CCP-I front side (Figs. 2B and 4, second panel).

Of the three strongly sH competing CCP-I mAbs, I.3.a was the only one for which exchanges with strong effects on binding were identified (Fig. 3A). The corresponding residues, R25 and D27, indicated in red in Fig. 4, center panel (three residues leading to slightly reduced binding are shown in yellow), are situated contiguously to the loop formed by residues 39–44 (arrowheads), in the center of the CCP-I front side. Remarkably, not only mAb I.3.a but also the other two group 3 mAbs completely lost reactivity against the P39-T44 mutant. Further exchanges causing weak effects on binding of I.3.a, I.3.b, and I.3.c were less informative. Thus, the epitopes of CCP-I-directed group 3 mAbs may include residues R25, D27, and the P39-T44 loop, all located on the front side of CCP-I, below the epitopes of group 2 mAbs (Figs. 2B and 4, third panel).

Last, both strongly sH competing CCP-II mAbs lost binding to mutant R69A (Fig. 3A, black squares in II.3.b and in II.3.a). mAb II.3.b also partially lost binding to mutants in which residues D70 and E103 were altered (Fig. 3B). Residues R69, D70, and E103 cluster in the lower part of CCP-II (Fig. 4, fourth panel).

It also has to be mentioned that few amino acid changes, most of which clustered in the linker peptide (Y61, R62, and E63, Fig. 3C), enhanced the binding of certain mAbs. These exchanges increased the accessibility of certain regions of the modules to different ligands, including not only certain mAbs but also sH and viral particles (Fig. 3B), possibly by destabilizing the CCP-I-II interface.

**MV and sH Binding and Fusion-support Function of Mutant Proteins**—We then analyzed binding of purified viral particles and sH to the mutant proteins. Complete loss of MV and sH binding was only observed with the P39-T44 control mutant, as described previously (27). Four proteins (E11A, K29A, P39A, D70A) partially lost binding to sH, two to purified virus (D70A, E84A) (Fig. 3, sH and MV columns). Only protein D70A showed reduced binding to both ligands. The most pronounced effect was caused by P39A, which reduced sH binding to about 50% of the standard CD46 level (Fig. 3A).

We also tested the fusion-support capacity of the mutant
proteins by cocultivation of cells transiently expressing these proteins with cells transiently expressing the MV F and H proteins (32). All mutant proteins supported cell fusion. Only cells transfected with the plasmid coding for mutant protein P39A showed slightly impaired syncitia formation (data not shown), confirming the importance of P39 not only for primary binding but also for later steps in MV cell entry.

P39, K29, and E11, as well as D70, map on the front side of CCP-I-II, either close to the interface or in the lower half of CCP-II (Fig. 4, last panel). Based on the three-dimensional model, residues K29 and D70 are separated by a distance of 29 Å. Both regions overlap with residue clusters R25/D27 and R69/D70, required for binding of mAbs strongly competing with sH.

**DISCUSSION**

Attachment of virus proteins to specific cell surface receptors is the first step in viral replication. The description of the molecular mechanism of virus attachment is required to understand cell entry and should guide the rational development of antiviral drugs. Several viral receptors belonging to different protein families have been identified, but only in a few proteins of the Ig family is a three-dimensional description of the receptor binding site available, the best studied case being the HIV receptor CD4. This paper describes mapping of the MV H protein binding site on the two membrane distal CCP modules of the MV receptor CD46, a member of the RCA protein family.

Until cocrysalts of virus attachment proteins and their receptors will be available, the identification of virus attachment sites has to be sought by other means, including mutagenesis. Then, either affinities of purified soluble receptors or binding efficiencies of recombinant receptors expressed on cell surfaces for their attachment proteins have to be determined. Although dependent on more parameters, we decided on using the latter approach as this reflects the natural situation of viral cell entry more appropriately. Moreover, we used a panel of monoclonal CD46 antibodies, directed against CCP-I or -II, inhibiting the sH-CD46 complex formation to different levels to localize the H attachment site indirectly.

An inherent difficulty in the mutagenesis approach is to distinguish between real contact residues and residues necessary to maintain the overall structure of the binding site. Indeed, the exclusive importance of particular residues of the CD4 CD2R loop for gp120 binding is still disputed (36). Several studies demonstrated severe impairments or even loss of HIV-gp120 binding when single residues of the CD2R loop were exchanged (37–40). However, these exchanges often added or reversed charges, possibly altering the native conformation of the binding site. Indeed, less severe substitutions at most of these positions caused only minor effects. Then, only 2 out of 52 mutants with preserved conformation, which remarkably did not contain exchanges of the CD2R loop, exhibited less than 30% binding activity compared with the standard receptor (41).

In our analysis, 5 out of 40 mutant CD46 proteins carrying single amino acid exchanges showed reduced binding to sH or purified MV. Previous studies revealed differences between dimeric sH and the tetrameric MV-associated H protein in conformations and their mode of binding to CD46. Therefore, we investigated the binding of both ligands to the mutant receptors. Indeed, exchanges E11A, K29A, and P39A only reduced sH binding but not MV binding. It was previously shown that CCP-III and -IV enhance MV but impair sH binding (23). It is likely that these two modules compensated for the slight reductions (binding at about 70% of standard CD46) we observed with E11A and K29A in sH binding when MV was used as ligand. Alternatively, due to their different conformations, sH and MV-associated H might contact different residues on CCP-I and -II (23).

Be that as it may, the fact that only minor effects on sH or MV binding with a few mutant receptors occurred shows that the H protein binding motif of CD46 well tolerates single residue exchanges in the CCP-I and -II modules. Therefore, the CD46/H interaction must be based on many contacting residues in CCP-I and -II. For several reasons, we are confident that residues E11, K29, P39, D70, and E84, which reduced sH or MV binding, are among the contacting residues. First, with the possible exception of P39, great care was taken not to alter the module conformations by the residue exchanges. We selected solvent accessible surface residues for the mutagenesis based on the three-dimensional model of CCP-I and -II and replaced them by small residues (alanine for charged and polar residues, serine for apolar residues). The efficient cell surface expression of 38 of the 40 mutant proteins and their unimpaired reactivity against conformation-dependent mAbs revealed that the conformations of the two CCP modules were preserved. Second, residues K29 and D70 are close to or even part of epitopes from mAbs that strongly inhibited the H/CD46 interaction, and residue P39 was covered by the P39-T44 loop exchange mutant, which has lost MV receptor activity (27). Finally, all five residues map to the same (front) side of CCP-I and -II (Fig. 4).

Further evidence for the front side containing the H protein attachment site comes from the epitope mapping we performed for mAbs directed against CCP-I or CCP-II. All these mAbs interfered with the CD46/H protein interaction, which is conceivable when a buried antigen area of 560–855 Å² as determined from crystal structures of antibody/protein antigen complexes (42), is considered. Nevertheless, we could distinguish between three groups of mAbs from a panel of seven CCP-I and two CCP-II mAbs inhibiting the complex formation between CD46 and H protein to different levels. As the strongly inhibiting group 3 mAbs reached 100% inhibition of the CD46-H complex formation, we believe that they bind at or close to the H protein attachment site. Their reactivity against the simian CD46 homologue, which is functional as MV receptor, further distinguished them from the other CD46 mAbs investigated and supports the hypothesis that they are directly competing with H protein binding (35). The moderately and slightly inhibiting group 2 and 1 mAbs are probably indirectly impairing the CD46-H complex formation. Indeed, group 2 also included a CCP-III/IV binding mAb, which can only indirectly interfere.

Based on the reactivity of the mAbs against the CD46 mutants, we were able to localize parts of the epitopes of at least one mAb from each of the three groups. The epitope of the slightly inhibiting group 1 mAb is localized on top of the back side of CCP-I (Fig. 4). Indeed, total loss of reactivity against CCP-I was observed with this mAb upon the single amino acid exchange E3A. Exactly this exchange is present in simian CD46, thus explaining the failure of this antigen to react against this mAb. The moderately inhibiting group 2 mAbs seem to have their epitopes on the upper front side of CCP-I, with K17 and, based on indirect evidence, the adjacent E21 as critical residues (Fig. 4, second panel, arrow on E21). Finally, the epitopes of the strongly inhibiting CCP-I mAbs reside in the lower part of the front side of CCP-I, close to the interface (Fig. 4, center panel).

Interestingly, the important residues of the I.3.a epitope, especially R25, are close to the loop 39P 4LATHHT44 (Fig. 4, center panel, arrows). This might explain why changing this loop against the corresponding segment of CD55, which is GEKDSV, results in loss of MV receptor function and complete loss of reactivity with all strongly inhibiting CCP-I mAbs (this paper and Ref. 27). It is likely that the charged residues within
GEKDSV neutralized the mAb binding site and caused local conformational perturbations.

While the map of antibody epitopes on CCP-I is quite distinguished, the situation on CCP-II is less clear, especially as only two mAbs were available that both inhibited the H/CD46 interaction strongly. Three residues were mapped as contact side for one of the mAbs. Remarkably, R69, which was the most critical residue for binding of both mAbs, is close to D70, which was critical for sH and MV binding. Moreover, R69, exactly as the epitopes of the strongly inhibiting CCP-I mAbs, maps to the front side of CCP-I/II (Fig. 4, fourth panel).

In summary, the back top side of CCP-I contains the epitope of the low inhibiting mAb while the front side of CCP-I/II contains epitopes of the mAbs interfering strongly with the CD46-H complex formation. Moreover, on the CCP-I front side, we can distinguish between epitopes of moderately inhibiting mAbs in the top and strongly inhibiting mAbs in the lower half.

An area covering the residues of epitopes belonging to strongly inhibiting mAbs and the putative H protein contact residues discussed before is delimited by a frame in the last top panel of Fig. 4. This area covers the lower half of the CCP-I and most of the CCP-II front side. It includes the N-glycosylation site at residue N80 (arrow in the last top panel), which was previously shown to be important for the MV receptor function (27, 28). The oligosaccharide chain linked to this residue may either form contacts or participate in maintaining a functional interface. Besides the oligosaccharide also, further amino acid residues in this area, which have not been included in the mutation, may contact the H protein (F35, I37, A79, T82, Y87).

Is there a continuous binding region or are there several binding sites within the putative H protein contact area? The latter possibility would require a cooperative binding mechanism based on two binding sites with moderate affinities that do not allow detection of H binding with the available assays when CCP-I or CCP-II are displayed individually on Ig do-


din. Indeed, the cooperative mechanism will explain more easily how group 1 and 2 mAbs can interfere with the CD46-H complex formation while having their epitopes outside the putative H protein contact area. Based on the data presented, we assume that one binding site is on the CCP-I front side close to residues R25, D27, and K29 while the other binding site localizes on the CCP-II front side at residues R69 and D70. Both sites would be separated by a distance of about 30 Å. A moderately inhibiting group 2 mAb will then bind directly above the binding site on CCP-I and thereby link two monomeric CD46 molecules, possibly facing each other by their H protein attachment sites. This will alter or at least fix the tilt and twist angles of the CCP-II interface. Although attachment of H to the binding site on CCP-II is possible, cooperativity and thus accessibility to the CCP-I site will be reduced due to steric hindrance and loss of flexibility. In the case of the slightly inhibiting mAb I.1.a, steric hindrance is unlikely as here the two CD46 molecules will be linked together facing each other with the backsides of their H protein binding sites. However, this will interfere with cooperative binding as it reduces the flexibility between CCP-I and -II. In agreement with this hypothesis, increased binding efficiencies were observed for certain mAbs and more importantly also for MV and sH when we increased the flexibility between CCP-I and -II by substituting amino acids in the linker peptide (Y61A, R62A, E63A) and thereby destabilizing the putative salt bridges R62-E8 and E63-K110 (27). Obviously, flexibility between CCP-I and -II, as well as their positioning to each other, is a critical parameter for H binding.

Independently from the presence of a continuous binding region or several cooperating binding sites, the data presented here show that the topology of the MV attachment site on its receptor differs principally from that of HIV, poliovirus, or rhinovirus on their receptors. For the latter viruses, the attachment site is mainly located at the tip of the membrane distal module. MV in contrast requires two membrane distal modules, of which the interface between the first and second module together with parts of the second module form the binding site while the tip of CCP-I is not required. Likewise, both other viral receptors from the RCA protein family also require more than one module for virus binding. For EBV, the two distal modules of CD21 and for echovirus modules II-IV of CD55 are required (15, 43). Thus, attachment to two or more modules might be a principal scheme when viruses use members of the RCA family as their receptors. However, mapping of the echovirus and EBV binding sites is yet to be awaited.

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Note Added in Proof—After acceptance of our manuscript two publications describing the mapping of the measles virus binding site on CD46 appeared: Manchester et al. (1997) Virology 233, 174–184 and Hau et al. (1997) J. Virol. 71, 6144–6154. Our results are in line with those of Hau et al.

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