Evidence for distinct complement regulatory and measles virus binding sites on CD46 SCR2

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Human CD46, or membrane cofactor protein, is a regulator of complement activation and is used as a cellular receptor by measles virus. Using a series of 13 single point mutants, the region of short consensus repeat (SCR) 2 domain involved in the regulation of complement activation was mapped to residues E84, N94, Y98, E102, E103, I104 and E108. Molecular modelling localized all residues, with the exception of E84, close to each other on the external lateral face of the molecule, away from the residues important for the binding of measles virus, which are localized on the top of the molecule. The E84 residues is localized in the SCR1-2 hinge and the deleterious effect of its substitution by an alanine residue could affect the relative orientation and/or tilt of SCR1 on SCR2. Taken together, the results suggest that the measles virus binding and cofactor activity of CD46 map to distinct areas on the SCR2 module.

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1 Introduction

Human CD46 (or membrane cofactor protein, MCP) is a glycoprotein expressed at the surface of all nucleated cells [1, 2]. It regulates complement activation and is used as a cellular receptor by measles virus (MV) [3, 4] and human herpesvirus 6 (HHV-6) [5].

As a member of the regulators of complement activation (RC4) family [6], CD46 acts intrinsically by preventing the amplification of the complement cascade on the cells on which it is expressed [7]. CD46 functions as a cofactor for the factor-I-mediated cleavage of the activated complement components C3b and C4b when they are covalently bound to the cell surface, owing to the activation of their internal thioester bonds [8, 9] (see [6] for review). CD46 is a potent inhibitor of the alternative pathway [10] and counteracts the amplification phase of C3b deposition [11], but displays a limited activity towards the classical pathway [10]. The crucial role of cell surface complement regulators has recently been highlighted by the report of the death *in utero* of mouse embryos by complement attack after the inactivation of the gene encoding the membrane regulatory protein Crry, which exhibits both CD46 and decay-accelerating factor-like activities [12]. The essential role of CD46 itself is also suggested by the fact that there are no reported CD46-deficient or defective expression conditions in humans. As a regulator of complement activation, CD46 is of clinical interest because of its role in protecting cancer cells [13] and enveloped viruses [14], and its possible use in xenotransplantation [15, 16]. The ectodomain of CD46 consists of four short consensus repeat (SCR) domains of 60–64 amino acids, linked to a membrane proximal Ser-Thr-Pro (STP)-rich region [6]. Studies using deletion mutants and SCR-specific antibodies have mapped both the C3b and C4b binding sites and the cofactor functional domain to the membrane-proximal SCR domains 2, 3 and 4 of CD46 [17, 18].

In addition to its role in regulating complement activation, human CD46 can also act as a cellular receptor for most MV strains, including laboratory and attenuated vaccine strains [19–22] and primary clinical isolates [23]. However, alternative cellular receptor(s) can also be used by MV following passaging in certain primate or human B lymphoblastoid cell lines [23, 24]. The *Mononegavirales* MV is responsible for an acute human pulmonary disease with high morbidity and mortality, killing over one million young children every year, mainly in developing countries. Infection is associated with a profound but

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transient cellular immunodepression. In rare cases, MV can induce lethal neuropathological disease, acute encephalopathy, measles inclusion bodies encephalitis or subacute sclerosis panencephalitis. CD46 mediates MV entry through the interaction of its ectodomain with that of the MV envelope glycoprotein hemagglutinin (H) [25]. This MV-H/CD46 interaction induces a multimolecular scaffold in which the MV fusion glycoprotein (F) initiates the fusion between the MV envelope and the plasma cell membrane at neutral pH. The H binding site on CD46 has been mapped to the first two N-terminal SCR domains [18, 26, 27]. Modelling of CD46 SCR 1–2 domains [28], which was recently proved to be largely correct following X-ray diffraction analysis of CD46 SCR1–2 crystals [29], together with H, MV and antibody binding studies on site-directed mutated CD46 protein [22, 30–32], indicated that the H protein interacts on one face extending from the top of CD46 SCR1 to the bottom of SCR2. Although dispensable for MV binding, the underlying SCR3 and 4 domains optimize this interaction [33], with SCR4 playing a major role [34]. The STP regions are not directly involved in CD46-mediated MV entry [35, 36]. Thus, the SCR2 domain of CD46 is the only region of the molecule that appears to have both complement cofactor and MV-H binding functions. To determine the individual residues of SCR2 that were important for the complement regulatory function of CD46, a series of 13 single point mutants were generated and the proteins expressed on CHO cells. Several residues were identified as being important. Using molecular modelling techniques, we demonstrate that these key residues mapped outside the previously defined MV-H binding site.

2 Results and discussion

2.1 Amino acid substitutions affecting CD46 cofactor activity

To delineate the SCR2 residues important for the complement regulatory function of CD46, the following mutant proteins R69A, D70A, E84A, Q88A, I92S, N94A, Y98A, E102A, E103A, I104S, E108A, K110A and K119A (see Fig. 1 for sequence location) were expressed in the CHO cell line. These mutants were selected because the residues were all located at the surface of the molecule and accessible to the solvent [28, 29].

To make a comparison of complement regulatory activity between the various mutated proteins, the relationship between the level of CD46 expression and cofactor activity was determined on two CHO cell lines expressing wild-type CD46 at high and low levels (Fig. 2, left panels). Compared with CHO cells, both levels of CD46 expression resulted in a similar reduction in C3b deposition after activation of the alternative pathway (Fig. 2, right panels). Individual clones of SCR2 mutants were then selected that expressed a similar level of CD46, which was at least as high as CHO.CD46^{low} (Fig. 3). Thus, any differences in regulation of C3b deposition could not be attributed to any minor differences in expression. These clones were then tested for their ability to control C3b deposition on CHO cells after activation of the alternative pathway. Two clones, E84A and Y98A showed the lowest inhibitory effect on C3b deposition with nearly half the level of that of wild-type CD46 (Fig. 4). Five other protein mutants, N94A, E102A, E103A, I104S and E108A were also less efficient than CD46 with a 20–30 % reduction in the C3b control efficiency. The remaining proteins mutants, *i.e.* R69A, D70A, Q88A, I92S, K110A and K119A displayed an activity similar to that of CD46 (Fig. 4). Similar results were observed in three separate experiments and, also, when using other cell clones or after transient expression. The lack of effect of the R69A substitution was rather unexpected since this mutation strongly affected the binding of the M75 and M177 antibodies [30], which are potent inhibitors of CD46 cofactor activity [18].

2.2 Residues affecting the CD46 complement regulatory activity are located away from those involved in the binding of MV

All of the substitutions which affected the complement regulatory activity of CD46, with the exception of E84, localized close to each other on the same lateral face of the SCR2 module according to the 3D structure [29] (Fig. 5 B, green residues). This is in marked contrast to all

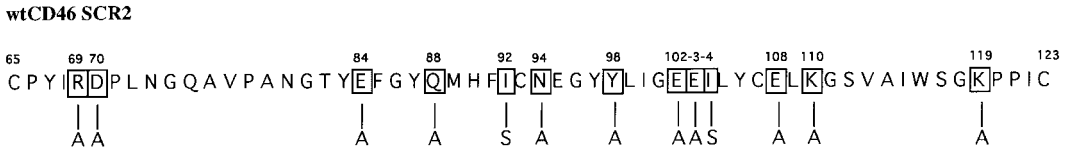

Fig. 1. Amino acid sequence of CD46 SCR2 and location of point mutations.

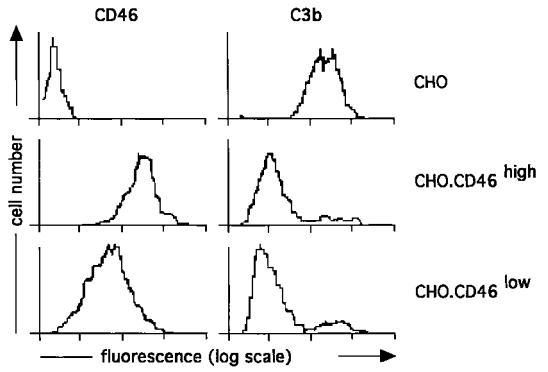

Fig. 2. C3b deposition profile on CHO, CHO.CD46^{high} and CHO.CD46^{low} cells after activation of the alternative complement pathway as determined by flow cytofluorometry.

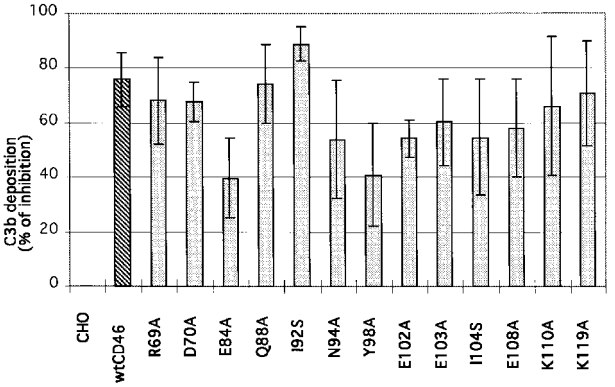

Fig. 4. C3b deposition inhibitory activity of CD46 proteins with point mutations.

the other residues which were localized elsewhere on the surface of the molecule. This suggests that the functional cofactor site of CD46 is located on the lateral face of the SCR2 module. The E84 residue is located within the SCR1/SCR2 hinge and is hydrogen bonded to the hinge residue R62. The lost of this bond after substitution with an alanine is likely to affect the relative orientation and/or the tilt of SCR1 on SCR2. Such a major structural change could affect the cofactor functional SCR2 domain by steric hindrance since the SCR1 module is dispensable for the cofactor activity and the size of the target C3b molecule (~ 170 kDa) is much larger than CD46 (~ 60 kDa). Interestingly, the R69 residue is located on the opposite upper side (Fig. 5 A, among the yellow residues). Thus, although R69 seemed to be located in the binding site of the M75 and M177 antibodies which displayed strong inhibitory activities, it is unlikely to be within the cofactor functional site of SCR2. As a consequence, the inhibitory activity of these two antibodies is unlikely to be related to a direct competition for binding to the functional cofactor site of SCR2.

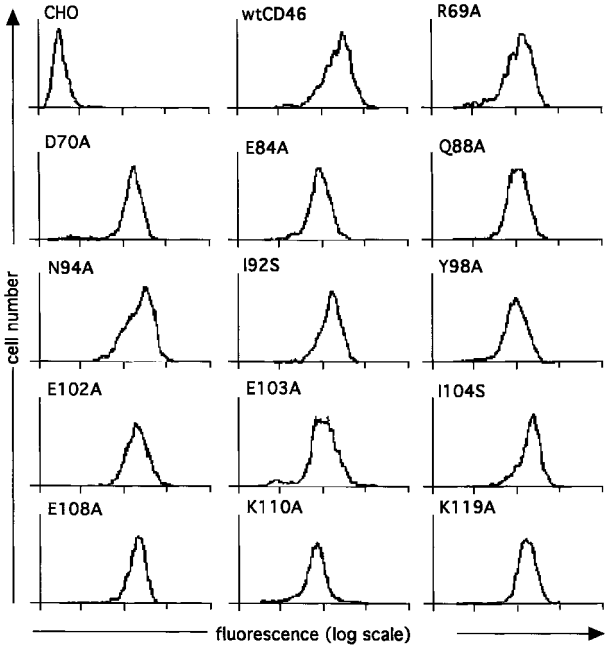

Fig. 3. CD46 expression level of clones expressing wild-type CD46 and CD46 with point mutations as determined by flow cytofluorometry. Note that the fluorescence scale is the same as in Fig. 2.

The binding site of MV and/or MV-H protein have been previously proposed to map to critical residues on both SCR1 and SCR2 modules. These include E11, K17, E24, R25, D27, F35, P39, R48, on SCR1, and Y67, P68, R69, D70, Y83 on the SCR2 module. These residues are all located towards the top of the molecule (Fig. 5 A, yellow residues), on an opposite face to those residues involved in the cofactor activity (Fig. 5 B, green residues). The substitution of E84 has been also shown to reduce MV reactivity as have the hinge residues Y61, R62 and E63, likely by changing the relative orientation of SCR1 and SCR2. From the crystallographic data, the CD46 molecules oligomerize in trimers. Interestingly, when both the putative MV binding and cofactor functional sites are visualized on SCR1–2 trimers, the MV binding site is located on the top of the trimers (Fig. 5 C, yellow residues) and the cofactor functional site on the external lateral side of the trimers (Fig. 5 C, green residues) as one would expect since SCR2 is only a part of the functional cofactor site, which involves also the C3b binding site(s) located on the underlying SCR3 and 4 modules [17, 18]. This lateral orientation of the cofactor functional site is compatible with the intrinsic activity of CD46 which acts only on C3b molecules covalently bond to the same cell surface.

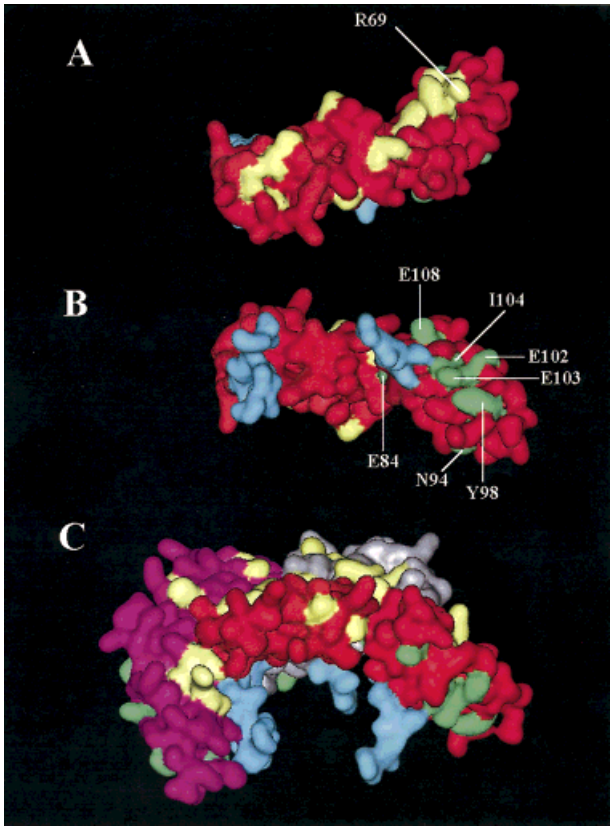

Fig. 5. Molecular surface of CD46 SCR1-2 monomer (A, B) and trimer (C). The 1CKL entry pdb file was used to compute the molecular surface using a soft function and a 1.4-Å probe radius. Top view (A) and view from bottom (B) of the monomer colored in red with the amino acid selections involved in MV-H binding (SCR1 E11, Y20, E24, R25, Y34, F35 residues and SCR2 Y67, I68, R69, D70 and Y83 residues) colored in yellow and those involved in CD46 cofactor activity (E84, N94, Y98, E102, E103, I104, E108) colored in green. In (C), side view of SCR1-2 trimers, one is colored in red, the second in pink and the third in grey. Residues involved in MV binding and complement regulatory functions of CD46 are colored in yellow and green, respectively. Sugar moieties are colored in blue.

3 Concluding remarks

We propose that the functional cofactor site of CD46 is located on the external lateral side of the SCR2 module away from the MV ginding site which mapped to the top side of the SCR1-SCR2 modules.

4 Materials and methods

4.1 Single amino acid SCR2 CD46 mutant cell lines, antibodies and serum

The wild-type cell-surface CD46 isoform used in this study includes STP C and cytoplasmic tail 2. Stable cell lines expressing CD46 with single amino acid substitutions in the SCR2 domain were derived by transfection of CHO cells using mutated cDNA previously described by Buchholz et al. [30] that had been inserted into the SFFV expression vector. Cell clones expressing similar levels of CD46 protein, as determined using an anti-CD46 SCR1 antibody, were selected. All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 6 % fetal calf serum (DMEM-6 % FCS), 10 μg/ml gentamycin, nonessential amino acids and 10 μg/ml of adenosine, deoxyadenosine and thymidine.

The following mouse mAb were used: anti-CD46 MC120.6 directed against SCR1 [30, 37], anti-C3b, iC3b and C3c WM1 (European Collection of Animal Cell Cultures, no. 92021211). PE-labelled anti-mouse IgG (H+L) was purchased from Immunotech.

Human serum was isolated from ice-clotted blood from two donors, stored as frozen aliquots at – 70 °C and used only once after thawing. Factor B depleted human serum was prepared by heating the serum for 20 min at 50 °C [38]. Rabbit anti-CHO serum was inactivated at 56 °C for 30 min prior to use.

4.2 Quantification of C3b deposition on the cell surface

The assay has been described previously [11]. In brief, cells were harvested, washed once with DMEM-6 % FCS and twice with DMEM+NaN_3 (0.05 %) (containing no FCS). Cells (2×10^5) were incubated at 37 °C for 30 min with human serum diluted in DMEM+NaN_3 (0.05 %) in a final volume of 60 μl in a round-bottom 96-well microplate. To selectively activate the alternative pathway, the incubation was performed in the presence of 20 mM $MgCl_2$ and 100 mM EGTA. Complement activation was stopped by rapid dilution with cold DMEM+NaN_3 (0.05 %) and centrifugation at 1200 rpm for 2 min. Following an additional two washes, the cell pellet was resuspended in 50 μl of the appropriate dilution of WM1 mAb and incubated at 4 °C for 30 min. After additional washing, the cells were resuspended in the appropriate dilution of the relefant PE-conjugate, incubated for 30 min, and then washed prior to the measure of the fluorescence labeling by flow cytofluorometry. Although the antibody used to detecte C3b could not distinguish between C3b and iC3b, and because the latter is derived from the former, the amount of bound antibody reflected the amount of C3b which was bound to cells. The level of C3b deposition was expressed as the mean fluorescence value and used to cal-

culate the percentage of deposition by defining the C3b deposition on control CHO cells as 100 %. The results were expressed as a mean of three separate experiments in percentage of inhibition of C3b deposition.

4.3 Positioning of amino acid mutants on the SCR1-2 3D-structure

The 3D structure of SCR1-2 was retrieved from the RSCB Protein Data Bank (accession code 1CKL) [29]. The Conolly molecular surface was calculated by using a probe radius of 1.4 Å with the help of the Weblabviewer software from MSI Inc.

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