The Distal Short Consensus Repeats 1 and 2 of the Membrane Cofactor Protein CD46 and Their Distance from the Cell Membrane Determine Productive Entry of Species B Adenovirus Serotype 35

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Received 23 February 2005/Accepted 22 April 2005

The human regulator of complement activation membrane cofactor protein (CD46) has recently been identified as an attachment receptor for most species B adenoviruses (Ads), including Ad type 3 (Ad3), Ad11, and Ad35, as well as species D Ad37. To characterize the interaction between Ad35 and CD46, hybrid receptors composed of different CD46 short consensus repeat (SCR) domains fused to immunoglobulin-like domains of CD4 and a set of 36 CD46 mutants containing semiconservative changes of single amino acids within SCR domains I and II were tested in binding and in Ad35-mediated luciferase transduction assays. In addition, anti-CD46 antibodies and soluble polypeptides constituting various CD46 domains were used in binding inhibition studies. Our data indicate that (i) CD46 SCR I or II alone confers low but significant Ad35 binding; (ii) the presence of SCR I and II is required for optimal binding and transgene expression; (iii) transduction efficiencies equivalent to that of full-length CD46 are obtained if SCR I and II are at an appropriate distance from the cell membrane; (iv) ablation of the N-glycan attached to SCR I has no influence on receptor function, whereas ablation of the SCR II N-glycan results in about a two- to threefold reduction of binding and transgene expression; (v) most putative Ad35 binding residues are located on the same solvent-exposed face of the SCR I or SCR II domain, which are twisted by about 90°; and (vi) the putative Ad35 binding sites partly overlap with the measles virus binding surface.

The species B serotypes of human Adenoviridae account for about 34% of all adenovirus (Ad) isolates typed and reported to the World Health Organization, second to species C Ads, which account for about 50% of the isolates (65). Species B Ads are divided into B1 serotypes, including Ad type 3 (Ad3), Ad7, Ad11, Ad16, Ad21, and Ad50, which predominantly infect the upper respiratory tract, and the B2 serotypes, including Ad11, Ad14, Ad34, and Ad35, which are associated with kidney and urinary tract infections (56, 65). Whereas species B1 Ads are commonly isolated, species B2 Ads account for fewer than 1% of the reported Ad isolates, with a seroprevalence of <5% for Ad11, Ad34, and Ad35 and 15 to 20% for Ad14 in a typical Western population (64). However, like all human adenoviruses, Ad11, Ad34, and Ad35 are frequently isolated from patients with compromised immune systems, such as AIDS patients or renal or bone marrow transplant patients (36).

Recently, recombinant species B Ads or fiber-swapped Ad vectors in which the fiber protein of the commonly used Ads is swapped with species B Ad fiber have gained interest for use in gene therapy and vaccination approaches. An important reason for the latter, besides the low seroprevalence of these viruses, which should allow accurate dose control, is that B Ads were suggested to bind to a different cell surface receptor (9, 11, 19, 52, 63). Accordingly, recombinant species B Ads (54, 64) or fiber-swapped Ad vectors (23, 24, 29, 51, 61) have an extended tropism compared to species C vectors and are able to efficiently infect hematopoietic and dendritic cells. Using different approaches, CD46 has been identified as an attachment receptor for species B Ad3 (62), Ad11 (59), and Ad35 (18). Additional viruses that bind to CD46 include species D Ad37 (70), the Edmonston strain of measles virus (MV) (12, 49), human herpesvirus 6 (55), and bovine viral diarrhea virus (42). In addition, CD46 has been reported to bind different bacteria (33). CD46 belongs to a family of regulators of complement activation (RCA), whose biological role is to prevent complement activation on autologous tissue by binding C3b and C4b and by acting simultaneously as a cofactor for the proteolytic factor 1 (34). Recent evidence also suggested an additional role in linking innate and acquired immunity by signaling events induced in macrophages and lymphocytes (reviewed in reference 53).

CD46 consists of (i) four amino-terminal copies of an approximately 60-amino-acid structural motif termed the short consensus repeat (SCR) (also called complement control protein repeat), (ii) one to three serine-threonine-proline-rich (STP) domains, (iii) a short region of unknown function, (iv) a transmembrane spanning domain, and (v) a carboxy-terminal...
cytoplasmic tail. Alternative splicing of the STP domain-encoding exons (STABC) and cytoplasmic tail exons (CYT1 and CYT2) gives rise to the four major splice variants C1, C2, BC1, and BC2 and to additional minor variants (34). Each SCR module is predicted to be about 35 Å long and to form a five-stranded β-barrel structure. Domains SCR I, SCR II, and SCR IV each contain one N-linked oligosaccharide, and the STP domains contain O-linked glycans. For MV, N-linked oligosaccharides do not participate in direct binding, although the sugar attached to N80 in SCR II was shown to be essential, suggestive of a stabilization function (37, 39, 46).

An increasing number of virus receptor binding sites are being defined at the molecular and structural levels, an important prerequisite to infer links between receptor binding and mechanisms of pathogenesis (58, 66, 67). Also, the superior gene transfer performance of serotype B fiber-containing vectors, in particular those of Ad35, underscores the importance of characterizing the molecular sites involved in binding of Ad35 to CD46 in more detail. To this end, a series of CD46 hybrid receptors consisting of different CD46 SCR domains and CD4 immunoglobulin (Ig)-like modules (5, 46) were used to assess Ad35 binding and Ad35-mediated transgene expression. To further define residues involved in Ad35 binding, a set of 36 CD46 mutants containing semiconservative changes of single amino acids within the SCR I and II domains (4) were tested. The results from these analyses were complemented with binding inhibition studies using anti-CD46 antibodies and soluble polypeptides comprising various CD46 domains.

MATERIALS AND METHODS

Cells, viruses, and screening with fiber-chimeric viruses. The A549 human lung carcinoma cell line was received from P. Sonderegger (Biochemical Institute, University of Zurich). The mouse fibroblast cell line Ltk− and melanoma cell line B16-F1 were received from J. Pavlovic (Medical Virology, University of Zurich). The baby hamster kidney cell line BHK-21 was provided by M. Bachmann (Cytos Biotechnology, Schlieren-Zurich, Switzerland). All cells, including simian COS7 cells and human embryonic retinoblastoma 911 cells (16) and PER.C6 cells (15), were grown in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum (FBS). The B16-C6 cell line, stably expressing the B1-C6 splice isoform, was generated as described for BHK-C6 cells (62).

Ad35 was radiolabeled as described for Ad2 (21, 48). Briefly, A599 cells were grown in 10-cm-diameter petri dishes to 90% confluency and wild-type (wt) Ad35 (64) was inoculated at a multiplicity of infection (MOI) of 10. In 10 hours postinfection (p.i.), the virus-containing medium was replaced by a labeling medium (Dulbecco’s modified Eagle’s medium, 10% dialyzed fetal calf serum, 150 μg/ml [3H]methyImidethione per plate [TRK686; Amersham]). Virus was harvested at complete cytopathic effect, 36 to 48 h p.i., and released from cells by three freeze/thaw cycles, Perc extraction, and two CsCl gradient ultracentrifugations. Specific activity (2.6 × 1015 cpm/viral particle [vp]) was determined by scintillation and measurement of viral proteins, with the assumption that 4 × 109 vp correspond to 1 μg protein.

To study whether the Ad5 fiber-chimeric viruses carrying the B group fiber 7, 11, 16, 35, or 50, and the C group fiber 5 as a control (24), make use of CD46, an infection experiment was performed with the B16-C6 cell line and its parental control. Infection was performed on 1 × 106 cells/well in a 24-well format with the Ad5 fiber-chimeric viruses carrying the luciferase transgene for 2 h at an MOI of 1,000 vp/cell. At 48 h postinfection, cells were lysed with a lysis buffer containing 1% Triton X-100, and luciferase expression was measured with a luciferase assay system (Promega) on a Luminoskan Ascent instrument (Thermo Labsystems).

Blocking of Ad5 binding. Blocking experiments were performed as described previously (62). Briefly, BHK-C6 cells were detached with phosphate-buffered saline (PBS)–20 mM EDTA, and 5 × 105 cells were incubated with monoclonal antibodies specific for CD46, control anti-human coxsackievirus B and Ad receptor (CAR) antibody E1-1, or soluble CD46 domain-containing polypeptides or with a 30-fold excess of unlabelled Ad35 on ice for 30 min. Subsequently, 0.3 μg of [3H]Ad35 was added to about 5 × 105 cells in a total volume of 200 μl on ice for 1 h (corresponding to 2,500 vp/cell). Cells were washed and analyzed for bound virus by using liquid scintillation counting. The data were normalized to the amounts of virus bound in the absence of inhibitors and represented as the average and standard deviation from three independent experiments. Statistical evaluation was performed using Student’s t test.

Construction of the soluble CD46ex-Fc has been described earlier (62). CD46ex-Fc comprises 295 amino acids of the mature extracellular domain, including the SCR I to IV domains, the STP and BC1, and the short region of unknown function, fused to the 232 amino acids of the human IgG1-Fc domain including the hinge, CH2, and CH3 regions. The CARex-Fc protein comprises the extracellular domain of coxsackievirus B and the Ad receptor (14). Expression and purification of CD46 proteins encompassing the SCR I domain (CD46-SCR I), the SCR I and II domains (CD46-SCR I-II), the SCR I to IV domains (CD46-SCR I-IV), and the whole extracellular CD46 domain of the BC splice form (CD46-BC) were similarly performed. Boundaries of SCR domains were adopted as described by Lublin et al. (35). As a starting expression vector a modified pcDNA3 (Invitrogen) was used, in which the endogenous cytomegalovirus promoter was replaced by the cytomegalo virus early-immediate promoter plus intron A from pVR1012 (44) and in which the influenza virus hemagglutinin signal peptide consisting of 15 amino acids followed by His, tag, a spacer, and the rTEV cleavage site (Invitrogen) had been introduced. The signal peptide-cleaved form of CD46-SCR I was expected to consist of 82 amino acids, including the N-terminal 20 amino acids of the His tag, spacer, and cleavage site and 62 amino acids of SCR I. Similarly, signal peptide-cleaved forms of CD46-SCR I-II, CD46-SCR I-IV, and CD46-BC consisted of totals of 145, 272, and 315 amino acids, respectively. All proteins were expressed in simian COS cells and purified from conditioned supernatants by using anti-CD46 antibodies against the extracellular domain of CD46 (Biotest, Frankfurt, Germany, Switzerland). The concentrations of purified proteins were determined by bicinchonic acid assay (Pierce) using bovine serum albumin as a standard. The purity of all proteins was judged to be >90% when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining.

Blocking experiments were performed as described previously (5, 46, 57). All chimeric proteins contained the signal peptide and were membrane anchored via the transmembrane sequence of CD46 (also the I-Il and I-IV constructs). In all constructs, a 5-amino-acid-long glycine-rich peptide separated the CD46 and CD4 parts, allowing for structural flexibility. The two constructs I-Il/I-4 dg1 and I-Il/I-4 dg2 contained single point mutations N490Q (I-I/4 dg1) and N800Q (I-I/4 dg2), deleting the N-glycosylation site contained in each of these domains. The I-Il/I-4 and I-I-I/4 constructs contained duplicated and exchanged intracysteine segments, encompassing SCR I plus T40 to I92 of the SCR II domain fused to C30 to E63 of SCR I in the first construct and SCR I fused to C1 to K30 of SCR I and C93 to V126 in the second. Likewise, all 36 CD46 single point mutants and one 6-amino-acid exchange mutant, P39- T44, containing six alanines replacing the endogenous sequence, were described earlier (4, 30). These constructs were based on the C2 splice isoform of CD46 and contained all four SCR domains.

Mouse Ltk− cells were grown in 10-cm-diameter petri dishes to 70 to 90% confluency and were transiently transfected using the vaccinia virus T7 expression system (17). Cells were infected with vaccinia virus VTF7-3 at an MOI of 10 to 15 for 40 min. The virus was removed, and cells were transfected with 25 μg of plasmid DNA encoding the different CD46 constructs and 33.5 μg Lipofectin, using the protocol of the manufacturer (Gibco-BRL, Paisley, United Kingdom). The cells were harvested about 20 h after transfection by being detached from the dish by PBS-EDTA treatment. After being washed twice with balanced salt solution (BSS)–2% FBS, the cells were aliquoted for cytometric analysis (see below), binding assay, or transgene expression experiments. For binding assays 3.5 × 105 to 5 × 105 cells were incubated with radiolabeled virus as described above. For transgene expression 5 × 105 cells/well were seeded in 12-well plates and allowed to attach for at least 5 h, followed by addition of 2,500 vp/cell of Ad35ΔE3.Luc (64). At 24 h p.i. cells were harvested, and luciferase activity was determined as described above. The luciferase activities were normalized to the lysate protein concentration. Binding efficiencies and transgene expression levels were obtained by dividing binding values (counts per minute of bound [3H]Ad35 minus counts per minute of cells alone) or transgene expression levels (relative light units of infected cells minus relative light units of cells alone) by the levels of hybrid surface expression. All measurements, including surface expression analysis, were done in triplicate and repeated at least three times.

Antibodies and flow cytometric analysis. CD46 SCR I-specific antibodies included E4.3 (PharMingen, Basel, Switzerland), MC120.6 (49), 13/42 (BMA Biomedical AG, Augst, Switzerland), Tra-2.101 (1), and MEM-258 (Serotec Ltd, Oxford, United Kingdom). The SCR II-specific antibody M177 was purchased from ProteinTech Group, Inc. (Chicago, IL) and used at a 1:500 dilution in PBS-EDTA. Mouse monoclonal antibodies to CD46 in E1-1 (1:100), E9-2 (1:100), and E21-2 (1:100) were obtained from PharMingen (San Diego, CA).
from HyCult Biotechnology, Uden, The Netherlands, and the SCR III/IV-specific antibody GB24 (1) was a generous gift from J. Atkinson, Washington University School of Medicine. Cell surface expression of the chimeric proteins was determined by using the SCR I-specific antibody E4.3 for all constructs containing SCR I or the SCR II-specific M177 for the two hybrids encoding only SCR II. CD46 single point mutants and the one 6-amino-acid exchange mutant P39-T44 were all quantified using the E4.3 antibody, except construct E2A, which was measured using M177. Mutant CD46 expression levels were in the range of 50 to 130% of wt CD46 expression, except for the E4.3 antibody, except construct E2A, which was measured using M177. Mutant CD46 expression levels were in the range of 50 to 130% of wt CD46 expression, except for the II/4 and II+II/4 constructs, which yielded only about 10% and 20% of the wt CD46 expression level, respectively. Control-antibody for E1-1 anti-CAR was described previously (14). Secondary fluorochrome conjugates sheep anti-rabbit and goat anti-mouse IgG-phycoerythrin were purchased from Serotec, Oxford, United Kingdom.

For cytotoxic immune analysis of adherent cells, subconfluent cells were washed with PBS and detached by treatment with PBS–20 mM EDTA. About 2 × 10^6 cells were incubated with 1 µg of antigen-specific antibodies in 250 µl of BSS (0.14 M NaCl, 1 mM CaCl2, 5.4 mM KCl, 0.8 mM MgSO4, 0.3 mM NaH2PO4, and 0.4 mM KH2PO4, pH 6.9) containing 5% FBS on ice for 30 min. Cells were washed by pelleting in BSS–2% FBS, incubated with 1 µg of phycoerythrin-labeled secondary conjugates, and washed again before cytofluorometric analysis.

RESULTS

Chimeric adenoviruses carrying B group fibers. Parental mouse B16 melanoma cells or stably transfected B16-CD46 cells expressing the BC1 splice form were incubated for 2 h with luciferase-expressing Ad5-Luc or Ad5-chimeric viruses at an MOI of 1,000 vp/cell. Cells were washed, and transgene expression was analyzed 2 days p.i. Luciferase activity is expressed in relative light units.

FIG. 1. CD46-expressing cells infected by chimeric adenoviruses carrying B group fibers. Parental mouse B16 melanoma cells or stably transfected B16-CD46 cells expressing the BC1 splice form were incubated for 2 h with luciferase-expressing Ad5-Luc or Ad5-chimeric viruses at an MOI of 1,000 vp/cell. Cells were washed, and transgene expression was analyzed 2 days p.i. Luciferase activity is expressed in relative light units.

Antibody- and SCR domain polypeptide-mediated blocking of Ad35 binding to CD46. To identify which SCR domains are involved in Ad35 binding to CD46, seven different monoclonal antibodies with specificity for individual SCR domains were tested for their ability to inhibit binding of [3H]Ad35 to CD46, as reported previously for Ad3 (62). BHK cells expressing the BC1 form of human CD46 were first incubated with monoclonal antibodies at various concentrations, followed by addition of [3H]thymidine-labeled Ad35 and determination of cell-associated virus by liquid scintillation counting (Fig. 2A). The two SCR I-specific monoclonal antibodies 13/42 and MEM-258, as well as the SCR II-specific M177, strongly inhibited virus binding in a concentration-dependent manner (Fig. 2A and B). Of note, as determined by analysis of binding to CD46 hybrid constructs, MEM-258 binds to an epitope of SCR I (data not shown) and not SCR IV, as suggested by the manufacturer’s information. The SCR III/IV-specific antibody GB-24 inhibited binding at an intermediate level of about 30% at the highest concentrations used. Finally, the three SCR I-specific antibodies E4.3, MCI20.6, and Tra-2 had significant but weak effects on Ad35 binding of 16%, 17%, and 21% inhibition, respectively, whereas the anti-CAR control antibody E1-1 had no significant effect on Ad35 binding (Fig. 2A and B). Together, these data suggest that mainly SCRs I and II are potentially involved in Ad35 binding.

Since the blocking data obtained thus far hinted that both the SCR I and SCR II domains were involved in Ad35 binding, we performed further binding experiments using soluble polypeptides carrying various CD46 domains. These proteins included CD46ex-Fc (62), which forms a dimeric protein containing two CD46 binding sites (similar to CARex-Fc, which leads to Ad5 aggregate formation through multivalency [14, 43; unpublished data]); soluble CD46-BC, comprising all four SCR domains plus the STP domains B and C; and four shorter polypeptides spanning the SCR I-IV, SCR I-II, and SCR I domains. Additional constructs encoding the SCR II domain or the SCR III-IV domains yielded only small protein amounts and could not be included in our analyses. We found that the soluble CD46ex-Fc protein inhibited binding of Ad35 to BHK-CD46 in a dose-dependent manner, with 20 µg/ml giving rise to 95% inhibition, whereas the control CARex-Fc protein had no significant effect on Ad35 binding (Fig. 2C). In the cases of CD46-BC, CD46-SCR I-IV, and CD46-SCR I-II, amounts of soluble CD46ex-Fc protein inhibited binding of Ad35 to BHK-CD46 in a dose-dependent manner, with 20 µg/ml giving rise to 95% inhibition, whereas the control CARex-Fc protein had no significant effect on Ad35 binding (Fig. 2C). In the cases of CD46-BC, CD46-SCR I-IV, and CD46-SCR I-II, amounts equimolar to that of CARex-Fc gave 90%, 88%, and 76% binding inhibition, respectively. In contrast, CD46-SCR I did not inhibit Ad35 binding. These data show that at least SCR II is needed for Ad35 binding.

Mapping Ad35 binding domains with hybrid CD46-CD4 proteins. To map the molecular sites involved in binding of Ad35 to CD46, a set of 17 hybrid receptors consisting of different CD46 SCR domains and CD4 Ig-like modules (5, 46) were used. Using a first set of four constructs, we tested binding and transduction efficiencies mediated by the four SCR domains fused to increasing numbers of CD4 Ig-like domains. The CD4 domains act as spacers, increasing the distance between the potential Ad35 binding site and the plasma membrane. Cells expressing the complete CD4 protein did not confer Ad35 binding (data not shown). The construct I-IV lacked the 43-amino-acid stretch including the serine/threonine-rich domains B and C and the short region of unknown
function UN. Constructs I-IV/4, I-IV/3-4 and I-IV/4/1-4 contained one, two, or four additional CD4-like Ig domains, respectively, where the roman numerals indicate the SCR domains and arabic numerals the CD4 domains. Single SCR domains have a size similar to that of Ig domains, and each Ig domain is expected to increase the distance to the membrane by about 30 Å (3). All four constructs bound virus with similar efficiencies, although I-IV/3-4 was slightly more efficient at virus binding (Fig. 3A). These results confirmed that the membrane-proximal stretch of 43 amino acids does not substantially influence virus binding. Interestingly, transgene expression varied drastically with the receptor length. Deletion of the BC sequence (43 amino acids) reduced transgene expression by about 50%. In contrast, increasing the receptor length by one or two Ig-like domains (construct I-IV/1-4) resulted in a threefold increase of transgene expression.

The second set of CD4-CD46-SCR hybrid constructs contained the two outer SCR domains SCR I-II, either alone (I-II) or fused to increasing numbers of CD4 Ig domains (I-II/4, I-II/3-4, and I-II/4/1-4). Two additional constructs including the I-II/4-type construct also contained the single point mutations N49Q (I-II/4 dg1) and N80Q (I-II/4 dg2), ablating the N-glycosylation sites of these domains. Remarkably, the CD46 SCR I-II domain added N terminally to CD4 domains 1 to 4 (construct I-II/1-4) yielded virus binding similar to that of CD46-expressing cells (Fig. 3B). The deletion of CD4 Ig-like domains (construct I-II/4 or I-II) yielded about 65% and 25% of wt CD46 binding, respectively, suggesting an important role of the CD4 spacer domains. Like for the previous SCR I-IV constructs, increasing receptor length in the SCR I-II constructs was paralleled by increased transgene expression, reaching a maximum of threefold (construct I-II/1-4) over that for CD46 alone. Ablation of the SCR I N-linked glycosylation did not significantly affect virus binding, whereas ablation of the SCR II N-linked glycosylation resulted in about a twofold

![Figure 2: Inhibition of Ad35 binding to CD46 by anti-CD46 antibodies and SCR domain polypeptides.](http://jvi.asm.org/)
binding reduction ($P = 0.005$) compared to construct I-II/4. Transgene expression in these two mutants reached an efficiency similar to that in CD46 wt cells, as reflected by similar amounts of virus binding and transgene expression, but was clearly lower than that in the similarly sized I-II/4 construct. Taken together, the data suggest that SCR I and II are sufficient for Ad35 binding and transgene expression. Moreover, the distance of these domains from the plasma membrane strongly influences transgene expression efficiency.

Using another set of seven constructs, we asked whether SCR I or SCR II alone confers Ad35 binding (Fig. 3C). Included as controls were CD46- and I-II/4 construct-expressing cells. Two of the tested constructs, I/4 and II/4, contained single SCR I and SCR II domains joined to the fourth CD4 Ig-like domain, resulting in a receptor length similar to that of the I-II construct. Virus binding to and transgene expression in cells expressing construct I or II fused to domain 4 of CD4 were not significantly different from those of cells expressing the I-II construct without CD4 domains (Fig. 3B). It should be noted that the large variation of the values for II/4 resulted from the unusually low expression levels seen with this construct. Likewise, the extended I+I/4 and II+II/4 constructs containing duplicated SCR I or SCR II domains were not significantly different from the constructs containing only one of these domains.

Finally, the two constructs I+II;I/4 and I+I;II/4, containing duplicated and exchanged intracysteine segments, and construct II+I/4, with an inverted order of the SCR I and SCR II domains, were tested. It is notable that the I+II;I/4 construct carries an additional N-linked sugar in the SCR II domain, whereas the I+I;II/4 construct is devoid of an N-linked sugar in its second SCR domain. Both binding and transgene expression of the I+II;I/4 construct were strongly reduced, to 5 and 6%, respectively, compared to the values for full-length CD46. The values obtained with the I+I;II/4 as well as with the II+I/4 construct were in the range of those with the other constructs tested in this series. In summary, the results suggest that the two N-terminal domains SCR I and II are sufficient for Ad35 binding and transgene expression and that binding was not confined to a single SCR domain. In addition, binding and viral transgene expression critically depended on the length of the

by dividing the $[^{3}H]$Ad35 binding values by surface expression levels and are shown in relation to that of the CD46-BC1 standard. Likewise, luciferase transgene expression measured by infection of the CD46-expressing cells with Ad35.E3.Luc at 24 h posttransduction was determined by normalizing to protein concentration and surface expression of the CD46 proteins, attributing 100% to the BHK cells expressing CD46 (BC1). Measurements were done in triplicate and repeated at least three times. Schematics indicate the following: SCR I, black; SCR II, crosshatched; SCR III, light grey; and SCR IV, white. The CD4 Ig-like domain is drawn as a loop. N-linked oligosaccharides of SCR domains are indicated, whereas the single oligosaccharide of the CD4 Ig-like domain is omitted, as this sugar is present in all constructs. (A) Function of four hybrid proteins containing SCR I-IV fused to increasing numbers of CD4 Ig-like domains. (B) Function of six hybrid proteins containing SCR I-II fused to increasing numbers of CD4 Ig-like domains or combined with mutations ablating N-linked oligosaccharide in SCR I (dg1) or SCR II (dg2). (C) Function of seven hybrid constructs consisting of single SCR domains, or containing duplicated or exchanged intracysteine segments, fused to a constant CD4 Ig-like domain.
receptor and hence on the distance of the SCR I and II domains from the membrane.

Mapping Ad35 binding residues with 36 single-amino-acid SCR I-II mutant proteins. To define residues involved in Ad35 binding, a set of 36 CD46 mutants containing semiconservative changes of single amino acids within the SCR I-II domains were tested (4). These mutations concerned residues predicted to be on the CD46 SCR I-II solvent-exposed surface (46) and consisted of replacements by small residues (alanine for charged and polar residues and serine for apolar residues). The use of such semiconservative changes is expected to be well tolerated and to have a minimal impact on protein conformation. Also included was a 6-amino-acid exchange mutant, P39-T44, containing six alanines replacing the endogenous sequence. This loop exchange was suggested to induce a conformational change, which resulted in loss of MV hemagglutinin binding and a complete or partial loss of binding of antibodies specific for SCR I (4). Analysis of Ad35 binding and transgene expression was performed as for the hybrid constructs and is summarized in Fig. 4. Among the 19 mutant CD46 proteins with alterations in the SCR I domain, a weak but significant binding reduction was found for the K15A, E24A, R25A, and

FIG. 4. Ad35 binding efficiencies mediated by 36 single-amino-acid SCR I-II mutant proteins. (A) Nineteen single-amino-acid mutants plus the exchange mutant P39-T44, containing six alanines replacing the endogenous sequence localized in SCR I, were analyzed as described for Fig. 3. (B) Seventeen single-amino-acid mutants of SCR II were similarly analyzed. The asterisks indicate the level of significance ($P < 0.05$ compared to wt CD46).
K29A mutations, with 22%, 36%, 22%, and 20% decreases in binding, respectively. These residues are situated on the same face of the SCR I solvent-exposed surface as the epitopes characterized by antibodies 13/42, with some of them contiguous (Fig. 5A). Interestingly, the mutant P39-T44, which had almost completely lost binding of several CD46 SCR I antibodies, including 13/42, as well as binding of MV hemagglutinin or whole MV, had only a marginal Ad35 binding loss ($P = 0.05$). A 45% reduction of Ad35 binding was monitored with the Y97A mutant, whose mutation resides in the lower half of the SCR II domain. Five further mutant sites demonstrated reduced binding in the range of 20 to 30%, including Q88A, N94A, Y98A, Y106A, and K119A. Of note, residues Q88, Y98, K119, and Y106 together with the antibody M177 epitope R69 are situated on a SCR II face that is twisted by about 90° compared to the SCR I binding surface (Fig. 5D and A, respectively). In contrast, N94 and Y97 are located on the opposite side of this face. Reduction of virus binding was accompanied by lower transgene expression in several but not all constructs.

**DISCUSSION**

A common feature of proteins with SCR domains is that they recognize virus particles through two or more of those domains. Thus, our findings that the two N-terminal domains of CD46 are sufficient for Ad35 binding is in line with the results obtained from studies of other pathogen RCA receptor interactions. For example, complement regulatory protein CR2/CD21, containing 15 or 16 tandemly arranged SCR domains, binds Epstein-Barr virus with its two external SCR modules (45). The three SCR II-IV modules of CD55, the
closest known relative to CD46, were identified as binding sites for echovirus 7 (7). For CD46 and MV, the two membrane-distal domains SCR I-II are sufficient for hemagglutinin binding, but virus particle attachment is enhanced by SCR III and IV domains (4, 5, 10, 26, 41). Human herpesvirus 6 binds to the SCR II and III domains (22). Finally, binding of Neisseria gonorrhoeae pili to host cells depends on determinants in the SCR III and STP regions (27), whereas the M protein of group A streptococci promotes adhesion to keratinocytes via SCRS III and IV (20). Interestingly, in many cases, the binding sites of the pathogen are different from, or overlap only partially with, the binding site of the natural ligand (1, 20, 41, 50).

As both Ad35 and MV bind to CD46 SCR I-II, it is conceivable that these unrelated viruses share common binding areas. Our data revealed common binding features but also clear differences between the bindings of Ad35 and MV to CD46 (Fig. 5). Binding inhibitions of the CD46-specific monoclonal antibodies commonly used in studies of both viruses revealed similar rankings. For binding inhibition of Ad35, antibodies 13/42 and M177 showed the strongest effect, GB24 an intermediate effect, and E4.3, MCI20.6, and Tra-2 a weak effect. Inhibition of MV soluble hemagglutinin (sH) binding was strongly inhibited by the antibodies 13/42 and M177, whereas MCI20.6 and Tra-2 showed a moderate effect and E4.3 and GB24 a weak effect (4). Thus, for both viruses, antibodies 13/42 and M177 had the strongest blocking effect, whereas the rankings of the other antibodies were exchanged.

Antibodies may exert a long-distance steric effect, as the distance between the two antigen-binding paratopes may vary between 150 Å in a relaxed conformation and 50 Å in a fully compressed conformation. The buried antigen area making contact with the antibody binding site consists of 560 to 855 Å², as determined from crystal structures of antibody/protein complexes (8). In comparison, virus receptor surface areas bury about a two- to threefold larger surface area (2, 6, 32). It has been proposed that strongly inhibiting monoclonal antibodies directly compete for the virus binding site, while the others indirectly hinder the virus receptor (4). This suggests that the two individual epitopes recognized by 13/42 and M177 are contained within the binding areas of both viruses. The binding site of 13/42 was not unequivocally mapped but was tentatively located on the front side below or adjacent to the epitopes of E4.3, MCI20.6, and Tra-2, including the residues D27, K31, and H43 and the P39-T44 loop (Fig. 5) (4). It is unknown if the MEM-258 antibody affects MV binding. The mutation R69A resulted in a complete loss and mutations D70A and E103A in a partial loss of SCR II-specific antibody M177 binding, indicating that at least R69 is contained within the epitope recognized by M177. Even if both viruses bind to the 13/42 epitope on SCR I and the M177 epitope on SCR II, the loop mutation P39-T44 has a detrimental effect on MV but only a weak effect on Ad35 binding, suggesting that binding to this particular region is not shared by the two viruses.

The comparisons of Ad35 and MV sH binding to CD46 single point mutants corroborated this general conclusion but revealed subtle differences. For Ad35 we found a robust reduction of 45% for mutation Y97A and weaker reductions in the range of 20 to 30% for mutations K15A, R25A, K29A Q88A, N94A, Y98A Y106A, and K119A. Binding of MV sH was inhibited by the mutation P39A (50%) and by the mutations E11A and K29A in SCR I and D70A in SCR II (25 to 50%) (4). Another study by Hsu et al. indicated that the double mutation ER24/25AA (adapted numbering) even reduced binding of MV to 20% (25). In addition, peptide inhibition studies combined with genetic modifications over longer stretches indicated that residues 45 to 48 and 94 to 97 are critical for MV binding (40, 46). Thus, only one mutation, K29A, resulted in equivalent reductions of binding to both viruses. The differential sensitivity of Ad35 and MV for binding to the N-glycan ablation mutant I-II/4 dg2 indicated an additional difference in binding behavior. The N-glycan N80 is situated in the SCR I-II interface and N49 on top of SCR I (Fig. 5). Ablation of the N80-glycan led to a more than 10-fold reduction of MV binding (25, 38), whereas for Ad35 we found only a weak 2-fold reduction.

For Ad35, our mapping results in combination with the three-dimensional structure of the outer SCR I+II fragment (6) define a large, glycans-free surface formed by two flexibly linked CD46 domains. The most distal SCR I domain contains the critical residues K15, E24, R25, and K29 but not the cluster of residues P39 to T44 (Fig. 5A to D [best seen in Fig. 5A]). Weaker inhibition effects of the SCR I antibodies E4.3, MCI20.6, and Tra-2 with critical residues E3, R48, and K17, respectively, all located on the upper third of the SCR I domain, suggest that these sites are outside of the putative Ad35 binding area. The critical binding site in SCR II included the Q88, Y106, Y98, and K119, as wells as sites close to R69 and E103, all located on the same surface (Fig. 5A to D [best seen in Fig. 5D]). The latter two sites are defined by antibody M177 inhibition but not by single point mutations. Critical residues N94 and Y97 are located on the face opposite to this binding region. Since these residues are located at the low end of the SCR II module, they may be involved in interdomain contacts and exert an indirect effect on virus binding. Our data indicate that the two putative Ad35 binding areas in SCR I and II of the crystal structure are not exactly on the same side of the molecule but are twisted by about 90°. For MV, the mapping results (4, 25, 40, 46) indicated a slightly shifted binding area extending from near the top of the first to the bottom of the second repeat, including the residues P39, K29, and E11 and the residue clusters R25/D27 and R69/D70 of SCR I-II, all located on the same side of the molecule (Fig. 5E to H [best seen in Fig. 5F]). As in the case of Ad35, both domains are free of carbohydrates and therefore are accessible for protein-protein interactions (6). The interdomain organization, which differs strikingly among the known SCR domain structures (6), may be variable in solution. Indeed, when the three-dimensional structure of the vaccinia virus complement control protein VCP was deduced, the molecule was found to have a rigid, rod-like structure with no intramolecular contacts between nonsequential modules but with a kink between modules I-III and module IV (47). In contrast, a nuclear magnetic resonance-derived structure of VCP SCR II-III alone indicated flexibility between the modules (28). Based on the findings that in RCA proteins more than one SCR domain is involved in protein-protein interactions and that some degree of interdomain flexibility is allowed, it was suggested that the contact surface between viruses and RCA receptors may consist of not a single, narrow, and continuous binding site but rather of several composite contact sites (4, 6). Such multimeric inter-
actions result in higher avidity, thereby increasing affinity between otherwise low-affinity partners (66).

The length of the chimeric CD46-CD4 receptor molecules modulates the efficiency of Ad35 uptake. In the case of MV, inserting an increasing number of Ig-like modules as spacer domains in the chimeric receptor gave a robust increase in MV binding, but fusion efficiency, i.e., productive uptake, was uncoupled and strongly reduced (5). Increased binding may be due to reduction of steric hindrance resulting from moving the binding site away from the membrane. For adenoviruses, proper orientation of the receptor binding site towards the viral ligand as well as secondary receptors can influence the binding efficiency (reviewed in reference 68). In the context of the virus particles, the length of the fiber protein influences the efficiency of viral binding, as shown for the knob of the CAR binding Ad5, which, when placed on the short shaft of another Ad serotype, resulted in a reduction of virus attachment (60, 69). Here we found a robust increase of Ad35 transgene expression when using chimeric receptors with extended lengths, although binding of Ad35 was not strongly influenced by the CD46 length, suggesting that postbinding events depend on proper spacing of viral attachment from the membrane.

The structure of the Ad3 fiber knob revealed an overall shape very similar to that of CAR binding fiber heads, although a more hydrophobic surface is exposed (13). No single exclusive structural motif that disfavors binding to CAR could be deduced, but rather several differences may account for the receptor switch. The CAR binding site is located on the knob side at the interface between two adjacent knob monomers. A strong variation of the ligand binding residues, together with water-filled cavities in the interface, resulting in a low topological complementarity between the two molecules, is a special feature of the Ad fiber-CAR receptor complex (2, 13). It will be interesting to see whether binding of species B Ad knobs to CD46 is based on similar structural features.

ACKNOWLEDGMENTS

We thank Leta Fuchs for technical assistance and F. Ochsenbein for help with graphic design.

This work was supported by grants from the Kanton Zürich (to S.H. and U.F.G.) and by grant 3100A0-103592 from the Swiss National Science Foundation (to S.H.). R.C. was supported by NIH grant R01 CA90636.

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