Species B adenovirus serotypes 3, 7, 11 and 35 share similar binding sites on the membrane cofactor protein CD46 receptor

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We recently characterized the domains of the human cofactor protein CD46 involved in binding species B2 adenovirus (Ad) serotype 35. Here, the CD46 binding determinants are mapped for the species B1 Ad serotypes 3 and 7 and for the species B2 Ad11. Ad3, 7 and 11 bound and transduced CD46-positive rodent BHK cells at levels similar to Ad35. By using antibody-blocking experiments, hybrid CD46–CD4 receptor constructs and CD46 single point mutants, it is shown that Ad3, 7 and 11 share many of the Ad35-binding features on CD46. Both CD46 short consensus repeat domains SCR I and SCR II were necessary and sufficient for optimal binding and transgene expression, provided that they were positioned at an appropriate distance from the cell membrane. Similar to Ad35, most of the putative binding residues of Ad3, 7 and 11 were located on the same glycan-free, solvent-exposed face of the SCR I or SCR II domains, largely overlapping with the binding surface of the recently solved fiber knob Ad11–SCR I–II three-dimensional structure. Differences between species B1 and B2 Ads were documented with competition experiments based on anti-CD46 antibodies directed against epitopes flanking the putative Ad-binding sites, and with competition experiments based on soluble CD46 protein. It is concluded that the B1 and B2 species of Ad engage CD46 through similar binding surfaces.

INTRODUCTION

The 51 serotypes of human adenoviruses (Ads) are classified into six species, A–F (Benkó et al., 2000; Horwitz, 2001), and species B Ads are divided further into B1 and B2 serotypes, for which tropism differs. The B1 group comprises Ad3, 7, 16, 21 and 50 and predominantly infects the upper respiratory tract, whereas the B2 group serotypes Ad11, 14, 34 and 35 are associated with kidney and urinary-tract infections, with fatal outcome in immunocompromised patients (Leen & Rooney, 2005; Schmitz et al., 1983; Wadell, 2000).

Species B Ads bind a different cell-surface receptor from most of the species A, C, D, E and F Ad serotypes, which bind to the coxsackievirus and adenovirus receptor (CAR) (Bergelson et al., 1997; Defer et al., 1990; Roelvink et al., 1998; Stevenson et al., 1995). Several groups have identified the membrane cofactor CD46 as an attachment receptor for species B serotypes, including Ad11 (Segerman et al., 2003b), Ad35 (Gaggar et al., 2003) and Ad3 (Sirena et al., 2004). CD46 belongs to a family of regulators of complement activation, whose biological role is to prevent complement activation on autologous tissue (Liszewski et al., 1991). Based on virus competition experiments and antibody-mediated blocking of various Ads on human cells, it has been suggested that more than one species B receptor exist (Segerman et al., 2003a; Sirena et al., 2004; Tuve et al., 2006), and it remains controversial whether CD46 functions as attachment receptor for all species B serotypes (Gaggar et al., 2003; Gustafsson et al., 2006; Marttila et al., 2005; Segerman et al., 2003b; Tuve et al., 2006). Additional Ads that bind to CD46 include species D Ad37 and 49 (Lemckert et al., 2006; Wu et al., 2004).

CD46 consists of (i) four amino-terminal copies of an approximately 60 aa structural motif termed short consensus repeat

Supplementary methods are available with the online version of this paper.
as virus-binding efficiencies in competition with anti-CD46 antibodies and a soluble, extracellular CD46 domain. The crystal structure of the species B Ad35 knob complexed with CD46 SCR I–II has been published; it revealed three contact areas along the entire length of the glycan-free side of the receptor (Persson et al., 2007). We show here that the binding sites of Ad3, 7 and 11 overlap largely with the earlier predicted binding site for Ad35. Moreover, these sites are in the binding surface of the resolved fiber knob Ad11–SCR I–II three-dimensional (3D) structure.

METHODS

Cells, viruses and enhanced green fluorescent protein (eGFP) expression analyses. Human A549 lung carcinoma and 911 embryonic kidney cells, the mouse fibroblast cell line Ltk− and the baby hamster kidney cell line BHK-21 expressing the BC1 splice variant of the hexon protein by the use of a highly purified wild-type (wt) Ad5 as standard. VP concentration of wt Ad5 itself was determined according to Maizel et al. (1968) and was found to be 1.3 × 1012 VP ml⁻¹. Concentrations determined were 7.1 × 10¹¹ VP ml⁻¹ for Ad3CMV-eGFP, 1.6 × 10¹¹ VP ml⁻¹ for Ad7CMV-eGFP and 6.6 × 10¹¹ VP ml⁻¹ for Ad11CMV-eGFP.

Antibodies and flow-cytometric analysis. Cytofluorometric analysis, CD46- and CAR-specific antibodies and secondary fluorescent chrome conjugates have been described previously (Ebbinghaus et al., 2003; Fleischli et al., 2005). For eGFP expression analysis, triplicates of 1 × 10⁵ cells were infected at an m.o.i. of 1, 10, 100 or 1000 VP. Medium was replaced 3 h post-infection (p.i.) and cells were analysed 2 days p.i. by flow-cytometric analysis.

Virus binding and blocking. Binding and blocking experiments were performed as described previously (Fleischli et al., 2005; Nagel et al., 2003).

Binding and transgene expression using hybrid CD46–CD4 and single point mutant proteins. Generation and composition of hybrid CD46–CD4 constructs, single point mutants and the 6 aa exchange protein P39–T44 were described previously (Fleischli et al., 2005). Transient expression of the hybrid CD46–CD4 and single point mutant proteins was accomplished in two cell types, either mouse Ltk− cells (for binding analysis) or hamster BHK cells (for transgene expression). Cells were grown to 70–90% confluence and were transiently transfected by using the vaccinia virus T7 expression system (Fuerst et al., 1986). Cells were infected with vaccinia virus T7F-3 at an m.o.i. of 10–15 for 40 min. The virus was removed and cells were transfected with plasmid DNA encoding the different CD46 constructs by using the protocol of the manufacturer (Polyplus-transfection SA). The cells were harvested about 20 h after transfection by detaching from the dish by PBS/EDTA treatment. After washing twice with Dulbecco’s modified Eagle’s medium containing 8% fetal bovine serum, the cells were aliquotted for cytomeric analysis (see above), binding assay or transgene expression experiments. For binding assays, between 3.5 × 10⁵ and 5.0 × 10⁵ cells were incubated with radiolabelled virus. For transgene expression, 5 × 10⁴ cells per well were seeded in 12-well plates and allowed to attach for at least 5 h, followed by addition of 750 VP Ad3CMV-Luc and Ad11CMV-Luc per cell. Twenty-four hours p.i., cells were harvested and luciferase activity was determined as described above. Luciferase activities were normalized to the lysate protein concentration. Binding efficiencies and transgene expression levels were obtained by dividing binding values (c.p.m. of bound ³H-labelled Ad−c.p.m. of cells alone) or transgene expression levels [relative luciferase units (RLU) of infected cells−RLU 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. Individual CD46 expression levels were similar in BHK and Ltk− cells; however, background luciferase levels were much lower in BHK cells.

RESULTS

All four B serotype viruses, Ad3, 7, 11 and 35, use CD46 as attachment receptor

We first confirmed and extended observations that CD46 serves as receptor for different species B Ads. A549 lung carcinoma cells, rodent BHK cells or BHK cells stably expressing the BC1 CD46 isoform (BHK-CD46) or CAR (BHK-CAR) (Sirena et al., 2004) were used for binding or transgene expression assays. For comparative binding
assays, cells were incubated with 1000 vp [³H]thymidine-labelled wt Ad3, 7, 11, 35 or species C Ad5 per cell, and the number of cell-associated particles was determined (Fig. 1a). A549, which expressed high levels of CD46 [mean fluorescence intensity (MFI) of 61.8], revealed 4–11% binding of the input viruses, with two- to threefold more Ad35- and Ad11-bound particles than for Ad3 and Ad7. BHK-CD46 cells, expressing about twofold more CD46 (MFI of 113), revealed a slightly higher Ad11 binding (increase of 22%) and a weakly reduced Ad3, 7 and 35 binding (decrease of 36, 27 and 8%, respectively) compared with A549 cells. This could be due to additional, alternative receptor usage of some species B serotypes in human A549 cells (see Discussion). In contrast, parental BHK and BHK-CAR cells bound six to 17 times less labelled species B viruses compared with BHK-CD46 cells. Similarly, high levels of Ad5 binding were found in A549 and BHK-CAR cells, but not in parental BHK or BHK-CD46 cells. When eGFP transgene expression was analysed following inoculation with increasing amounts of recombinant Ad3, 7, 11 and 35 expressing eGFP, a robust 1–2 log increase was documented in BHK-CD46 cells compared with the parental BHK or BHK-CAR cells (Fig. 1b). Of note, although binding of serotype B Ads was similar in

![Fig. 1. CD46-dependent binding and transgene expression of species B adenoviruses. (a) Human A549 cells, rodent BHK cells and CD46- or CAR-transfected BHK cells (5 × 10⁵) were incubated on ice with 1000 vp of the indicated [³H]-labelled species B Ad serotypes. After incubation for 2 h, the cells were washed and cell-associated radioactivity was determined. Mean values and SD of triplicates from one representative experiment are shown. (b) For expression analyses, cells were incubated with eGFP-expressing Ad3, 7, 11, 35 and 5 vectors at increasing m.o.i.s of 1, 10 and 100 vp per cell for A549 and 1, 10, 100 and 1000 vp per cell for BHK cells. eGFP expression was analysed 2 days p.i. by flow cytometry. Results are expressed as mean fluorescence intensity (MFI).]
BHk-CD46 and A549 cells, transgene expression was about 30- to 50-fold lower in BHk-CD46 cells than in A549 cells. A similar effect was found when BHk-CAR cells and Ad5-based eGFP vector were utilized. Thus, the findings that CD46 serves as a receptor for species B Ads 3, 7, 11 and 35 are in full agreement with our earlier identification of CD46 as an Ad receptor with chimeric Ad5 vectors harbouring the fibers of Ad7, 11, 16, 35 or 50 (Fleischli et al., 2005) or Ad3-based eGFP vectors (Sirena et al., 2004, 2005).

**Antibody- and soluble CD46-mediated blocking of species B Ads binding to CD46**

To check whether Ad3, 7 and 11 bind the same distal CD46 SCR domains I and II that are involved in Ad55 binding (Fleischli et al., 2005; Gaggar et al., 2005; Sakurai et al., 2006), seven mAbs with specificity for individual SCR domains were tested for their efficacy to inhibit binding of 3H-labelled Ads to CD46, as reported previously for Ad3 (Sirena et al., 2004) and Ad35 (Fleischli et al., 2005). To this end, BHk-CD46 cells were first incubated with mAbs at various concentrations, followed by addition of [3H]thymidine-labelled Ads and determination of cell-associated virus (Fig. 2a; summarized for all four viruses in Fig. 2b).

The two SCR I-specific mAbs 13/42 and MEM-258, as well as the SCR II-specific mAb M177, strongly inhibited binding of all four serotypes in a concentration-dependent manner. The SCR III/IV-specific mAb GB-24 inhibited Ad3 and 7 binding efficiently, to about 70–80% at the highest concentrations used, compared with about 30% for Ad11 (and Ad35; Fleischli et al., 2005). Finally, the three SCR I-specific mAbs E4.3, MCI20.6 and Tra-2 had generally weaker, but variable, effects on binding of the various serotypes. E4.3 revealed an intermediate 52% inhibition of Ad3 and 7 binding, but essentially no effect on Ad11 and 35 binding. The MCI20.6 antibody had no effect on Ad3 or 7 binding and a significant but weak effect on Ad11 and 35 binding. Finally, the Tra-2 antibody had a stronger effect on Ad3 and 7, with 35% inhibition, than on Ad11 and 35 binding, with 12 and 21% binding inhibition, respectively. Together, these data suggest that, similar to Ad35, mainly SCRs I and II are involved in Ad3, 7 and 11 binding, but that subtle differences in the binding sites may exist.

To characterize the binding affinities of the three different Ads further, we studied the blocking capacity of soluble CD46ex–Fc (Sirena et al., 2004), which forms a dimeric protein containing two CD46 binding sites, similar to CARex–Fc, which leads to Ad5 aggregate formation through multivalency (Ebbinghaus et al., 2001; Meier et al., 2005). We found that the soluble CD46ex–Fc protein, which contains the complete extracellular receptor domain, inhibited binding of all three Ads to BHk-CD46 cells in a dose-dependent manner (Fig. 2c), whereas the control CARex–Fc protein had no significant effect on species B binding (shown only for Ad3). However, CD46ex–Fc protein was about 10-fold more potent at blocking Ad11 (and Ad35; Fleischli et al., 2005) binding compared with Ad3 or 7 binding (Fig. 2c).

**Mapping Ad3-, 7- and 11-binding domains with hybrid CD46–CD4 proteins**

We next mapped the amino acids of CD46 involved in binding Ad3, 7 and 11 by using a set of ten CD46 hybrid receptors. The hybrid receptors consisted of different CD46 SCR domains fused to CD4 Ig-like modules (Buchholz et al., 1996; Mumenthaler et al., 1997). 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 (Fig. 3a, c, e). Binding and luciferase transgene expression were normalized to the cell-surface expression levels of the construct, which could vary by up to threefold. The CD4 domains function as spacers, increasing the distance between the potential CD46-binding site of Ad and the plasma membrane. Single SCR domains have a similar size to Ig domains and each Ig domain is expected to increase the distance to the membrane by about 30 Å (Bork et al., 1996). Overall, no dramatic differences were found for the four constructs when binding of the three viruses was analysed. A small reduction of Ad3 and 11 binding was noticed for the shortest I–IV construct, as well as for Ad7 and the I–IV/4 construct. Construct I–IV/3–4 was slightly more efficient at binding Ad3 and 11. Ad3 and 11-mediated transgene expression increased with receptor length (Ad7 was not included in these analyses). Increasing the receptor length by one, two or four Ig modules increased the Ad3 transgene expression to 150–250% of that of wt CD46, and the latter two constructs also resulted in similarly enhanced Ad11-mediated reporter expression (Fig. 3c, e).

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, I–II/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 site of these domains. We found that an extension of the CD46 SCR I–II construct by two (construct I–II/3–4) or four (construct I–II/1–4) CD4 domains resulted in virus binding similar to that of CD46-expressing cells for all three viruses (Fig. 3b, d, f). This was paralleled by increased transgene expression, reaching maximally about 150% (construct I–II/3–4 and Ad3) or 170% (construct I–II/1–4 and Ad11) of that of CD46 alone. The shorter constructs I–I and I–I/II yielded about 25–50% of wt CD46 binding with all three viruses and a low reduction of transgene expression. Ablation of neither SCR I nor SCR II N-linked glycosylation affected virus binding or transgene expression significantly compared with construct I–II/4. As for Ad35, the data suggest that SCR I and II are sufficient for Ad3, 7 and 11 binding and transgene expression, and that the distance of these domains from the plasma membrane influences transgene expression efficiency.
Mapping Ad3-, 7- and 11-binding residues with 36 single amino acid SCR I–II mutant proteins

To define residues involved in Ad3, 7 and 11 binding, a set of 36 CD46 mutants containing semi-conservative changes of single amino acids within the SCR I–II domains was tested (Buchholz et al., 1997). These mutations involved residues predicted to be on the CD46 SCR I–II solvent-exposed surface (Mumenthaler et al., 1997) and consisted of replacements by small residues (alanine for charged and polar residues; serine for apolar residues). Use of such semi-conservative changes is expected to be tolerated well.
Fig. 3. Ad3, 7 and 11 binding efficiencies and transgene expression mediated by CD46–CD4 hybrid proteins. Proteins were expressed transiently in Ltk− cells (for binding assays) and BHK cells (for transgene expression) by using the vaccinia virus T7 system. CD46 surface-expression levels of the various CD46–CD4 hybrid constructs were normalized to the surface-expression levels of CD46-BC1. Binding efficiencies were calculated by dividing the 3H-labelled Ad 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 Ad3CMV-luc and Ad11CMV-luc 24 h post-transduction 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. CD46 expression levels are indicated for Ltk− cells only, but were similar for BHK cells. Schematics indicate the following domains: SCR I (black), SCR II (hatched), 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. (a, c, e) Function of four hybrid proteins containing SCR I–IV fused to increasing numbers of CD4 Ig-like domains. (b, d, f) 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). Note that transgene expression with Ad7 was not included and that CD46 surface-expression levels are depicted only once in (a) and (b), and are identical in (c), (d), (e) and (f).
and to have minimal impact on protein conformation. Also included was a 6 aa exchange mutant, _39PLATHT_44, containing six alanines replacing the endogenous sequence. Analysis of Ad3, 7 and 11 binding was performed as described for the hybrid constructs, and binding data are summarized in Fig. 4. Not all of the alterations described to decrease Ad35 binding (Fleischli et al., 2005) resulted in similar binding reduction of Ad3, 7 and 11. Among the 19 mutant CD46 proteins with alterations in the SCR I domain, a weak but consistent binding reduction for all four Ad serotypes was documented for the K29A mutations, with about 20% decrease in binding. Both closely and more distantly located residues influenced binding of some, but not all, Ads. For instance, mutations in the K15, E24 and R25 residues reduced binding of Ad11 and 35, but not of Ad3 or 7. Mutation of residue K31 affected Ad7 and 11, but not Ad3 or 35. Similarly, alterations of P39, P39–T44 and L40 affected variably three of the four viruses. These residues are situated on the same face of the SCR I solvent-exposed surface as the epitopes characterized by antibody 13/42, some of them contiguous (Fig. 5a). A second hot spot was found in the SCR II domain around Y97, which resides in the lower half of the SCR II domain. Like Ad35 (Fleischli et al., 2005), Ad3, 7 and 11 bound 40–60% less efficiently to the Y97A mutant than to wt. An additional binding reduction of all three Ads was found with the K110A and K119A mutations. Both of these residues are located on the SCR II face containing the antibody M177 epitope R69 and D70.

**DISCUSSION**

Receptor binding can be a major determinant of viral tropism, and the characterization of receptor-binding mechanisms is of fundamental importance for the development of vectors for targeted gene transfer. Here, we supply evidence that both Ad species B1 and B2 utilize

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**Fig. 4.** Ad3, 7 and 11 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 analysed as described in the legend to Fig. 3. (b) Seventeen single amino acid mutants of SCR II were analysed similarly.
CD46 as an attachment receptor for entry into cells, and document how species B1 interacts with CD46.

The role of CD46 as an attachment receptor for all species B serotypes has been controversial. Marttila et al. (2005) interpreted their data as indicating that CD46 represents a functional receptor for all species B viruses, but not for Ad3 and 7. These authors suggested that Ad3 and 7 bind to an elusive common species B virus receptor (Gustafsson et al., 2006; Marttila et al., 2005; Segerman et al., 2003a, b). These conclusions were based mainly on immunostainings of infected human A549 cells pre-incubated with a polyclonal anti-CD46 antibody or CHO cells expressing the BC1 isoform of CD46. The dynamic range of the infectious readout and the anti-CD46 antibody blocking effect was not defined, and CHO cells expressing only a few CD46 mutants were tested. In studies by Gaggar et al. (2003) and Tuve et al. (2006), all tested species B Ads except for Ad3 and 7 were found to bind to CHO cells expressing the C2 isoform of CD46. In contrast, we measured Ad3 and 7 binding and transgene expression in different rodent cells, including BHK, CHO, L929 and B16 cells. These cells stably express all four major splice forms of human CD46 (this study; D. Sirena, unpublished data; Fleischli et al., 2005; Sirena et al., 2004). We assessed the efficiency of cell entry and gene delivery by eGFP expression by using flow cytometry, which has a higher dynamic range than immunostaining experiments. The discrepancy with respect to the findings of Tuve et al. (2006) and Gaggar et al. (2003) may be attributed to higher CD46 expression levels in our transfected rodent cells compared with the other studies. We measured an eightfold increase of Ad35 binding, compared with a twofold increase reported by Tuve et al. (2006). We do not exclude the possibility that additional receptor(s) for species B serotypes exist, particularly because antibody-blocking experiments in human cells revealed only partial Ad3, 7, 11, 14 and 16 inhibition (Marttila et al., 2005; Segerman et al., 2003b; Sirena et al., 2004; Tuve et al., 2006), unlike in CD46-expressing rodent cells. The analysis of species B serotype interactions with rodent cells expressing high CD46 levels reduces the complexity of virus–receptor interactions. Based on CD46 antibody-blocking studies in K562 (Sirena et al., 2004) or HeLa (Tuve et al., 2006) cells, which revealed a maximum 40% blocking of Ad3 binding, we suggest that the Ad–CD46 interactions may reflect about 40% of Ad3 binding to human cells. In contrast, B2 serotypes may predominantly use CD46 as receptor (Tuve et al., 2006).

Our results demonstrate clearly that the species B Ads 3, 7 and 11 engage CD46 through binding surfaces similar to those described previously for Ad35 (Fleischli et al., 2005; Gaggar et al., 2005; Sakurai et al., 2006). This is based on antibody competition, soluble CD46 competition, binding assays to cells expressing truncated CD46 molecules, and single amino acid mutants of CD46. The proposed glycans-free surface formed by the two flexibly linked CD46 SCR I–II domains (Fig. 5a) overlaps well with the binding surface of the recently resolved fiber knob Ad11–SCR I–II 3D structure, with three contact areas, A, B and C (Fig. 5b) (Persson et al., 2007). Area A, with major contact-forming CD46 residues R25, 34FYI37 and 41ATHT44, overlaps with the epitope of the strongly virus-inhibiting 13/42 antibody, which includes residues D27, K31 and H43 and the P39–T44 loop (Buchholz et al., 1997). The epitope of the second strongly blocking antibody MEM-258 is contained in SCR I, but has not been mapped at the amino acid level (Fleischli et al., 2005; Sakurai et al., 2006). Strongly inhibiting antibodies may compete directly for the virus-binding site (Buchholz et al., 1997). Area A binding also explains our results with single point mutants of CD46, where changes in K29, localized apart from residues 34FYI37, resulted in weak but consistent reduction of binding for all four viruses. In line with these results, we found reduced binding of some, but not all, species B viruses for residues K15, E24, R25, P39, L40 and loop mutation P39–T44. These patterns of Ad binding to the
mutated CD46 receptor correlated well with our antibody-mapping results.

The epitopes of the moderately binding-inhibiting SCR I antibodies are located in the upper third of the SCR I structure, adjacent to the epitope of antibody 13/42 or binding area A. They include the E4.3 antibody, which binds to an epitope containing the critical E3 residue (Fig. 5a; not visible as on opposite site). At the highest concentration used, E4.3 blocked Ad3 and 7 by 52 and 61%, respectively, whereas a weak inhibition of 16% for Ad35 and no significant inhibition of Ad11 were documented. MCI20.6, a second antibody recognizing a different epitope on the SCR I tip with the critical R48 residue, had no effect on Ad3 or 7 and a very weak effect on Ad11 and 35 binding, with 14 and 17% blocking, respectively. Tra-2, a third antibody interacting with SCR I through K17 on the upper third of SCR I, reduced binding by 34, 35, 19 and 21% to Ad3, 7, 11 and 35, respectively. As weakly and moderately inhibiting mAbs may exert their effects indirectly, hindering the virus receptor by steric long-range effects, rather than by overlapping the virus-binding site directly (Buchholz et al., 1997), it was anticipated that these epitopes do not colocalize with the virus-binding sites.

Due to lack of antibodies recognizing epitopes in binding area B, as well as a lack of single point mutants for this area containing $62$RETC65 as major contact residues, this SCR I–II interface site could not be defined further here. Area C, with major contact-forming residues $68$IRDPL72 (Persson et al., 2007), is confirmed by an overlap with the binding site of M177, which maps to the residues R69, D70 and E103 of the lower part of SCR II (Buchholz et al., 1997). The two single point mutants K110A and K119A, with less strong but consistent binding reduction, are located near R69. The same holds true for the single point mutant Y97A, which revealed the highest impact on virus binding for all four serotypes. Of note, residue Y97 is part of the 6 aa exchange mutant 96–101 (adapted numbering), and residue K119 is part of the 5 aa exchange mutant 118–122, both causing complete loss of Ad35-mediated transgene expression (Gaggar et al., 2005). As Y97 is located at the lower end of the SCR II module, it may be involved in interdomain contacts and exert an indirect effect on virus binding (Liszewski et al., 2000). This is particularly interesting because the SCR III–IV domains are positioned at an angle of about 90° relative to SCR I–II (Persson et al., 2007). In the absence of SCR III–IV structural data, the variable effects of the GB24 antibody, recognizing epitopes in SCRIII–IV (Liszewski et al., 2000), on different Ad serotypes are difficult to interpret. Differences in blocking efficiencies of the moderately and weakly blocking antibodies could hint towards the exact binding area. The fact that the binding interface can vary to some extent among the different Ad serotypes has been documented for the CAR-binding serotypes (reviewed by Law & Davidson, 2005). An additional interesting finding is that the Ad3/Ad7 pair and Ad11/Ad35 pair have similar binding patterns. This could relate to differences in the fiber sequences, particularly the two unique hydrophobic residues 240 and 296 shared only by Ad3 and 7 (Chroboczek et al., 1995; Marttila et al., 2005).

Whether such minor binding differences can explain the difference in blocking activity of soluble CD46ex–Fc for Ad3 and 7 is unclear and remains to be investigated. The dimer structure of CD46ex–Fc may not be responsible for this difference, as soluble, extracellular CD46 proteins devoid of Fc showed identical behaviour (data not shown). It is perhaps more likely that Ad3 and 7 bind to an oligomeric form of CD46 that is different from the oligomer bound by Ad11 and 35. Alternatively, an unknown factor could hetero-oligomerize with CD46 and thus modulate the affinities of CD46 for Ad3 and 7, or Ad11 and 35. This differential effect of CD46ex also implies that levels of CD46 are crucial for distinguishing binding of Ad species B1 and B2 serotypes.

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