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Adenovirus 11p downregulates CD46 early in infection

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ABSTRACT

Adenovirus 11 prototype (Ad11p), belonging to species B, uses CD46 as an attachment receptor. CD46, a complement regulatory molecule, is expressed on all human nucleated cells. We show here that Ad11p virions downregulate CD46 on the surface of K562 cells as early as 5 min p.i. Specific binding to CD46 by the Ad11p fiber knob was required to mediate downregulation. The complement regulatory factors CD55 and CD59 were also reduced to a significant extent as a consequence of Ad11p binding to K562 cells. In contrast, binding of Ad7p did not result in downregulation of CD46 early in infection. Thus, the presumed interaction between Ad7p and CD46 did not have the same consequences as the Ad11p–CD46 interaction, the latter virus (Ad11p) being a promising gene therapy vector candidate. These findings may lead to a better understanding of the pathogenesis of species B adenovirus infections.

Introduction

Human adenoviruses (Ad) are nonenveloped, double-stranded DNA viruses. They have been divided into seven species (A–G) currently containing 52 recognized types (Benkö et al., 2000; Jones et al., 2007; Wadell et al., 1980). Human adenoviruses have a broad spectrum of tropism, and different types can cause the same or distinct infections. The outer protein capsid structure mainly consists of three polypeptides: the hexon, the penton base, and the fiber (Berk, 2005; Maizel et al., 1968). There is one pentameric penton base with a protruding trimeric fiber situated at each of the 12 vertices of the virus particle, while most of the icosahedral capsid is made up of hexons (Hong and Engler, 1996; Valentine and Pereira, 1965; Xia et al., 1995). The major determinant for attachment of Ads to host cells is the C-terminal knob domain of the fiber protein (Arnberg, 2009). Ad11p and the majority of other species B serotypes except perhaps Ad3 and Ad7 use CD46 as a cellular receptor (Gaggar et al., 2003; Marttila et al., 2005; Segerman et al., 2003). However, it has also been suggested recently that Ad7p and Ad3 can also bind to CD46 and use similar binding sites on the molecule as used by other species B serotypes (Fleischli et al., 2007; Sirena et al., 2004).

CD46, also known as membrane cofactor protein (MCP), is a complement regulatory protein that is expressed on all nucleated cells (Riley-Vargas et al., 2004). C3b and C4b are natural ligands to CD46 (Oksjoki et al., 2007). CD46 mediates their breakdown, acting as a cofactor for serine protease factor I, and thus protects host cells from homologous complement attack (Oksjoki et al., 2007; Riley-Vargas et al., 2004). Other important cell surface receptors that regulate complement activity are CR1, CR1q, CD55, and CD59 (Oksjoki et al., 2007). A number of pathogens including human herpesvirus 6 (HHV-6), the Edmonston strain of measles virus, bovine viral diarrhea virus (BVDV), *Neisseria gonorrheae*, *Neisseria meningitides*, and *Streptococcus pyogenes* all use CD46 as a receptor (Cattaneo, 2004). Human CD46 is alternatively spliced into several isoforms, resulting in a varying number of extracellular domains and two different cytoplasmatic tails, CYT1 and CYT2 (Seya et al., 1999). CD46 seems to be constitutively recycled from the cell surface via clathrin-coated pits and transported to perinuclear multivesicular bodies (Crimeen-Irwin et al., 2003).

Measles virus (MV) and Ad35 binding to CD46 leads to downregulation of CD46 expression on the cell surface (Gill et al., 2003; Gill et al., 2005; Russell, 2004; Sakurai et al., 2007). Binding of MV to CD46 on epithelial cells leads to rapid internalization, whereas binding of *N. gonorrheae* results in shedding of CD46. The biological implications of modulation of CD46 expression are not fully understood (Dempsey et al., 1996; Riley-Vargas et al., 2004). Antibody crosslinking of CD46 induces macropinocytosis, followed by rapid intracellular degradation. MV-mediated downregulation of CD46 alters MHC class I or class II presentation of antigens (Gerlier et al., 1994a,b). Binding of Ad16 and Ad35 to CD46 has been shown to mediate reduction of IL-12 levels in IFN- or lipopolysaccharide-stimulated human peripheral blood mononuclear cells (Iacobelli-Martinez et al., 2005).

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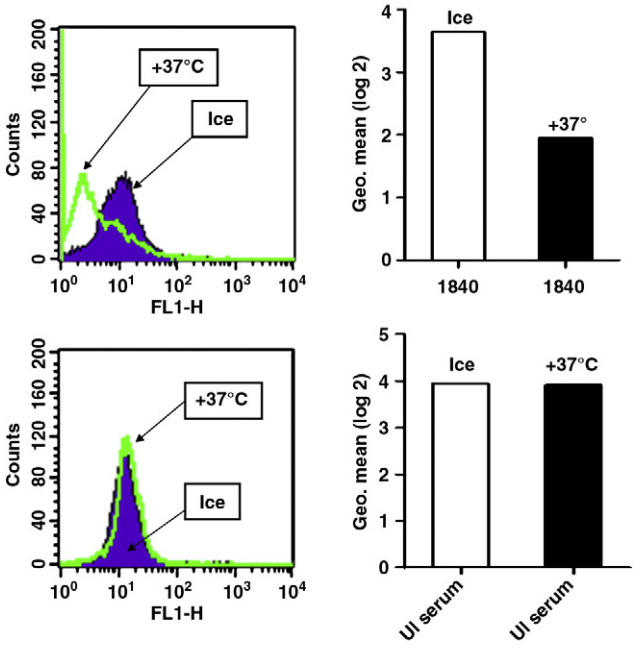

Fig. 1. Polyclonal antiserum downregulates CD46 at the cell surface. Polyclonal anti-CD46 antiserum (1840) downregulated CD46 at the K562 cell surface. K562 cells were incubated with this antiserum (1840) or unimmunized rabbit serum (UI serum) for 4 h at 37 °C or on ice. Histograms show CD46 levels that were detectable at the cell surface after incubation with antiserum. Bar graphs show the geometric means (Geo. Means) of histograms. The data shown here are representative of two independent experiments with duplicate samples.

Studies in transgenic mice have shown that human CD46 CYT1 inhibits contact hypersensitivity reactions in mouse T cells, while CD46 CYT2 increases them (Marie et al., 2002). Also, internalization of CD46 makes cells more susceptible to complement-mediated lysis (Marie et al., 2002; Schneider-Schaulies et al., 1996; Schnorr et al., 1995). It has been shown recently, however, that CD59 inactivation—or combined CD55 and CD59 inactivation—sensitizes lung cancer cell lines to complement-mediated lysis, while CD46 inactivation does so to a lesser extent (Ajona et al., 2007). Recently, however, reported results showed that modified Ad35 rfiber (Ad35rfiber^{++}) with increased affinity for CD46 sensitizes tumor cells of lymphoma disease for complement-dependent cytotoxicity (Wang et al., 2010).

We set out to determine if CD46 is downregulated by binding of Ad11p virions. Fleischli et al. (2007) have recently shown that Ad7 and Ad3 virions share a low-affinity binding site on CD46 with other CD46 binding adenoviruses. Thus, we wanted to investigate the role of the possible low-affinity interaction between Ad7p virions and CD46 in the context of CD46 downregulation. We show here using flow cytometry that cell surface CD46 levels are rapidly downregulated by the presence of Ad11p virions. We found that specific binding between the fiber knob of Ad11p and CD46 is required for down-regulation of CD46. In addition, the cell surface levels of the closely related complement regulatory factors CD55 and CD59 vary greatly among cell lines used. In this regard, exposure to Ad11p virions clearly reduced CD55 and CD59 on K562 cells but such downregulation effect was barely discernible on A549 cells.

Results

Adenovirus 11p virions downregulate CD46 expression on the cell surface

To determine whether CD46 levels are downregulated by Ad11p virions, we employed a flow cytometry assay that detects cell surface CD46 levels on K562 cells (see Materials and methods). It has been shown previously that serum crosslinking of CD46 induces down-regulation by macropinocytosis (Crimeen-Irwin et al., 2003). To establish that the same mechanism was present in the clone of K562 cells used in the experiments, we repeated the antibody cross-linking experiment originally performed by Crimeen-Irwin et al. (2003). In short, polyclonal rabbit serum against CD46 was added to K562 cells and incubated for 4 h at 37 °C or on ice. Cells were then stripped (Crimeen-Irwin et al., 2003), and cell surface CD46 levels were detected by FACS after incubation with monoclonal FITC-conjugated anti-CD46 antibody. Polyclonal rabbit antiserum (1840) to CD46 (incubated for 4 h) induced almost total downregulation of CD46 on the K562 cell surface at 37 °C (Fig. 1). In contrast, the CD46 levels of the control cells incubated on ice were not affected by serum crosslinking (Fig. 1). Also unimmunized rabbit serum failed to downregulate CD46 (Fig. 1). We then proceeded to determine whether Ad11p virions could cause downregulation of CD46 expression on the K562 cell surface. Increasing amounts of Ad11p virions were added to K562 cells resuspended in 1% FCS RPMI medium at 30 min and 4 h (37 °C) (Fig. 2). Maximal surface downregulation of CD46 was observed after 30 min and at a concentration of 8×10^4 virions/cell (Fig. 2). Having established the amount of virions/cell

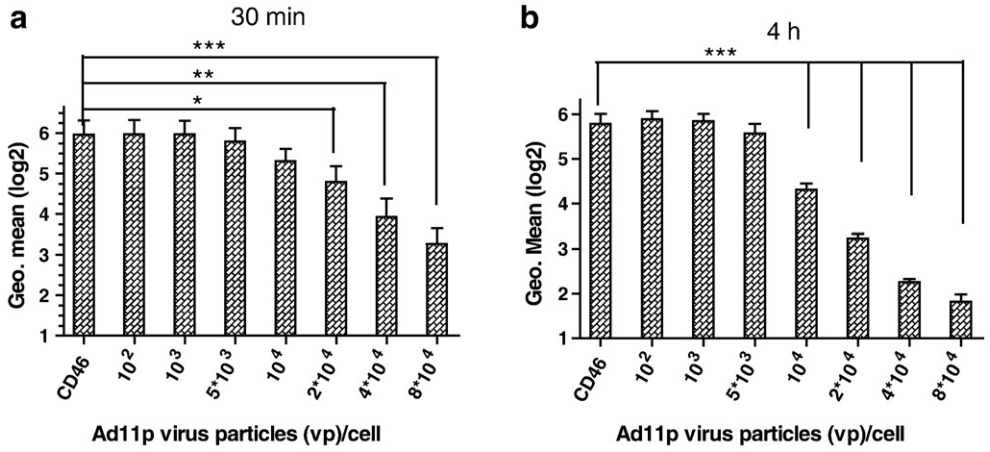

Fig. 2. CD46 level is downregulated on the K562 cell surface by treatment with Ad11p virions. K562 cells were incubated with increasing amounts of virus particles per cell for two different lengths of time, 30 min, or 4 h. The results shown here are means ± standard errors of means (SEM) of duplicate samples from two independent experiments. Significant differences from the results of incubations with different amounts of virions/cell are shown: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

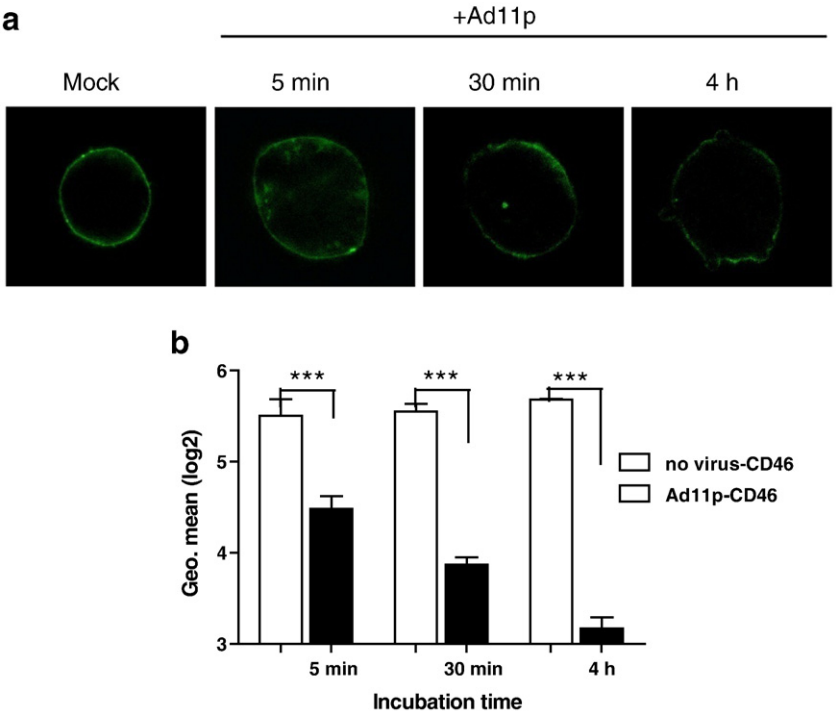

Fig. 3. Levels of CD46 on the K562 cell surface is rapidly downregulated by Ad11p virions. K562 cells were incubated with 4×10^4 Ad11p virus particles per cell for 5, 30 min, or 4 h at 37 °C. (a) Epifluorescence images of CD46 downregulation with Ad11p were acquired on an Olympus CellR imaging station (Olympus Biosystems) equipped with an inverted microscope (IX81; Olympus), a 100×1.4 A Planapochromat objective, and a cooled CCD camera (Orca ER; Hamamatsu Photonics). (b) The results are means ± standard errors of means (SEM) of duplicate data from two independent experiments. No virus–CD46 represents the CD46 levels on cell surfaces when no virus particles were added, and Ad11p-CD46 represents the CD46 levels on cell surfaces when virus particles were added. Significant differences from results of incubations with no virus present (control–CD46) are shown above each bar: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

needed for maximal downregulation, we then proceeded to investigate the time dependency of the downregulation of CD46 expression by Ad11p (Fig. 2).

A significant downregulation of CD46 by Ad11p virions could be detected already after 5 min at 37 °C, and increased in a time-dependent fashion to reach a maximum at 4 h as seen by inspection through an inverted microscope or by flowcytometric analysis (Figs. 3a and b). The antibody MEM-258 was used to detect CD46 at the cell surface after virus binding. This antibody has been shown to interact with the SCR1-2 region of CD46. Since this is the binding region for species B adenoviruses (Fleischli et al., 2007), we performed additional experiments in which cell surface CD46 levels after virus binding were detected with polyclonal antiserum to CD46 (H-294) or monoclonal antibody (E4.3) (Materials and methods), both antibodies

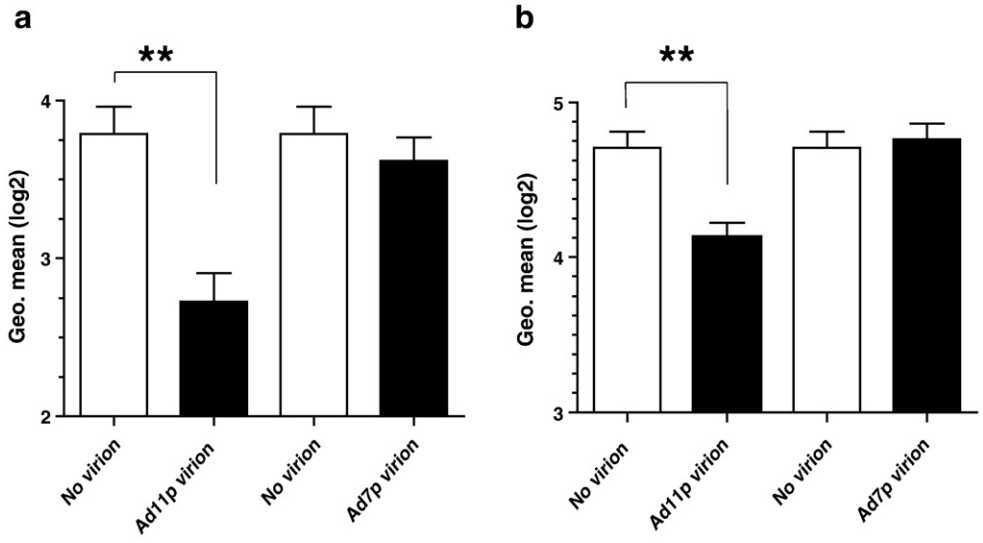

Fig. 4. Downregulation of cell surface CD46 by Ad11p is also detected with different antibodies. K562 cells were incubated with Ad11p or Ad7p virions for 30 min at 37 °C. CD46 was detected with polyclonal anti-CD46 antibody (H-294) (a) or monoclonal anti-CD46 antibody (169-1-E4.3; Ancell) (b). The data shown here are means ± standard errors of means (SEM) of duplicate data from three independent experiments. No virion indicates CD46 level when no virus particles were added; Ad11p virion or Ad7p virion indicates CD46 level when Ad11p or Ad7p virus particles (4×10^4 particles per cell) were added. Significant differences from the results of incubations with no virus present marked as control cells are shown above each bar: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

recognize different epitopes of CD46 molecules. With the additional poly- or monoclonal antibodies, a corresponding downregulation of CD46 was observed 30 min after incubation with Ad11p virions (Fig. 4). Thus, we concluded that the observed downregulation of CD46 was not simply due to blockage of the target epitopes for the detecting antibody as a result of virus binding (Fig. 4). In summary, these results show that CD46 levels on the K562 cell surface is downregulated by Ad11p virions in a dose- and time-dependent manner.

CD46, CD55, and CD59 on K562 cells and only CD46 on A549 cells were downregulated by Ad11p virions to a significant extent

It has been shown previously that downregulation of CD46 expression sensitizes cells to complement-mediated lysis (Krantic et al., 1995), and also that CD55 and CD59 are of importance for protecting cells against complement (Ajona et al., 2007). In a recent publication, Fleischli et al. (2007) showed that Ad3 and Ad7p bind to CD46. We have previously shown that the fiber knobs of Ad7p do not bind to CD46 in the same fashion as Ad11p fiber knobs (Gustafsson et al., 2006). Thus, we wanted to investigate the biological significance of the binding affinity of Ad7p for CD46. We used a flow cytometry assay to determine the effect of incubation of K562 or A549 cells with Ad11p or Ad7p virions on the levels of CD46, CD55, and CD59 at the cell surfaces. We began by establishing the normal control, levels of CD46, CD55, and CD59 on K562 and A549 cells (Fig. 5a). Incubation of K562 cells with Ad11p virions caused significant downregulation of CD46 at the cell surface, whereas Ad7p virions did not ($P<0.001$ for 30 min, 4 h and 8 h of incubation) (Figs. 5b and c). CD55 and CD59 levels were also significantly reduced at 4 h or 8 h, compared to untreated K562 cells (Figs. 5b and c). Incubation of K562 cells with Ad7p virions did not lead to any significant downregulation of CD46, or CD59 at 30 min, 4 h or at 8 h ($P>0.05$) but did downregulate CD55 to some extend at 4 h or 8 h (Figs. 5b and c).

The same experiment was then carried out with A549 cells (Figs. 5d and e). Again, CD46 levels at the cell surface were downregulated by Ad11p virions at 30 min, 4 h, and 8 h; however, a general downregulation of CD55 and CD59 level was observed at the 8 h time point (Figs. 5d and e). The extent of CD59 downregulation was smaller than that of CD46 downregulation at the same time

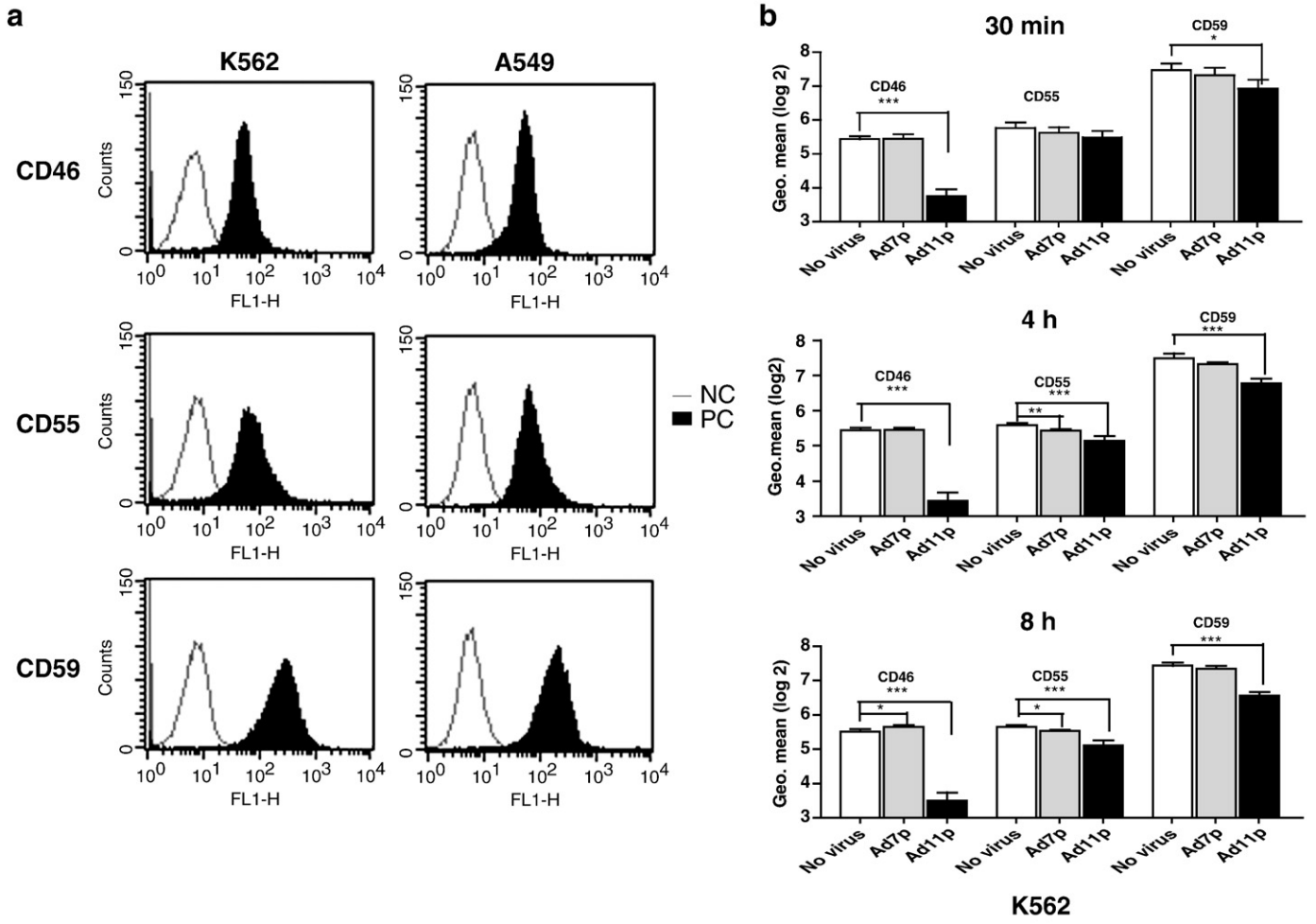

Fig. 5. (a) Cell surface levels of CD46, CD55, and CD59 on K562 and A549 cells. Filled histograms (PC) represent cell surface expression of CD46, CD55, or CD59. Open histograms (NC) represent cell autofluorescence when isotype IgG FITC-conjugated negative control antibody was added. (b) Ad11p virions but not Ad7p virions downregulate cell surface levels of CD46 on K562 cells. Columns represent cell surface expression of CD46, CD55, or CD59 after incubation with Ad11p or Ad7p virions (at 4×10^4 virions per cell) for the indicated times. The data shown here are means ± standard errors of means (SEM) of duplicate samples from two different experiments. No virus represents CD46, CD55, and CD59 levels when no virus particles were added; and Ad7p or Ad11p represents CD46, CD55, and CD59 levels when virus particles were added. Significant differences from results of incubations with no virus present are shown: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (c) Histograms show the levels of CD46, CD55, and CD59 on K562 cell surface after incubation with Ad11p and Ad7p for 30 min, 4 h or 8 h respectively. (d) Ad11p virions specifically downregulate cell surface levels of CD46 on A549 cells very early in infection. Bars represent cell surface expression of CD46, CD55, or CD59 after incubation with Ad11p or Ad7p virions (at 4×10^4 virions per cell) for the prescribed times. The data shown here are means ± standard errors of means (SEM) of duplicate samples from two different experiments. No virus represents receptor levels when no virus particles were added; and Ad7p or Ad11p represents CD46, CD55, and CD59 levels when virus particles were added. Significant differences from results of incubations with no virus present are shown: $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (e) Histograms show the levels of CD46, CD55, and CD59 on A549 cell surface after incubation with Ad11p and Ad7p for 30 min, 4 h, or 8 h.

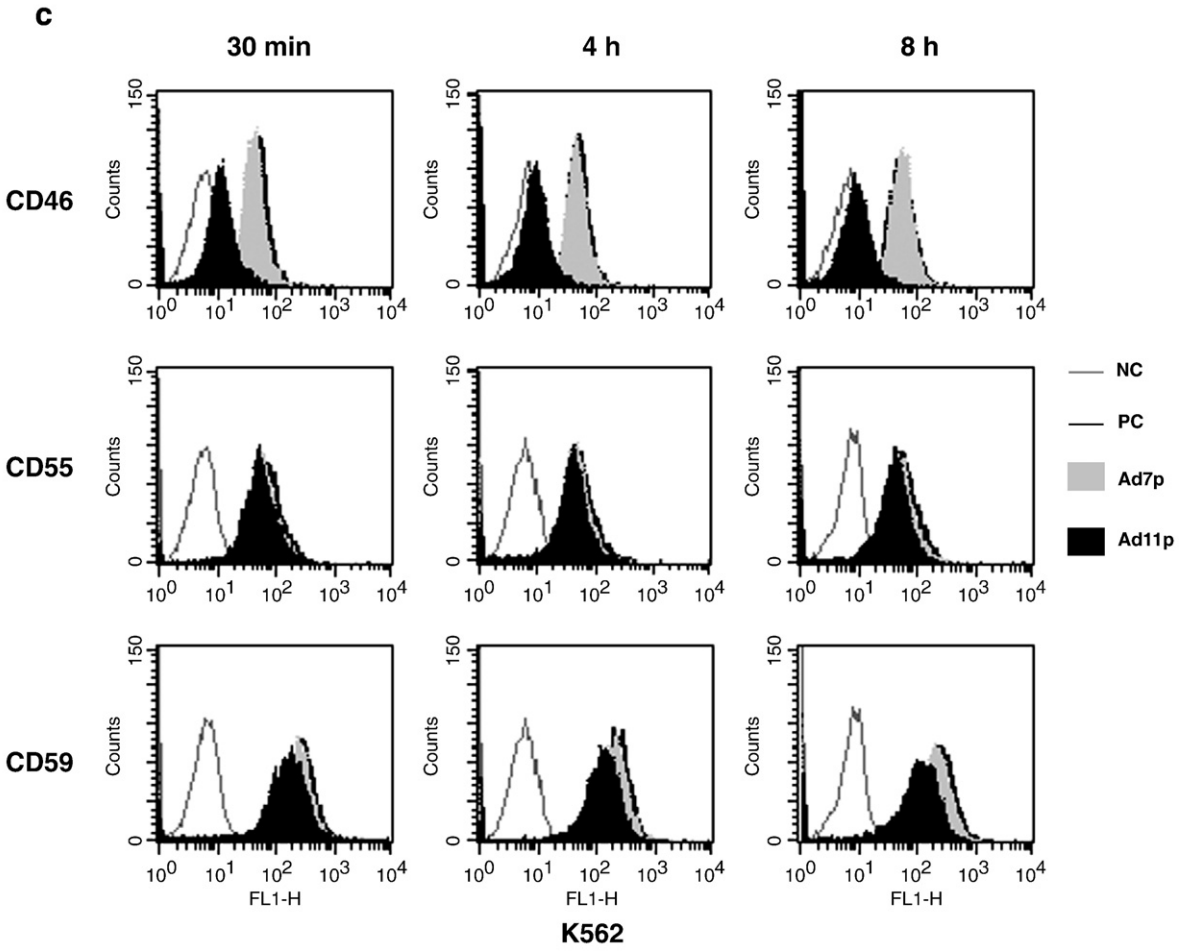

Fig. 5 (*continued*).

points. Ad11p virions, but not Ad7pp virions, mediate specific downregulation of CD46 on A549 cells at 30 min and 4 h. At 8 h, however, there was general downregulation of CD46, CD55, and CD59 on A549 cells after incubation with either Ad11p or Ad7p virions (Figs. 5d and e).

Binding of the Ad11p fiber knob to CD46 is required for downregulation of CD46 at the cell surface

The main adenovirus-specific determinant for virus binding to the cell surface is the fiber knob (Bewley et al., 1999; Philipson et al., 1968; Stevenson et al., 1997; Valentine and Pereira, 1965). We therefore hypothesized that the recombinant Ad11p fiber knob (Ad11pwt-rFK) should be sufficient to downregulate CD46 at the cell surface. In our experiments, we also included a mutant form of the Ad11pwt-rFK (11p-R279Q-rFK) that has previously been shown not to bind to CD46 or to bind to CD46 with an affinity similar to that of Ad7pwt-rFKs (Gustafsson et al., 2006). K562 cells were incubated for 30 min or 8 h at 37 °C with Ad11wt-rFK, Ad7pwt-rFK (Fig. 6a), or with Ad11pwt-rFK, Ad11p-R279Q-rFK (Fig. 6b). Ad11wt-rFK but not Ad7pwt-rFK caused significant downregulation of CD46 on K562 cells already at 30 min and to a somewhat greater extent at 8 h (Figs. 6a and b). Ad11p-R279Q-rFK also affected the CD46 levels when incubated with K562 cells at 30 min and to a greater extent at 8 h (Fig. 6b). The downregulation of CD46 mediated by Ad11p-R279Q-rFK was less prominent at both time points than that caused by Ad11wt-rFK. In contrast, Ad7pwt-rFK did not cause any significant CD46 downregulation at any time point. The results of this experiment show that the presence of recombinant fiber knobs from wild-type Ad11p (not only whole virions) is sufficient to cause downregulation of CD46 expression. The phenomenon therefore appears to be caused by Ad11p fiber knobs.

Discussion

Ad11p uses the ubiquitous CD46 molecule as a receptor for attachment (Marttila et al., 2005; Segerman et al., 2003), the expression of which can be upregulated on solid tumors (Maizel et al., 1968; Russell, 2004). Furthermore, there is a low seroprevalence of Ad11p in the general adult population. Consequently, Ad11p is a strong vector candidate for gene therapy (Stone et al., 2005; Vogels et al., 2003). We have also characterized the region in the recombinant fiber of Ad11p that mediates binding to CD46, by mutation studies (Gustafsson et al., 2006). These results have been verified by cocrystallization of Ad11p rFK with CD46 (Persson et al., 2007). Binding of Ad11p to CD46 mediates a conformational change in the SCR1 and SCR2 domains (Persson et al., 2007). Ad35, measles virus, and the *N. gonorrheae* pilus induce downregulation of CD46 after attachment (Gill et al., 2003; Krantic et al., 1995; Sakurai et al., 2007).

Binding of Ad11p to CD46 is somewhat different from that of Ad35 to CD46. The FG loop of the Ad35 fiber knob is closer to SCR1 of CD46 and the IJ loop is shorter in Ad35 (Wang et al., 2007), thus being positioned further away from SCR2 than the corresponding loop of Ad11. Also, four important amino acid positions (Phe242, Arg279, Ser282, and Glu302) have been identified in Ad35, whereby a mutation in any one of these can prevent CD46 binding. In contrast, only a single mutation at position 279 (normally also Arg) in the Ad11p fiber knob appears to be critical for binding to CD46 (Gustafsson et al., 2006).

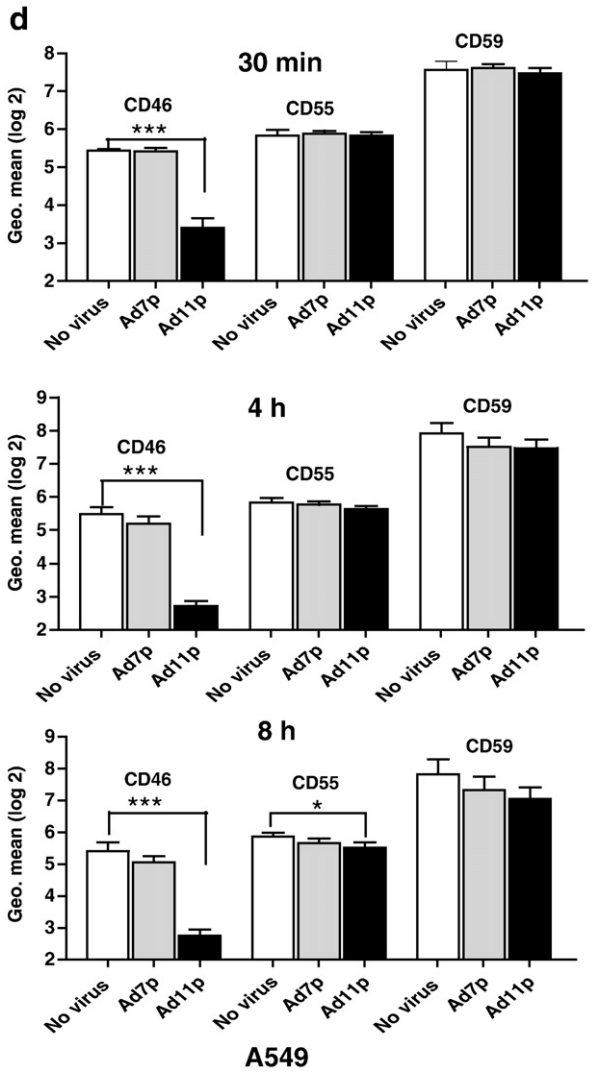

Fig. 5 (*continued*).

Fleischli et al. (2007) have shown that low-affinity binding of Ad3 and Ad7p to CD46 is plausible. We therefore set out to investigate whether Ad11p or Ad7p can downregulate CD46 expression at 37 °C. CD46 was found to be specifically downregulated by Ad11p virions but not by Ad7p virions. This was observed at early stages of infection on both A549 and K562 cells. We also found that recombinant fiber knobs from Ad11p downregulate CD46 expression at the cell surface, but Ad7p failed to significantly downregulate CD46 at concentrations of up to 4×10^4 virions per cell. If Ad7p and Ad11 do indeed share CD46 as an attachment receptor, differences in binding to CD46 could give rise to different downstream immunomodulatory signals. Since CD46 probably has an important role in the control of inflammation, this could provide an explanation for the aggressive respiratory tract pathogenicity of Ad7p in younger individuals and the severe urinary tract infections caused by Ad11 in immunocompromised individuals (Astier, 2008; Choi et al., 2006; Leen and Rooney, 2005; Manalo et al., 1971).

Since CD46 protects against autologous complement lysis of cells (Russell, 2004), it is also of importance to investigate the downregulation of CD46 by Ad11p virions, as this adenovirus serotype may well be used as a vector system for gene therapy in the future. We have confirmed the expression of complement regulatory factors (CD46, CD55, and CD59) on the surface of K562 and A549 cells. We also demonstrated that the relative densities of the factors are similar on the cell lines studied and the expression profile is CD59>CD55>CD46, which corresponds to the levels of these factors on pancreatic cells, oral squamous cells, and breast carcinoma cells (Ravindranath and Shuler, 2007). We have also shown here that Ad11p specifically downregulated CD46 expression on the surface of K562 and A549 cells and apparently reduced the levels of CD55 and CD59 on K562 cells and also late on in infection of A549 cells. We noted that binding of Ad7p to A549 cells caused a partial downregulation of CD46, and also of CD55 and CD59. This was most prominent after 8 h of incubation. Ad11p caused significant downregulation of CD46 in A549 cells at 30 min but also showed the same general downregulation of CD46, CD55, and CD59 after 8 h as compared to Ad7p. Since both serotypes replicate in A549 cells, we hypothesize that the experimental conditions and lytic infection cycle of the virus leads to a general downregulation of receptors at 8 h. The mutant fiber knobs of Ad11p (Ad11p-R279Q-rFK) were also found to downregulate CD46 to some extent but not as efficiently as the downregulation mediated by wild-type recombinant fiber knobs (Ad11pwt-rFKs). We propose that this may be a function of possible differences in affinity for CD46, Ad7pwt fiber knobs having the lowest affinity and Ad11p-R279Q-rFK having a somewhat higher affinity but not being able to bind to CD46 as efficiently as Ad11pwt fiber knobs while still perhaps managing to create the necessary conformational change or dimerization of the receptor required for down-regulation.

Studies have shown that downregulation of CD46 expression makes cells more exposed to complement challenge (Schneider-Schaulies et al., 1996). CD46 acts as a cofactor for inactivation and cleavage of C3b and C4b by serine protease factor I (Russell, 2004; Schneider-Schaulies et al., 1996); it thus brings about inhibition of the complement cascade by preventing an upstream event leading to C5b formation. CD55 accelerates the decay of C3 and C5 convertase, thus inhibiting the formation of C3b and C5b (Oksjoki et al., 2007). CD59 in turn prevents assembly of the membrane attack complex (MAC) (Oksjoki et al., 2007). In a recent publication, there is some evidence for a partial compensation of the protective function of CD46 by CD55 and CD59 (Ajona et al., 2007). This might suggest that the Ad11p-mediated downregulation of CD46 does not per se render the host cell totally unprotected against complement challenge. CD46 expression is upregulated on breast cancer cells, while the levels of CD55 and CD59 are downregulated (Madjd et al., 2005; Rushmere et al., 2004). This would in theory provide a tumor type with complement regulation resting mainly on CD46 levels. It is however interesting that binding of Ad11p to K562 cells does significantly affect the cell surface levels of CD55 and CD59, as this would make the host cell sensitive to cell lysis mediated by the complement system. Thus, downregulation of CD46 after infection could sensitize certain tumor cells to complement-mediated lysis, providing an additional tumor-lytic effect. With the high accessibility of breast tumors for *in situ* administration, the use of adenovirus vectors as novel adjuvant therapy seems at least theoretically feasible.

In this paper, we have shown that Ad11p virions and recombinant fiber knobs downregulate CD46 expression in a time- and dose-dependent manner. We have also demonstrated that the cell surface levels of CD55 and CD59 down-regulation vary greatly in various cell lines and at different time points p.i. Ad11p virions induced a more effective downregulation in the K562 cells of hematopoietic origin than in the epithelial A549 cells. These results are in agreement with the results of previous studies on downregulation of CD46 expression by Ad35 (Sakurai i et al., 2007), whereas the moderate effect with Ad7p perhaps provides a clue to the biological relevance of low-affinity binding to CD46.

Materials and methods

Cells, viruses, antibodies, and recombinant fiber knobs (rFKs)

(i) **Cells**. Human respiratory epithelial A549 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO) containing 5% fetal calf serum (FCS), HEPES, and penicillin–streptomycin (all from Sigma). K562 cells were

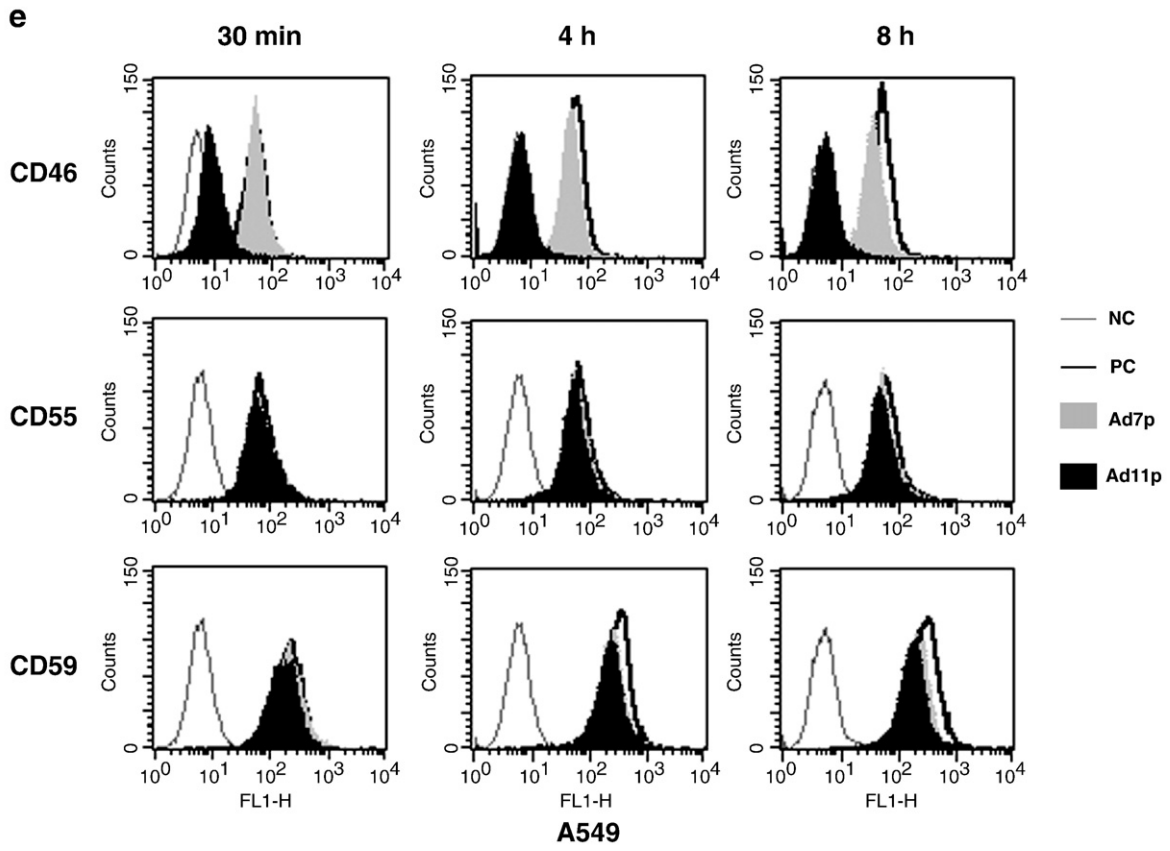

Fig. 5 (*continued*).

established from a chronic myelogenous leukemia in terminal blast crisis, and the cells are multipotential blasts that spontaneously can differentiate into progenitors of the erythrocytic, granulocytic, and monocytic series (Lozzio et al., 1981). K562 cells were grown in RPMI (Sigma) containing 10% fetal calf serum (FCS), HEPES, and penicillin–streptomycin.

(ii) **Viruses.** Adenovirus type 11p (prototype strain Slobitski) and 7p (prototype strain Gomen) were grown in A549 cells and purified in CsCl gradients as previously described (Mei et al., 1998). The quality of each viral preparation was controlled by a ratio of infectious particles versus physical particles (IP/PP). In this study, the ratio of IP/PP was 1:72 for Ad11p and 1:23 for Ad7p viral preparation. Virion concentration was determined by spectrophotometry (at 260 and 330 nm) according to the following: one unit of absorbance at $A_{260} - A_{330}$ is equivalent to 10^{12} particles/ml or 280 μg of viral particles per milliliter (Segerman et al., 2000). Both viruses were typed according to their DNA restriction enzyme patterns as previously described (Adrian et al., 1986).

(iii) **Antibodies**. The 1840 rabbit antiserum, raised against the recombinant extracellular domain of CD46, was a kind gift from Dr. Sarah M. Russell (Peter MacCallum Cancer Center, Trescowthick Research Laboratories, East Melbourne, Australia). Unimmunized rabbit serum was obtained from rabbit 471 on January 29, 1969 (Prof. G Wadell, Department of Virology, Umeå University, Sweden). Mouse monoclonal MEM-258-FITC antibody against human CD46 (Biolegend, San Diego, CA), mouse monoclonal antibody conjugated with FITC against anti-human CD46 (169-1-E4.3; Ancell); polyclonal anti CD46 antibody H-294 (Santa Cruz Biotechnology, Inc.), mouse monoclonal FITC-conjugated antibody against human CD55, CD59 (BD Pharmigen, San Diego, CA), and mouse monoclonal isotype IgG FITC-conjugated control antibody, X0927 (DAKO A/S), were all used according to the manufacturers' recommendations in concentrations described below.

(iv) **Recombinant fiber knobs (rFKs)**. DNA isolation and manipulation were performed by standard techniques. DNA fragments encoding the fiber knob of Ad11p and Ad7p were amplified by PCR with primers described previously (Gustafsson et al., 2006), preserving the last shaft motif and the trimerization signal. Point-mutated constructs of Ad11p-R279Q-rFK were created using the Quick Change kit (Stratagene, La Jolla, CA) according to the manufacturer's guidelines, using previously described primers. All PCR products were cloned into the pET-3d expression vector (Novagen, Madison, WI) containing an N-terminal His_6 tag motif by using previously described methods (Gustafsson et al., 2006). The identity of the construct was confirmed by nucleotide sequence analysis. Proteins were expressed in *Escherichia coli* (strain BL21) and purified using nitrilotriacetic acid (NTA) agarose according to the guidelines of the manufacturer (Qiagen, Hilden, Germany). All recombinant proteins were visualized by Coomassie brilliant blue staining of both denaturing (SDS–) and native polyacrylamide gels (not shown).

Flow cytometric assay of cell surface levels of CD46, CD55, and CD59

Adherent A549 cells were harvested with phosphate-buffered saline (PBS) containing 0.05% (wt./vol.) EDTA. Cells were allowed to recover for 1 h in DMEM containing 5% FCS (5% FCS DMEM) under constant agitation and they were then washed twice with 1% FCS DMEM. Suspension cultures of K562 cells were harvested by centrifugation and washed twice with 1% FCS RPMI medium. They were then resuspended at 4×10^4 cells per tube, in 1.5-ml eppendorf tubes. After centrifugation at $290 \times g$ for 5 min at 4 °C or 37 °C, the supernatant was removed, and virus suspension or fiber knob protein

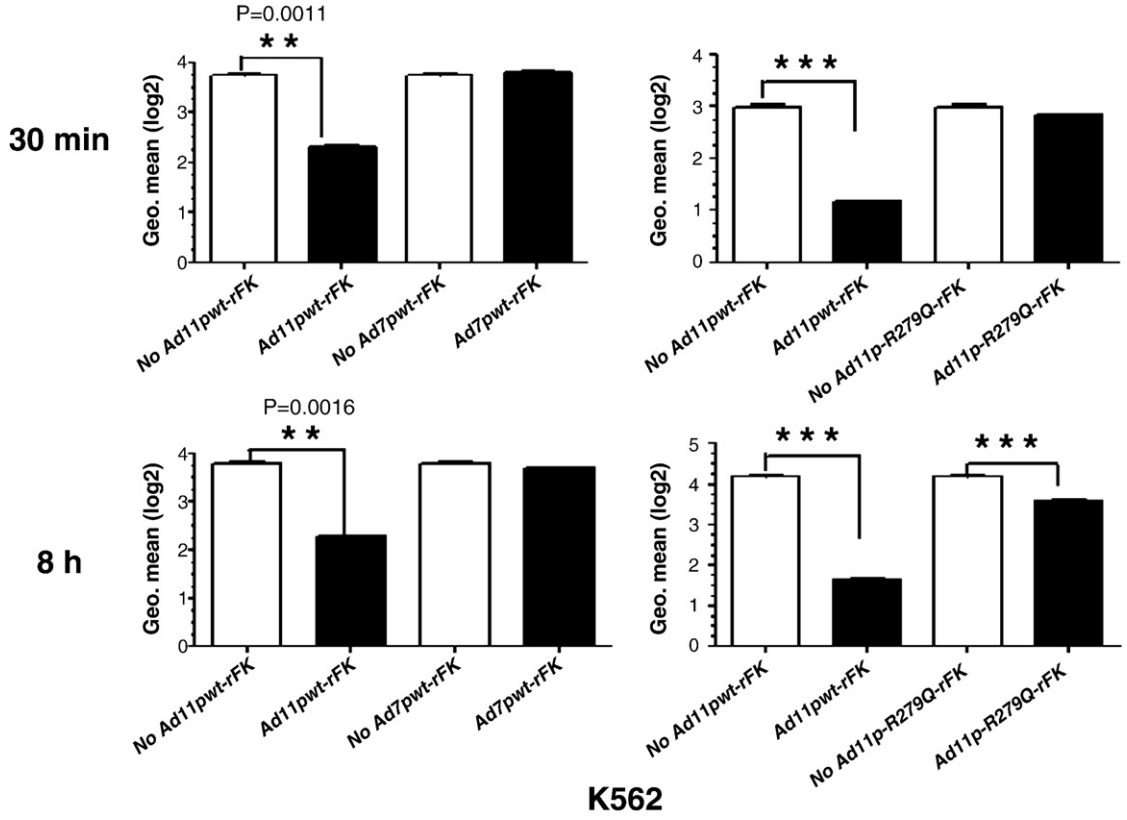

Fig. 6. Downregulation of CD46 levels at the cell surface by Ad11p requires binding by Ad11p recombinant fiber knobs. Ad11pwt-rFK was compared with Ad7pwt-rFK, or Ad11pwt-rFK was compared Ad11p-R279Q-rFK fiber knobs (5 pg/cell). The rFKs were incubated with K562 cells at 37 °C for 30 min or for 8 h. No virus indicates no Ad11pwt-rFK, No Ad7pwt-rFK, or No Ad11p-R279Q-rFK denote K562 cells incubated without fiber knobs, while Ad11pwt-rFK, Ad7pwt-rFK, or Ad11p-R279Q-rFK denotes K562 cells incubated with different fiber knobs. The results are means ± standard errors of means (SEM) of duplicate data from two representative experiments. Significant differences from results with No Ad11pwt-rFK, No Ad7pwt-rFK, or No Ad11p-R279Q-rFK are shown: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

was added at selected concentrations in a total volume of 200 µl (RPMI or DMEM with 1% FCS). After incubation of the tubes at 37 °C with constant agitation for the described time intervals, the cells were pelleted at 290×*g* for 5 min at 4 °C or 37 °C and washed with 150 µl of 1% FCS PBS. The cells were then incubated for 30 min on ice with FITC-conjugated mouse monoclonal antibodies against CD46, CD55, CD59 diluted 1:50 (CD46, CD55, isotype IgG) or 1:25 (CD59) in 100 µl of 1% FCS PBS per tube. Thereafter, the cells were washed as described above and analyzed by FACScan flow cytometer (Becton Dickinson). The measurements consisted of 10,000 events per sample. Data were then analyzed using the LYSYS II software (Becton Dickinson).

For experiments with rabbit polyclonal antibody against CD46 (H-294) or rabbit polyclonal antiserum (1840), the same procedure was followed until cells were pelleted at 290×*g* and washed with FLOW buffer, containing 1% (wt./vol.) glucose (Glc), 0.01% NaN_3 (wt./vol.), and 1% paraformaldehyde (PFA) (wt./vol.) in PBS, pH 7.4). H-294 or 1840 antiserum was then added at dilutions of 1:100 or 2 µg/ml, respectively, and incubated for 30 min on ice. The cell pellets were washed with FLOW buffer and then resuspended in 300 µl of FITC-conjugated swine anti-rabbit IgG diluted 1:40 (Dako Cytomation, Danmark A/B) in FLOW buffer and incubated for 30 min in the dark on a shaker. The cells were washed and analyzed by a FACScan flow cytometer as described above. Control experiments of CD46 downregulation by 1840 antiserum were performed as described above with the exception that cells were incubated with antiserum (1840 or unimmunized rabbit serum) for 4 h, washed, and then stripped of antibodies according to previously established protocols (Crimeen-Irwin et al., 2003).

Statistical analysis

Statistical analyses were performed with GraphPad Prism software version 4.03 (GraphPad Software, La Jolla, CA). The effect of Ad11p and Ad7p viron or fiber knob (FK) on the CD46 levels was evaluated by Student's *t* test, namely two-sample (unpaired) *t*-test.

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