

# Integrin $\alpha V\beta 6$ is a high-affinity receptor for coxsackievirus A9

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Coxsackievirus A9 (CAV9), a member of the genus *Enterovirus* in the family *Picornaviridae*, possesses an integrin-binding arginine-glycine-aspartic acid (RGD) motif in the C terminus of VP1 capsid protein. CAV9 has been shown to utilize integrins  $\alpha V\beta 3$  and  $\alpha V\beta 6$  as primary receptors for cell attachment. While CAV9 RGD-mutants (RGE and RGDdel) are capable of infecting rhabdomyosarcoma (RD) cell line, they grow very poorly in an epithelial lung carcinoma cell line (A549). In this study, the relationships between CAV9 infectivity in A549 and RD cells, receptor expression and integrin binding were analysed. A549 cells were shown to express both integrins  $\alpha V\beta 3$  and  $\alpha V\beta 6$ , whereas  $\alpha V\beta 6$  expression was not detected on the RD cells. Native CAV9 but not RGE and RGDdel mutants bound efficiently to immobilized  $\alpha V\beta 3$  and  $\alpha V\beta 6$ . Adhesion of CAV9 but not RGE/RGDdel to A549 cells was also significantly higher than to RD cells. In contrast, no affinity or adhesion of bacterially produced VP1 proteins to the integrins or to the cells was detected. Function-blocking antibodies against  $\alpha V$ -integrins blocked CAV9 but not CAV9-RGDdel infectivity, indicating that the viruses use different internalization routes; this may explain the differential infection kinetics of CAV9 and RGDdel. In an affinity assay, soluble  $\alpha V\beta 6$ , but not  $\alpha V\beta 3$ , bound to immobilized CAV9. Similarly, only soluble  $\alpha V\beta 6$  blocked virus infectivity. These data suggest that CAV9 binding to  $\alpha V\beta 6$  is a high-affinity interaction, which may indicate its importance in clinical infections; this remains to be determined.

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## INTRODUCTION

Coxsackievirus A9 (CAV9) is a significant human pathogen that causes infections of the central nervous system, myocarditis and respiratory illnesses. Like other enteroviruses, CAV9 particles consist of an icosahedral capsid composed of 60 copies of each of the capsid proteins (VP1 to VP4) that enclose an infectious RNA genome, approximately 7.5 kb in length. The VP1 protein has a C-terminal extension, which contains a functional arginine-glycine-aspartic acid (RGD) motif (Chang *et al.*, 1989) originally found in some extracellular matrix proteins shown to interact with integrins (Ruoslahti & Pierschbacher, 1987). CAV9 interacts with integrins  $\alpha V\beta 3$  and  $\alpha V\beta 6$  via an RGD motif (Roivainen *et al.*, 1991, 1994; Williams *et al.*, 2004). Similar functional RGD motifs have been identified in some other picornaviruses such as echovirus 9 Barty strain (an enterovirus; Zimmermann *et al.*, 1997), human parechoviruses 1, 2, 4, 5 and 6 (HPEVs; Hyypiä *et al.*, 1992; Ghazi *et al.*, 1998; Benschop *et al.*, 2006) and foot-and-mouth disease virus (FMDV; Mason *et al.*, 1994). Other suggested receptors with possible roles in post-attachment and/or internalization of CAV9, include GRP78 (Hsp70-5) and  $\beta_2$ -microglobulin/major histocompatibility complex I protein(s) (Triantafilou *et al.*, 1999, 2000a, 2002).

Sequence analysis of CAV9 isolates indicated that the RGD motif is conserved in clinical isolates and may, therefore, be a significant pathogenicity determinant (Santti *et al.*, 2000). It has also been shown that CAV9 lacking the RGD motif (RGD mutant) is attenuated in a mouse model (Harvala *et al.*, 2003). Somewhat surprisingly, RGD mutants efficiently infect rhabdomyosarcoma (RD) cells, indicating that there are both RGD-dependent and -independent attachment and internalization mechanisms (Hughes *et al.*, 1995; Roivainen *et al.*, 1996; Triantafilou *et al.*, 1999; Williams *et al.*, 2004). The putative RGD-independent receptor that mediates CAV9 infection in RD cells has not been identified, nor is it known whether RGD mutants infect the cells independently of integrins.

In the present study, integrin interactions of CAV9 and the RGD mutants were analysed using *in vitro* assays with two cell lines that have different susceptibility to the virus infection. Integrins  $\alpha V\beta 3$  and  $\alpha V\beta 6$  were shown to be expressed on the cell surface of the A549 cell line, whereas the RD cells were deficient of the  $\beta 6$  subunit. CAV9 also bound more efficiently to A549 cells than to RD cells. Integrin  $\alpha V\beta 6$  but not  $\alpha V\beta 3$  bound efficiently to immobilized CAV9, and  $\alpha V\beta 6$  was also capable of neutralizing the infectivity. Taken together, these data indicate that  $\alpha V\beta 6$  is a high-affinity receptor for CAV9.

## METHODS

**Cell lines and infections.** Human epithelial lung carcinoma (A549), rhabdomyosarcoma (RD) and African green monkey kidney (GMK) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% fetal calf serum (FCS) and 10 µg gentamicin ml<sup>-1</sup>. Virus infections were performed in DMEM supplemented with 1% FCS and gentamicin.

**Viruses and integrins.** Wild-type CAV9, CAV9-RGD-mutants (CAV9-RGE, CAV9-RGDdel) (Hughes *et al.*, 1995) and adenovirus 5 (Ad5) were taken from laboratory collections. Viruses were purified in sucrose gradients (Abraham & Colonna, 1984). Purified integrin  $\alpha V\beta 3$  was obtained from Chemicon (Millipore) and  $\alpha V\beta 6$  was produced and purified in Chinese hamster ovary (CHO) cells (Weinacker *et al.*, 1994; Williams *et al.*, 2004).

**Antibodies.** The primary monoclonal antibodies (mAbs) specific to different receptors or their subunits that were used are as follows:  $\alpha V$  (L230; ATCC),  $\alpha V\beta 3$  (MAB1976Z; Chemicon),  $\alpha V\beta 5$  (MAB1961Z; Chemicon) and  $\alpha V\beta 6$  (MAB2077Z and MAB2074Z; Chemicon). The rabbit antiserum against integrin  $\alpha V\beta 3$  was a kind gift from Merja Roivainen (National Public Health Institute, Helsinki, Finland). CAV9-specific antibodies were from laboratory collections. Alexa Fluor-488 monoclonal mouse or polyclonal rabbit and Alexa Fluor-546 polyclonal rabbit antibodies were used as secondary antibodies in detection (Molecular Probes).

**Growth curves.** Confluent A549 and RD cells were infected with native CAV9 and RGD-mutants (CAV9-RGE and CAV9-RGDdel; Hughes *et al.*, 1995) (m.o.i. of 1) in a 96-well plate and incubated on ice for 1 h. Wells were washed and incubated for 0–24 h at 37 °C. Samples were collected every 2 h and freeze-thawed three times before plaque assay. Plaque assays were performed in a 24-well plate using confluent RD monolayers. Briefly, viruses were attached to cell surfaces at room temperature for 15 min in Hanks' solution and washed. CMC solution (0.5%) containing MEMII medium (10 µg gentamicin ml<sup>-1</sup>, 0.03% glutamine, 0.6% glucose, 20 mM MgCl<sub>2</sub>, 1% inactivated FCS and 20 mM HEPES) was added and cells were incubated for 2 days before staining with crystal violet containing 5% formaline and 10% ethanol.

**Antibody blocking assay.** Function-blocking  $\alpha V$ -,  $\alpha V\beta 3$ -,  $\alpha V\beta 5$ - and  $\beta 1$ -integrin antibodies (1.5 µg), in serum-free MEM medium (Gibco) containing 10 µg gentamicin ml<sup>-1</sup>, 1 mM MgCl<sub>2</sub> and 1 mM MnCl<sub>2</sub>, were added onto confluent RD cells in 96-well plates and the cells were incubated for 1 h at room temperature. The antibodies were removed and the cells were infected with CAV9 and CAV9-RGDdel. After incubation for 1 h at room temperature, viruses were removed, the cells were washed and incubated at 37 °C overnight. Detached cells were freeze-thawed three times before plaque assay. Plaque assays were performed in confluent GMK cell monolayers in 12-well plates using the protocol described above.

**Flow cytometry.** mAbs were used to detect integrins  $\alpha V$ ,  $\alpha V\beta 6$ ,  $\alpha V\beta 3$  and  $\alpha V\beta 5$  on A549 and RD cells (see above). Cells were suspended in PBS, primary antibodies were added and incubated for 15 min at 4 °C. The cell pellets were washed, incubated with the secondary antibodies and, after washing, the cell pellets were suspended in PBS. Flow cytometric measurements were done with a FACSCalibur flow cytometer (Becton Dickinson) and 10 000 cells were analysed in each assay.

**Cloning and expression of viral VP1 proteins.** PCR primers were generated to clone the VP1 region of CAV9 (GenBank accession no. D00627; nt 2448–3353) and CAV9-RGD mutants (RGE and RGDdel; Hughes *et al.*, 1995) into pET15b (Novagen). Ad5 penton protein

cloned similarly to pET15b was used as a control. VP1-pET15b constructs were transformed into T7 Express cells (New England Biolabs) and protein production was induced with 1 mM IPTG for 3 h. Following that, the cells were lysed with Bugbuster lysis buffer (Novagen) containing 0.5 mg lysozyme ml<sup>-1</sup> and 1 µl benzonase and protease inhibitors ml<sup>-1</sup>. The lysates were vortexed and centrifuged (10 000 g, 15 min, 4 °C) and protein pellets were dissolved in 8 M urea prepared in Tris-buffered saline with 10 mM 2-mercaptoethanol and dialysed against PBS supplemented with 1 mM MgCl<sub>2</sub>. Protein concentrations were determined (BCA Protein Analysis kit; Pierce) and samples were run on SDS-PAGE gels and stained with Coomassie brilliant blue.

**Cell adhesion assay.** Binding of A549 and RD cells to CAV9, RGD mutants or VP1 proteins was analysed using a cell adhesion assay. CAV9, RGDdel or VP1 proteins (300 ng) in PBS were used to coat 96-well plates (Costar High Binding) which were incubated overnight at 4 °C. Wells were washed (1 mM MgCl<sub>2</sub> and 1 mM MnCl<sub>2</sub> in PBS), blocked with 1% BSA prepared in wash solution for 1 h at room temperature and washed again. Cells were detached with preheated (37 °C) 2 mM EDTA, washed twice and suspended in 5 ml serum-free DMEM. The virus-coated wells were overlaid with A549 or RD cells (10<sup>5</sup>), incubated at 37 °C for 45–60 min and washed. Cell attachment was visualized by staining with crystal violet. Absorbance was measured with a spectrophotometer at 590 nm. The attachment of Ad5 and Ad5 penton protein to A549 cells was determined previously (unpublished data), and they were used as a positive controls. BSA was used as a negative control.

**In vitro integrin binding assays.** Binding of CAV9 and CAV9 RGD mutants to  $\alpha V\beta 3$  and  $\alpha V\beta 6$  integrins was analysed in a solid-phase integrin binding assay. Integrins  $\alpha V\beta 3$  and  $\alpha V\beta 6$  (300 ng) in coating buffer (1 mM MgCl<sub>2</sub> and 1 mM MnCl<sub>2</sub> in PBS) were used to coat 96-well plates (Costar High Binding) and incubated overnight at 4 °C. Wells were washed three times with coating buffer, blocked with binding buffer (1% BSA in PBS with 1 mM MgCl<sub>2</sub> and 1 mM MnCl<sub>2</sub>) for 1.5 h at room temperature and washed before addition of virus. Plates were incubated with virus for 2 h at room temperature. Wells were washed with coating buffer and then incubated with virus-specific antiserum and secondary anti-mouse/rabbit horseradish peroxidase conjugate. Wells were stained with H<sub>2</sub>O<sub>2</sub> and absorbance was read at 450 nm. The same protocol was used when integrin binding to immobilized viruses was determined, but instead of integrins, the plates were coated with virus, overlaid with integrins and detected by using non-functional blocking antibodies (MAB2074Z against integrin  $\alpha V\beta 6$  and rabbit antiserum against integrin  $\alpha V\beta 3$ ).

**Plaque neutralization assay with soluble integrins.** Native CAV9 (1000 p.f.u.) and 20 ng or 200 ng of integrins  $\alpha V\beta 3$  and  $\alpha V\beta 6$  were mixed and incubated at 37 °C for 1 h. Confluent GMK cells were washed with Hanks' solution and cells were covered with Hanks' solution supplemented with 6% FCS. GMK cells were first infected with virus–integrin complexes at room temperature for 15 min, followed by washing and addition of 0.5% CMC solution. Cells were incubated for 2–4 days at 37 °C before staining with crystal violet and plaque counting. The experiment was repeated twice.

## RESULTS

### CAV9 infectivity in A549 and RD cell lines, and integrin expression in the cells

CAV9 receptor identification, cell binding and internalization in various cell lines have been analysed in a number of studies, and some of the results obtained with  $\alpha V\beta 3$  have

been contradictory (Hughes *et al.*, 1995; Triantafilou *et al.*, 2000b; Williams *et al.*, 2004). Therefore, infectivity and growth properties of CAV9 and RGD mutants on A549 and RD cells were first analysed with respect to integrin expression. CAV9 infectivity in A549 cells was found to be dependent on an intact RGD motif, while in RD cells, CAV9 infection proceeded independently of RGD. CAV9 was also capable of multiplying efficiently in both A549 and RD cells (Fig. 1), whereas the RGD mutants only multiplied efficiently in RD cells, suggesting different receptor use and/or internalization mechanisms.

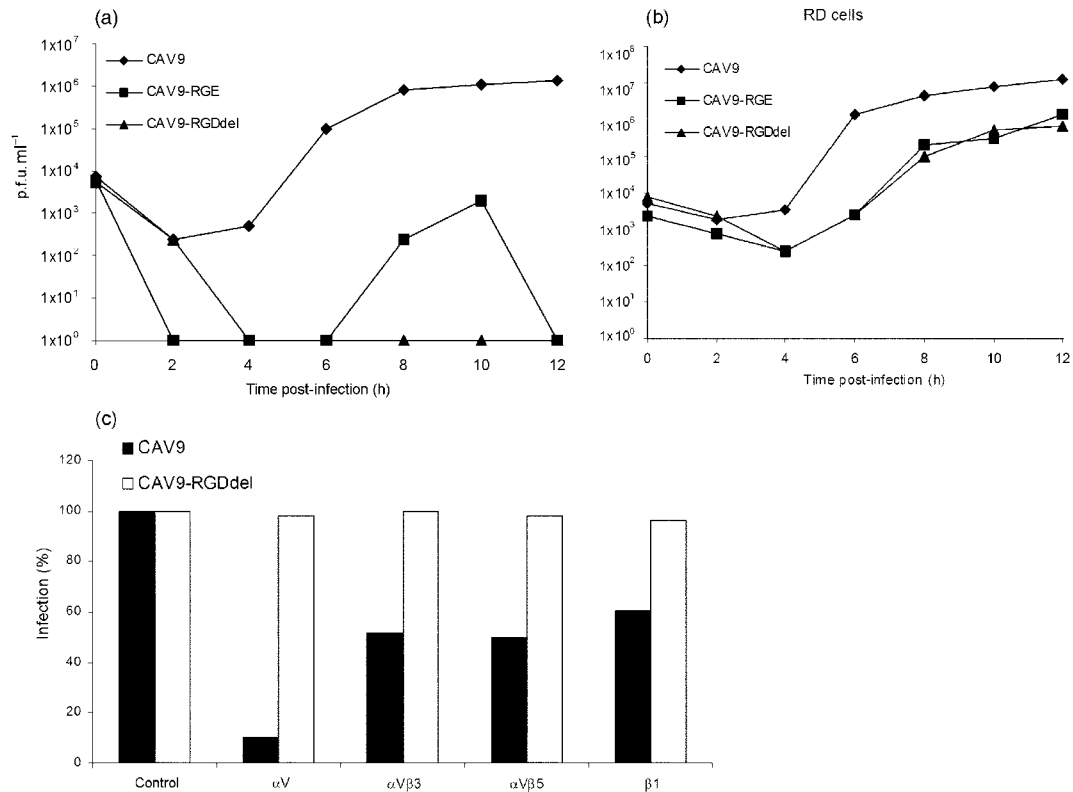
Function-blocking mAbs (FB-mAbs), known to block the interaction of the integrins with their physiological ligands, were used to study the importance of different integrin subunits in the initiation of infection in RD cells (Fig. 1c). FB-mAb against  $\alpha V$  had an almost complete blocking effect, as determined by plaque assay. Inhibition of CAV9 infection was also observed with  $\alpha V\beta 3$ ,  $\alpha V\beta 5$  and  $\beta 1$  FB-mAbs, although to a lesser extent. None of these antibodies blocked the infectivity of CAV9-RGDdel.

To elucidate the differences in the expression pattern of integrin types between the two cell lines, FACS analysis using antibodies specific to  $\alpha V$ ,  $\alpha V\beta 3$ ,  $\alpha V\beta 5$  and  $\alpha V\beta 6$  were

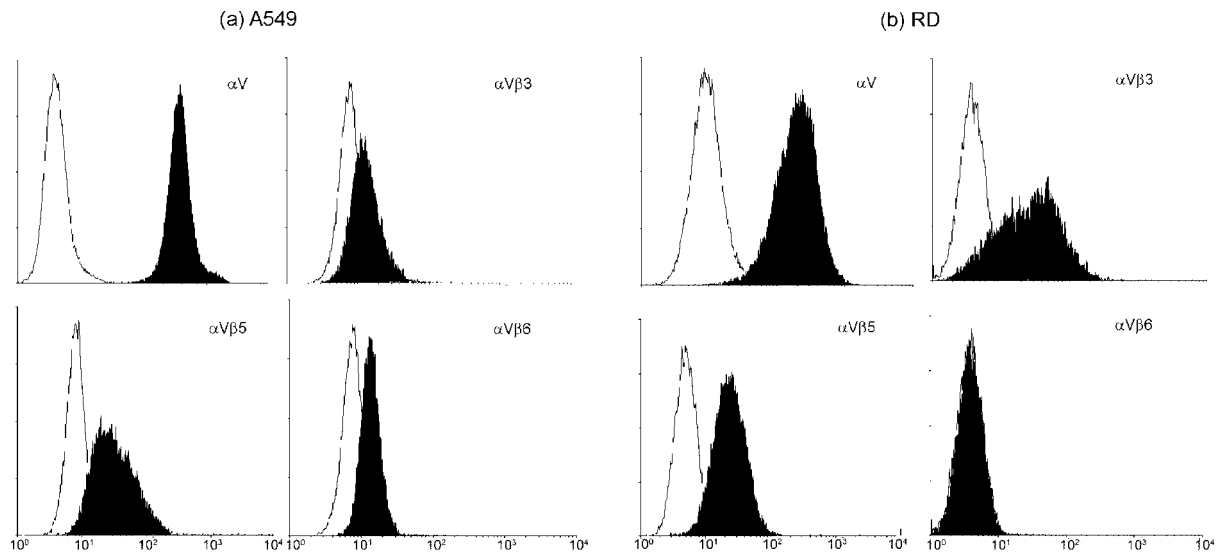
performed. The cell surface expression of  $\alpha V\beta 3$  was practically the same between A549 and RD cell lines but, interestingly,  $\alpha V\beta 6$  was expressed only on the surface of A549 cells (Fig. 2). This was also supported by the lack of detection of  $\alpha V\beta 6$  by immunofluorescence microscopy on RD cells, and quantitative RT-PCR also indicated that there was no  $\alpha V\beta 6$  expression in RD cells (data not shown).

### Cell adhesion assay

As flow cytometric analysis indicated that integrin  $\alpha V\beta 6$  was expressed only on A549 cells, a cell adhesion assay was performed to investigate CAV9 binding to A549 and RD cells. The A549 cells adhered efficiently to immobilized Ad5 and CAV9 but not to the CAV9-RGD mutants. When immobilized viruses were overlaid with the RD cells, the adhesion to Ad5 and CAV9 was significantly lower, but still higher than to CAV9-RGDdel (Fig. 3a). The adherence of the cells to individual CAV9 VP1 proteins and Ad5 penton protein expressed in bacteria was also analysed. (Fig. 3b). Ad5 penton protein bound efficiently to A549 cells, indicating that the purified protein was functionally active. However, CAV9 VP1 proteins did not adhere to A549 and RD cells (Fig. 3c). These data indicate clear differences in



**Fig. 1.** Infectivity of CAV9, CAV9-RGE and CAV9-RGDdel in A549 (a) and RD (b) cells. Viruses were infected for up to 12 h and samples were collected for plaque assay. CAV9 infected both cell lines while CAV9-RGE and CAV9-RGDdel were highly infectious only in RD cells, suggesting RGD-dependent entry in A549 and RGD-independent entry in RD cells. (c) Blocking of CAV9 and CAV9-RGDdel infection by FB-mAbs. Data indicate the mean of two separate experiments.



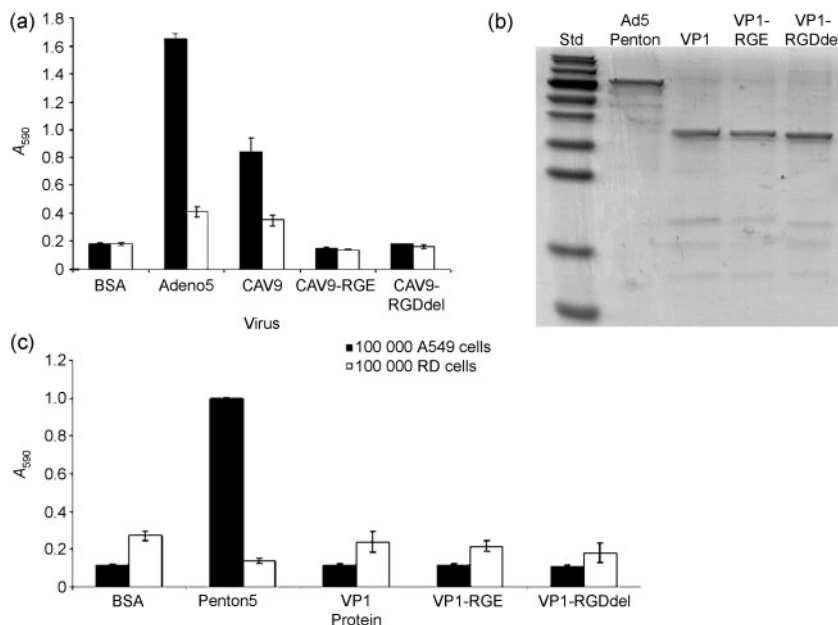
**Fig. 2.** Flow cytometric analysis of integrin expression on the surface of A549 (a) and RD (b) cells. Antibodies specific to  $\alpha V$ ,  $\alpha V\beta 3$ ,  $\alpha V\beta 5$  and  $\alpha V\beta 6$  were used to detect the corresponding integrin receptors on the surfaces of both A549 and RD cells. Integrin  $\alpha V\beta 6$  was not visible on RD cells. Secondary antibody controls (white plots) and detection of integrins by specific antibodies (black plots) are shown.

interaction of CAV9 with A549 or RD cells. The data suggest that CAV9 is dependent on the RGD motif for high-affinity binding and infectivity of A549 cells, and suggest that this binding is mainly mediated via integrin  $\alpha V\beta 6$ , since there is no expression of  $\alpha V\beta 6$  on RD cells, which bind CAV9 inefficiently (Figs 1b and 3a). Moreover, the data indicate that single VP1 proteins alone are not sufficient for virus attachment onto the cell surface. This

may be due to improper folding of CAV9-VP1 proteins or due to additional sites on the virus surface that contribute to overall binding of the virus to the integrins.

### Integrin binding assay

It was demonstrated that adherence of CAV9 to RD cells is RGD-independent, suggesting that either CAV9 infection



**Fig. 3.** Adherence of immobilized CAV9 (a) or purified VP1 proteins (b and c) to A549 and RD cells. CAV9 adhered to A549 cells more efficiently than to RD cells, while CAV9-RGDdel possessed low adhesion to both cell lines (a). Bacterially produced CAV9-VP1 proteins (b and c) did not adhere to A549 or RD cells (c). Ad5 and penton protein, known to adhere to A549 cells, were used as positive controls, and BSA was used as a negative control. Data indicate mean  $\pm$  SD of three separate experiments

in these cells occurs without interacting with integrin  $\alpha V\beta 3$  or the binding is of low affinity. Integrins  $\alpha V\beta 3$  and  $\alpha V\beta 6$  were immobilized onto wells of microtitre plates, and CAV9 or RGD mutants were overlaid for avidity assay (Fig. 4a, b). CAV9 but not RGD mutants bound to both integrins efficiently, supporting the previous findings that integrin binding *in vitro* occurs via an RGD motif (Hughes *et al.*, 1995; Williams *et al.*, 2004). In contrast, when immobilized virus was overlaid with integrins, a clear difference in affinity between CAV9 and  $\alpha V\beta 3$  or  $\alpha V\beta 6$  was observed. Soluble  $\alpha V\beta 6$  bound efficiently to CAV9, while soluble  $\alpha V\beta 3$  exhibited little or no affinity to CAV9 (Fig. 4c). Overall, our data suggest that CAV9 possesses high affinity to  $\alpha V\beta 6$  but not to  $\alpha V\beta 3$ .

### Effect of soluble integrins $\alpha V\beta 3$ and $\alpha V\beta 6$ on CAV9 infectivity

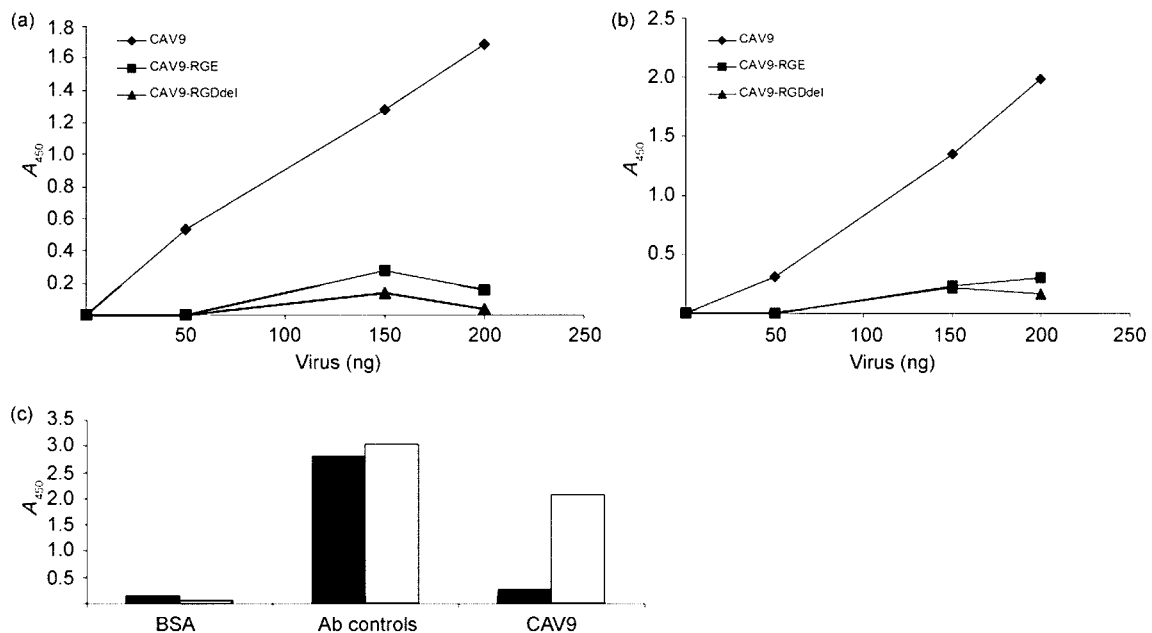
Previously, it has been shown that  $\alpha V\beta 6$  blocks CAV9 infectivity on GMK cells (Williams *et al.*, 2004). To determine whether integrin  $\alpha V\beta 3$  possessed similar properties as  $\alpha V\beta 6$ , excess amounts of integrins were incubated with CAV9 and infectivity was determined by plaque assay (Fig. 5). Soluble  $\alpha V\beta 3$  did not inhibit, but rather enhanced CAV9 infectivity. In contrast, soluble  $\alpha V\beta 6$  blocked CAV9 infectivity, which is consistent with the high affinity of integrin  $\alpha V\beta 6$  to CAV9. In conclusion, soluble  $\alpha V\beta 6$  efficiently inhibited virus infectivity indicating that  $\alpha V\beta 6$  is

a high-affinity receptor for CAV9 in RGD-dependent cellular entry.

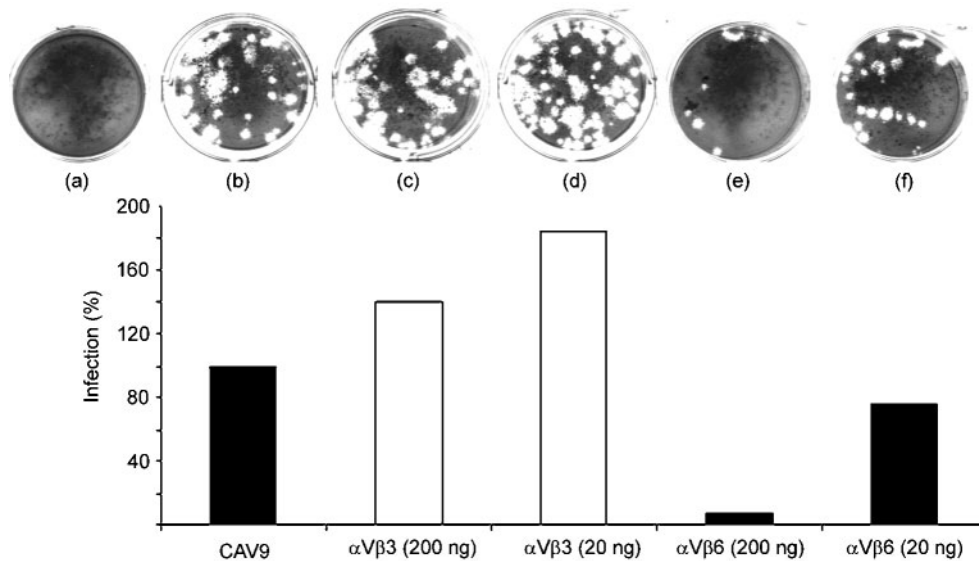
## DISCUSSION

Virus entry into host cells is the key requirement for cellular infectivity, and involves several steps that are essential for the virus life cycle. Enteroviruses are known to use various receptors for cell binding and internalization. These include decay-accelerating factor, to which some coxsackie B viruses bind, while coxsackie-adeno-receptor (CAR) functions as the receptor for internalization (Bergelson *et al.*, 1997). CAV9, a member of B group enteroviruses, is different, in that it has an RGD motif in the C terminus of VP1 capsid protein, through which the virus primarily binds to and utilizes integrins  $\alpha V\beta 3$  and  $\alpha V\beta 6$  as receptors (Roivainen *et al.*, 1994; Triantafilou *et al.*, 2000b; Williams *et al.*, 2004). GRP78 and  $\beta_2$ -microglobulin have been suggested as co-receptors in the viral internalization process, possibly functioning in a post-attachment step (Triantafilou *et al.*, 1999, 2002).

While CAV9 binding to integrin  $\alpha V\beta 3$  and  $\alpha V\beta 6$  receptors has been demonstrated using various cell models (Roivainen *et al.*, 1994; Hughes *et al.*, 1995; Triantafilou *et al.*, 1999, 2000b; Triantafilou & Triantafilou, 2003; Williams *et al.*, 2004), the receptor interactions have not been analysed fully in parallel. Moreover, CAV9 has been shown to enter some cell lines, such as RD, independently



**Fig. 4.** *In vitro* binding of CAV9 and RGD mutants to integrins  $\alpha V\beta 3$  (a) and  $\alpha V\beta 6$  (b). CAV9 bound to both immobilized integrins with similar affinity, whereas RGD mutants had no affinity to either integrin. In contrast, integrin  $\alpha V\beta 6$  (white bars) but not  $\alpha V\beta 3$  (black bars) bound to immobilized CAV9 (c). These data suggest that  $\alpha V\beta 6$  is a high-affinity receptor for CAV9. BSA was used as a negative control and integrins labelled directly with antibodies (Ab controls in c) were used as positive controls. Data indicate the mean of two separate experiments.



**Fig. 5.** Blocking of CAV9 infection with soluble  $\alpha V\beta 3$  and  $\alpha V\beta 6$  integrins. CAV9 (1000 p.f.u.) was incubated with 200 ng or 20 ng of integrin (c–f) for 1 h and the complex was inoculated onto GMK cells. Uninfected cells (a) and cells infected with CAV9 (100% infection; b) were used as controls. The data indicate that  $\alpha V\beta 6$  efficiently inhibited CAV9 infectivity in GMK cells, whereas  $\alpha V\beta 3$  enhanced CAV9 infectivity. Data indicate the mean of two separate experiments. The graph indicates infection compared with the control and images above the graph are photographs of individual wells, infected as described above.

of the RGD motif, suggesting that the virus uses both integrin-dependent and -independent internalization routes for cellular entry (Hughes *et al.*, 1995). We have investigated interactions of CAV9 in lung carcinoma (A549) and RD cell lines. CAV9, but not CAV9 from which the RGD motif was mutated (RGDdel), infected A549 efficiently, while RD cells were susceptible to both viruses (Fig. 1a, b). Function-blocking antibodies to  $\alpha V$ - and  $\alpha V\beta 3$ -integrins prevented CAV9 infectivity in RD cells (Fig. 1c), indicating that  $\alpha V$  integrins mediate CAV9 internalization. In contrast, CAV9-RGDdel infectivity was not affected by FB-mAbs, suggesting that integrins are not absolutely required for internalization and that an alternative internalization pathway(s) exists. Previous data (Triantafilou *et al.*, 1999), showing that integrin  $\alpha V\beta 3$ -specific antibodies prevent approximately 50% of native CAV9 infection in RD cells, support the idea that interactions with  $\alpha V$ -integrins are of importance for CAV9. Although it has been previously reported that CAV9 and CAV9-RGDdel infect RD cells in the same manner (Hughes *et al.*, 1995), a slight difference in infection kinetics was observed in our study. This may be explained by the use of a different receptor(s). Interestingly,  $\beta 1$ - and  $\alpha V\beta 5$ -antibodies also blocked CAV9 infectivity in RD cells, raising the possibility that these can also act as receptors (Fig. 1c).

Analysis of cell surface receptors by specific antibodies indicated that A549 cells express both  $\alpha V\beta 3$  and  $\alpha V\beta 6$  while RD cells lack  $\alpha V\beta 6$  expression (Fig. 2). We also determined  $\alpha V\beta 6$  levels using quantitative RT-PCR, as it

was recently demonstrated that RD cells are heterogeneous and express CAR receptor in subcellular populations at levels which cannot be detected by flow cytometry (Carson *et al.*, 2007). However, no expression of integrin  $\alpha V\beta 6$  was detected (data not shown). Nevertheless, RD cells were much more susceptible to CAV9 infection than A549, indicating that lack of  $\alpha V\beta 6$  did not interfere with the infection process (data not shown).

We investigated the affinity of CAV9 to A549 and RD cells further by overlaying immobilized virus with cells and measuring cell attachment. A549 cells attached significantly more efficiently to CAV9, while RD cells had no clear affinity. In addition, the data indicated that cells possessed no adherence to a single CAV9-VP1 protein (Fig. 3). This may be due to improper folding of CAV9-VP1 proteins, or the presence of secondary integrin-binding sites on the virus surface that contribute to overall binding of the virus to integrins and/or co-receptors. Triantafilou *et al.* (2000b) demonstrated that CAV9 and RGDdel attached efficiently to CHO cells overexpressing  $\alpha V\beta 3$ . While these data were challenged by Williams *et al.* (2004), who demonstrated that CAV9 did not bind or infect SW480 cells overexpressing  $\alpha V\beta 3$ , there is still room for the possibility that CAV9 binds to integrins using two sites – via the RGD motif and another unknown site. Overall, these data suggest, albeit indirectly, a relationship between virus infectivity, cell attachment and receptor expression, in which  $\alpha V\beta 6$  may play a dominant role.

It has previously been shown that CAV9 binds to immobilized  $\alpha V\beta 3$  and  $\alpha V\beta 6$  (Williams *et al.*, 2004). This

interaction indicates that there is an avidity between virus and integrin molecules which may lead to clustering on the cell surface, whilst affinity is purely a measure of binding of a single integrin to immobilized virus. It has been demonstrated that RGD-blocking peptides bind more efficiently to  $\alpha V\beta 6$  than  $\alpha V\beta 3$ , thus implying that CAV9– $\alpha V\beta 6$  affinity may be stronger than that of CAV9– $\alpha V\beta 3$  (Williams *et al.*, 2004). In this work, the avidity of  $\alpha V\beta 3$  and  $\alpha V\beta 6$  to CAV9 was the same (Fig. 4a, b), while affinity was much higher between CAV9 and  $\alpha V\beta 6$ . In addition, only  $\alpha V\beta 6$  blocked CAV9 infectivity, whereas  $\alpha V\beta 3$  had no blocking effect (Fig. 5). These data indicate that  $\alpha V\beta 6$  acts as a high-affinity receptor for CAV9.

The role of high-affinity receptor interactions in the life cycle of CAV9 can be predicted. Recently, Duque *et al.* (2004) demonstrated that integrin  $\alpha V\beta 6$  does not bind to an FMDV RGD-mutant and integrin  $\alpha V\beta 3$  does not bind efficiently to FMDV, even though it has been shown to utilize this integrin as a functional receptor to mediate infection (Neff *et al.*, 1998). It was suggested (Duque *et al.*, 2004) that the role of the high-affinity receptor,  $\alpha V\beta 6$ , may be to capture virus and help in the initiation of replication within susceptible cells, while  $\alpha V\beta 3$  could play a role in disseminating the virus to the sites of replication. This may be significant in a clinical situation, as in the case of FMDV, it has been suggested that  $\alpha V\beta 6$  serves as the major receptor for epithelial tropism (Monaghan *et al.*, 2005). The epithelial distribution of integrins with respect to CAV9 infectivity in native tissues remains to be studied.

The importance of integrin binding via the RGD motif in the virus life cycle is far from clear. It has been demonstrated that CAV9 is dependent on RGD in some cell lines such as A549, while in RD cells, RGD is not essential. There are also echovirus 9 isolates that are capable of binding to  $\alpha V\beta 3$  irrespective of whether they possess RGD in the VP1 or not (Paananen *et al.*, 2003). Thus, there is also evidence of RGD-independent integrin binding. In RD cells, it has been suggested that other receptors like GRP78 and  $\beta_2$ -microglobulin are needed for CAV9 internalization, but it is not known whether integrins are needed or not (Triantafyllou *et al.*, 1999, 2002). In this study, CAV9-RGDdel did not bind to immobilized integrins. In addition, both CAV9 and CAV9-RGDdel possessed low avidity to RD cells, which lack the high-affinity integrin receptor,  $\alpha V\beta 6$ . Thus, it is likely that infection of CAV9 and also CAV9-RGDdel into RD cells occurs either without integrin binding or via other co-receptors (Triantafyllou *et al.*, 1999, 2002). The role of the co-receptors in the infectious cycle of CAV9-RGDdel is evident, since pathogenesis of CAV9 and CAV9-RGDdel differed in mice, but both viruses were viable (Harvala *et al.*, 2003).

In conclusion, CAV9 seems to possess the ability to modulate its receptor use depending on the cell line it infects. The clinical relevance of this ability is unclear, but it may represent the quasispecies nature of virus infection

in human tissues (Vignuzzi *et al.*, 2006) and may, therefore, be crucial for the virus infection process in multicellular organisms. This is supported by the fact that all of the clinical CAV9 isolates from five decades possessed the RGD motif (Santti *et al.*, 2000). Since integrin  $\alpha V\beta 6$  is primarily expressed on epithelia, while  $\alpha V\beta 3$  is found on muscle cells, the high affinity of  $\alpha V\beta 6$  to CAV9 may be essential in facilitating virus infection; to develop clinically significant disease, the dominant virus form must possess an RGD motif in order to bind integrins in native cell lines, which may reside in key points of the viral infection route in human tissues (such as nasal epithelia, colon epithelia/endothelia and central nervous system cells). Interaction studies may ultimately lead to antiviral strategies which target viral proteins and/or their receptors by molecular mimicry. The importance of integrins in cancer biology has ultimately resulted in the generation of panels of integrin inhibitors, and it remains to be demonstrated whether such molecules are useful against CAV9 and other integrin-binding viruses.

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