Rotaviruses interact with $\alpha4\beta7$ and $\alpha4\beta1$ integrins by binding the same integrin domains as natural ligands

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Group A rotaviruses are major intestinal pathogens that express potential $\alpha4\beta1$ and $\alpha4\beta7$ integrin ligand sequences Leu–Asp–Val and Leu–Asp–Ile in their outer capsid protein VP7, and Ile–Asp–Ala in their spike protein VP4. Monkey rotavirus SA11 can use recombinant $\alpha4\beta1$ as a cellular receptor. In this study a new potential $\alpha4\beta1$, $\alpha4\beta7$ and $\alpha9\beta1$ integrin ligand sequence, Tyr–Gly–Leu, was identified in VP4. It was shown that several human and monkey rotaviruses bound $\alpha4\beta1$ and $\alpha4\beta7$, but not $\alpha9\beta1$. Binding to $\alpha4\beta1$ mediated the infectivity and growth of monkey rotaviruses, and binding to $\alpha4\beta7$ mediated their infectivity. A porcine rotavirus interacted with $\alpha4$ integrins at a post-binding stage to facilitate infection. Activation of $\alpha4\beta1$ increased rotavirus infectivity. Cellular treatment with peptides containing the $\alpha4$ integrin ligand sequences Tyr–Gly–Leu and Ile–Asp–Ala eliminated virus binding to $\alpha4$ integrins and infectivity. In contrast, rotavirus recognition of $\alpha4$ integrins was unaffected by a peptide containing the sequence Leu–Asp–Val or by a mutation in the VP7 Leu–Asp–Val sequence. VP4 involvement in rotavirus recognition of $\alpha4\beta1$ was demonstrated with rotavirus reassortants. Swapping and point mutagenesis of $\alpha4$ surface loops showed that rotaviruses required the same $\alpha4$ residues and domains for binding as the natural $\alpha4$ integrin ligands: mucosal addressin cell adhesion molecule-1, fibronectin and vascular cell adhesion molecule-1. Several rotaviruses are able to use $\alpha4\beta7$ and $\alpha4\beta1$ for cell binding or entry, through the recognition of the same $\alpha4$-subunit domains as natural $\alpha4$ ligands.

INTRODUCTION

Rotaviruses are a major cause of gastroenteritis through intestinal epithelial cell infection and they can spread extraintestinally (Iturriza-Gomara *et al.*, 2002; Mossel & Ramig, 2003). Many group A rotaviruses bind to the $\alpha2\beta1$ integrin via its ligand sequence Asp–Gly–Glu (DGE), which projects from the VP5* subunit of the spike protein VP4. These viruses use the outer capsid protein VP7 to interact with integrins $\alpha x\beta2$ and $\alpha v\beta3$ during cell entry (Coulson *et al.*, 1997; Dormitzer *et al.*, 2004; Graham *et al.*, 2003; Guerrero *et al.*, 2000; Hewish *et al.*, 2000). Rotavirus infectivity in intestinal and kidney cell lines is inhibited by at least 65 % with combinations of antibodies or ligand peptides directed to these integrins (Coulson *et al.*, 1997; Graham *et al.*, 2003, 2004; Guerrero *et al.*, 2000). Integrins are heterodimeric cell adhesion molecules (Hynes, 2002) that act as receptors for many viruses (Triantafilou *et al.*, 2001). Cellular sialic acids, gangliosides and other carbohydrates are also implicated as rotavirus receptors (Ciarlet *et al.*, 2002; Rolsma *et al.*, 1998). Heat-shock protein 70 has a role in rotavirus cell entry (Lopez & Arias, 2004).

Integrins and their natural ligands are essential for epithelial cell adhesion to the extracellular matrix, immune responses and lymphocyte trafficking (Hynes, 2002; Mittelbrunn *et al.*, 2004). The $\alpha4$ integrin subunit pairs with either $\beta1$ or $\beta7$ to form part of a subfamily that also includes $\alpha9\beta1$ (Hynes, 2002). The $\alpha4\beta1$ integrin is expressed on mesenchymal cells in the intestinal lamina propria. These cells are important for mucosal inflammation and repair (Choy *et al.*, 1990; Pender *et al.*, 2000; Powell *et al.*, 1999). The $\alpha4$ integrins are highly expressed on B and T lymphocytes, dendritic cells,

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monocytes and natural killer cells (Hynes, 2002; Pender *et al.*, 2000). Intestinal epithelial cells express $\alpha9\beta1$ (Basora *et al.*, 1998; Yokosaki *et al.*, 1999). The intestinal homing receptor, $\alpha4\beta7$, binds to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on post-capilliary venules. In contrast, $\alpha4\beta1$ preferentially engages fibronectin and vascular cell adhesion molecule-1 (VCAM-1) to induce intestinal mesenchymal cell migration (Pender *et al.*, 2000) and control B- and T-lymphocyte development, proliferation and survival (Mittelbrunn *et al.*, 2004; Tidswell *et al.*, 1997).

Protection from rotavirus reinfection is primarily mediated by rotavirus-specific intestinal immunoglobulin A (IgA) in animals (Franco & Greenberg, 1999), and correlates with this IgA in humans (Coulson *et al.*, 1992). The presence of rotavirus-specific, antibody-secreting cells in the intestine correlates with their detection in blood (Brown *et al.*, 2000; Yuan *et al.*, 1996). Human rotavirus-specific, antibody-secreting B cells and $CD4^+$ T cells predominantly express $\alpha4\beta7$ (Gonzalez *et al.*, 2003; Rott *et al.*, 1997). In mice, rotavirus-specific B cells require $\alpha4\beta7$ for protective immunity (Williams *et al.*, 1998; Youngman *et al.*, 2002), and memory T cells strongly express $\alpha4\beta7$ (Rose *et al.*, 1998; Rott *et al.*, 1997). Thus, effective rotavirus immune responses depend on MAdCAM-1 recognition by lymphocytes expressing $\alpha4\beta7$.

Potential ligand sequences for $\alpha4\beta1$ and $\alpha4\beta7$ are found in the outer capsid proteins of most rotaviruses. The sequences Ile–Asp–Ala (IDA) and Leu–Asp–Val (LDV) in fibronectin that are important for $\alpha4\beta1$ and $\alpha4\beta7$ recognition (Chan *et al.*, 1992; Komoriya *et al.*, 1991; Ruegg *et al.*, 1992; Sharma *et al.*, 1999; Wayner & Kovach, 1992) are present in VP5* and the disintegrin-like domain of VP7, respectively. This VP7 domain also contains the sequence Leu–Asp–Ile (LDI) that is closely related to LDV (Coulson *et al.*, 1997; Hewish *et al.*, 2000). Simian rotavirus SA11 binds cell surface-expressed $\alpha4\beta1$, facilitating infection and replication (Hewish *et al.*, 2000). Mouse polyomavirus also contains LDV sequence, and uses $\alpha4\beta1$ as an entry factor (Caruso *et al.*, 2003).

Two aims of this study were to determine if human and porcine rotaviruses interact with $\alpha4\beta1$ and to detect any rotavirus recognition of $\alpha4\beta7$. The presence in rotavirus VP5* of the Tyr–Gly–Leu (YGL) sequence used by osteopontin to bind $\alpha4$ and $\alpha9$ integrins (Barry *et al.*, 2000; Green *et al.*, 2001; Yokosaki *et al.*, 1999) is reported here for the first time, leading to the hypothesis that this YGL sequence might mediate rotavirus recognition of $\alpha4$ integrins. As $\alpha4$ and $\alpha9$ integrins share ligand specificity, rotavirus usage of $\alpha9\beta1$ was examined. The roles of rotavirus IDA, LDV, LDI and YGL sequences in $\alpha4$ integrin recognition were studied here for the first time in cells with demonstrable $\alpha4$ expression.

Integrin subunits combine extracellularly to form a head and two legs. These domains exhibit structural rearrangements between the 'closed', low affinity conformation and the 'open', high affinity activated state (Hynes, 2002; Xiao *et al.*, 2004). The N-terminal $\sim$440 aa of integrin α-subunits contain regions important for ligand binding and consist of seven sequence repeats that are predicted to fold into a β-propeller domain in the integrin head (Springer, 1997). Propeller blades are connected by loop structures. Loops that are critical for $\alpha4$ adhesion to VCAM-1, fibronectin and MAdCAM-1 are grouped in the upper face of the β-propeller (Higgins *et al.*, 2000; Irie *et al.*, 1997; Ruiz-Velasco *et al.*, 2000). Another hypothesis tested here was that rotaviruses bind $\alpha4\beta1$ through the recognition of the same $\alpha4$ sequences as natural $\alpha4$ ligands.

METHODS

Antibodies, proteins and peptides. Monoclonal antibodies (mAbs) P4C2 ($\alpha4$), 8A2, 4B4 ($\beta1$) and MOPC21 were obtained as before (Coulson, 1997; Coulson *et al.*, 1997; Hewish *et al.*, 2000). mAbs AK7 ($\alpha2$) and Fib27 ($\beta7$) were purchased from Becton Dickenson PharMingen, P4G9 ($\alpha4$) from Chemicon and Y9A2 ($\alpha9$) from Serotec. mAb RA3-6B2 (rat anti-mouse B220) was provided by J. Allison, Dept. of Microbiology and Immunology, The University of Melbourne, Australia. BSA and fibronectin were obtained and used as described previously (Graham *et al.*, 2003). Peptides Ser–Val–Val–Tyr–Gly–Leu–Arg (SVVYGLR), Val–Arg–Val–Gly–Leu–Tyr–Ser (VRVGLYS), Ile–Asp–Ala–Pro–Ser (IDAPS), Ile–Glu–Ala–Pro–Ser (IEAPS), Glu–Ile–Leu–Asp–Val–Pro (EILDVP), Glu–Ile–Leu–Glu–Val–Pro (EILEVP), Asp–Gly–Glu–Ala (DGEA) and Gly–His–Arg–Pro (GHRP; $\geqslant$90 % pure by HPLC) were purchased from Auspep. Peptide SVVYGLR also was provided by A. Albiston, Howard Florey Institute of Experimental Physiology and Medicine, Melbourne, Australia.

Cell lines and viruses. The generation and maintenance of the cell lines utilized in this study and monitoring of their cell surface integrin expression by flow cytometry were carried out as described previously (Eto *et al.*, 2000; Hewish *et al.*, 2000; Higgins *et al.*, 2000; Irie *et al.*, 1995, 1997; Londrigan *et al.*, 2000). The cell lines utilized were: human K562, Caco-2 and rhabdomyosarcoma (RD), hamster CHO B2 and CHO K1, and monkey MA104 cells; K562 cells transfected with cDNA encoding human integrin subunits $\alpha3$ ($\alpha3$-K562), $\alpha4$ ($\alpha4$-K562), $\alpha9$ ($\alpha9$-K562) and empty vector (PBJ-K562); CHO B2 cells transfected with cDNA encoding human $\alpha4$ integrin-subunit (CHO B2 $\alpha4$), mutant $\alpha4$ in which $\alpha4$-subunit loops were replaced with the corresponding $\alpha5$ integrin-subunit regions R1 (residues 40–52), R2 (residues 112–131), R3a (residues 151–164), R3b (residues 181–189), R3c (residues 186–191), R4 (residues 237–247) and R5 (residues 282–288) (CHO B2 $\alpha4$ R1, CHO B2 $\alpha4$ R2, CHO B2 $\alpha4$ R3a, CHO B2 $\alpha4$ R3b, CHO B2 $\alpha4$ R3c, CHO B2 $\alpha4$ R4 and CHO B2 $\alpha4$ R5, respectively) and human $\alpha4$ and $\beta7$ ($\alpha4\beta7$-CHO B2); CHO K1 cells transfected with cDNA encoding human $\alpha4$ (CHO K1 $\alpha4$) and $\alpha4$ with point mutations Y120A/G130A (CHO K1 $\alpha4$ Y120A/G130A), Y187A (CHO K1 $\alpha4$ Y187A) and G190A (CHO K1 $\alpha4$ G190A), and human $\alpha4$ and $\beta7$ ($\alpha4\beta7$-CHO K1). The presence of the expected epitopes on recombinant human $\alpha4$ and $\beta7$ expressed on the cell surface was confirmed by flow cytometry using mAbs P4C2 and P4G9 for wild-type and mutant $\alpha4$, respectively, and mAb Fib27 for $\beta7$. The origins of the rotaviruses that were used in this study, and their cultivation in MA104 cells, have been described previously (Graham *et al.*, 2003; Kirkwood *et al.*, 1993).

Virus binding, infectivity and growth assays. The assays used to determine the titre of infectious rotavirus bound to cells, and measure peptide and mAb inhibition of virus binding were carried out as described previously (Coulson *et al.*, 1997; Graham *et al.*, 2003, 2004; Hewish *et al.*, 2000; Londrigan *et al.*, 2003). Briefly,

washed cells were incubated at 37 °C with peptides (0·5 mM) at pH 7·5 for 1 h or mAbs (10 μg ml^{-1}) for 2 h. This antibody concentration was determined by flow cytometry to be the lowest saturating concentration. In the presence of peptide or antibody, cells were cooled on ice for 20 min. Trypsin-activated virus (m.o.i. of 3·5 unless otherwise stated) was cooled to 4 °C and allowed to attach to cells on ice for 1 h. Cell-bound virus titres were determined by indirect immunofluorescent staining of infected MA104 cells, and expressed as focus-forming units per ml (f.f.u. ml^{-1}) (Hewish *et al.*, 2000). The effects of peptides at 0·5 mM (unless otherwise stated), and mAbs on virus infectivity (m.o.i. of 0·02) and growth (m.o.i. of 0·35) were measured by a fluorescence focus reduction-assay (Coulson *et al.*, 1997; Graham *et al.*, 2004; Hewish *et al.*, 2000; Londrigan *et al.*, 2003). For mock infection, cell cultures were inoculated with lysates of confluent, uninfected MA104 cells that had been trypsin-treated and diluted as for the virus infectivity activation. The error bars on the graphs presented represent the standard deviation (SD).

Flow cytometric assay of virus infectivity. The proportion of PBJ-K562 and α4-K562 cells that were rotavirus-infected was determined by flow cytometry, as these cells showed non-adherent growth. Cells (5×10^5) that had been infected with trypsin-activated rotaviruses RRV, CRW-8, Wa or RV-5 (m.o.i. of 10) for 16 h were washed once with PBS containing 1 % (v/v) fetal calf serum (PBS/FCS). Cells were permeabilized by methanol fixation for 7 min at −20 °C and washed with PBS/FCS. Cell-associated viral antigen was detected with rabbit antiserum to RRV, using as a negative control normal rabbit serum in which antibodies to rotavirus were present at a titre of $<1:100$ by ELISA (Graham *et al.*, 2003). Cells were further processed for flow cytometry using a two-step stain. This flow cytometric method was validated using rotavirus-infected MA104 cells, by comparison with visual counting of infected cells in fixed monolayers stained by indirect immunofluorescence with the same antiserum (Hewish *et al.*, 2000).

RESULTS

Potential ligand sequences for α4β1, α4β7 and α9β1 in rotavirus

The YGL integrin ligand sequence was identified in 164 of 168 (98 %) of VP4 sequences in the Entrez Database (NCBI), in VP5* at aa 448–450. The YGL sequence in osteopontin binds α4β1 and α9β1, and antagonizes α4β7 binding by MAdCAM-1 (Barry *et al.*, 2000; Green *et al.*, 2001; Yokosaki *et al.*, 1999). In 60 of 156 (39 %) of rotaviruses, VP4 contains the IDA sequence in VP5* at aa 538–540. Also, in 272 of 605 (45 %) of rotaviruses, VP7 sequences contain Leu–Asp–Val–Thr (LDVT) at aa 237–240 and 78 % contain Leu–Asp–Ile–Thr (LDIT) at aa 269–272 (Coulson *et al.*, 1997). The sequences present in rotaviruses studied here are indicated in Table 1.

Rotavirus proteins involved in α4β1 recognition

Rotavirus binding to human α4β1 was examined using an assay developed previously that detected SA11 binding to α4β1 on K562 cells, and binding of many rotaviruses to α2β1 on a range of cell types (Graham *et al.*, 2003, 2004; Hewish *et al.*, 2000; Londrigan *et al.*, 2003). RRV purification did not alter its binding or infectivity using α4β1 on CHO K1 cells, so clarified virus cell harvests were used as before. Rotaviruses Wa, RV-5, SA11 and RRV that use α2β1, αxβ2 and αvβ3 for cell binding and entry also bound α4β1 (Table 1). Strain K8 that uses α2β1, αxβ2 and αvβ3, and integrin-independent virus CRW-8 did not bind α4β1. The rotavirus strain, ST-3, does not use α2β1, αxβ2 or αvβ3, but did bind α4β1. Thus, for 5 of 7 rotaviruses, α4β1 usage correlated with that of other integrins. The two P serotype 1A rotaviruses Wa and F45 differed in their ability to bind α4β1, showing that the P type did not entirely determine α4β1 usage. Wa and RV-5 lack IDA, and Wa, RV-5 and ST-3 lack LDVT, indicating that these sequences were not essential for α4β1 binding. CRW-8 and K8 have YGL and LDIT but did not bind α4β1. However, all rotaviruses that bound α4β1 possessed YGL and LDIT.

The identity of the viral gene(s) involved in α4β1 usage was examined using laboratory-generated reassortants that contained a heterologous VP7 gene segment. Several of these reassortants were used previously to map rotavirus recognition of other integrins (Graham *et al.*, 2003). In three reassortants, the α4β1-binding phenotype segregated with VP4 (Table 1). A further three reassortants bound α4β1, consistent with the phenotypes of their parental viruses. Rotavirus usage of α4β1 did not co-segregate with any other viral gene.

Rotavirus binding to α4β1 and α4β7 depended on the ligand-binding domain of α4 and was inhibited by integrin ligand peptides

The ability of rotaviruses to bind recombinant human α4β7 was tested by comparing virus binding to CHO B2, α4-CHO B2 and α4β7-CHO B2 cells. Flow cytometric histograms of α4 expression, determined with mAb P4C2, were identical for α4-CHO B2 and α4β7-CHO B2 cells, so their cell surface levels of α4 were indistinguishable. The relative linear median fluorescence intensity (MFI) of α4 expression was calculated from these histograms, using previously described methodology (Graham *et al.*, 2003), as 3·5 and 3·2, respectively. As expected, only α4β7-CHO B2 cells expressed the β7-subunit, which was detected with anti-β7 antibody Fib27. The MFI of β7 expression on α4β7-CHO B2 cells was 2·8. CHO B2 cells did not express α4 or β7, as they showed an MFI of 1·0 for these integrin subunits. α4-CHO B2 cells did not express β7 (MFI of 0·8). Similar numbers of α4-K562 cells and K562 cells transfected with α4β7 bind fibronectin CS-1 and VCAM-1 (Guerrero-Esteo *et al.*, 1998; Munoz *et al.*, 1997; Ruiz-Velasco *et al.*, 2000). Thus, alteration of the α4β1 activation state on CHO cells by β7 expression is unlikely.

SA11, RRV and Wa bound to human α4 combined with hamster β1 on α4-CHO B2 cells, but bound to a higher level to α4β7-CHO B2 cells that expressed both human α4 combined with hamster β1, and human α4β7 (Fig. 1). Thus, these viruses bound to both α4β7 and α4β1 on α4β7-CHO B2 cells. Similar results were obtained using α4β7-CHO K1 and α4-CHO K1 cells (data not shown). SA11 binding to α4-K562 cells was abolished by cellular treatment with mAb

Table 1. Relation of recombinant $\alpha4\beta1$ binding by laboratory-adapted rotaviruses and their reassortants to their possession of potential $\alpha4$ ligand sequences

Rotavirus*	Serotype†		Sequence present				Binding to $\alpha4\beta1$ (mean % of control ± SD)‡
	G (VP7)	P (VP4)	LDV (VP7)	LDI (VP7)	YGL (VP4)	IDA (VP4)	
Si/SA11	3	5B[2]	+	+	+	+	**220 ± 27**
K8 × SA11	1	5B[2]	−	+	+	+	**245 ± 22**
RV-5 × SA11	2	5B[2]	−	+	+	+	**334 ± 77**
ST-3 × SA11	4	5B[2]	−	+	+	+	**188 ± 8**
F45 × SA11	9	5B[2]	+	ND	+	+	**206 ± 16**
Si/RRV	3	5B[3]	+	+	+	+	**233 ± 13**
RV-5 × RRV	2	5B[3]	−	+	+	+	**158 ± 9**
Hu/Wa	1	1A[8]	−	+	+	−	**206 ± 5**
Hu/K8	1	3[9]	−	+	+	−	102 ± 23
Hu/RV-5	2	1B[4]	−	+	+	−	**225 ± 22**
Hu/ST-3	4	2A[6]	−	+	+	+	**153 ± 11**
Hu/F45	9	1A[8]	+	ND	+	−	97 ± 6
Po/CRW-8	3	9[7]	−	+	+	+	99 ± 7
RV-5 × CRW-8	2	9[7]	−	+	+	+	99 ± 10

*Laboratory-adapted rotaviruses are shown with strain name preceded by the species of origin. Si, Simian; Hu, human; Po, porcine. In reassortants (shown in italics), VP7 and VP4 originated from the first- and last-named virus, respectively (Lazdins *et al.*, 1995; Nagesha *et al.*, 1989; Nagesha & Holmes, 1991).

†Serotypes were designated previously (Ciarlet *et al.*, 2002; Hoshino *et al.*, 1998; Kirkwood *et al.*, 1998). P serotype precedes P genotype, which is shown in square brackets.

‡Infectious rotavirus binding to $\alpha4\beta1$ is expressed as a percentage of the binding to PBJ-K562 cells, and positive levels of binding are in bold. PBJ-K562 cells bound $1{\cdot}0 \times 10^4$–$1{\cdot}4 \times 10^5$ f.f.u. ml^{-1} of virus, depending on the strain. This shows that 2–28 % of these cells bound virus, and that 0·6–8 % of input virus was bound, depending on the virus strain. Cellular treatment with mAb P4C2, as described in Fig. 3, reduced F45 × SA11 binding to $\alpha4$-K562 cells to 102 ± 4 % of control, but had no effect on F45 × SA11 binding to PBJ-K562 cells. Therefore, the binding of F45XSA11 measured was specific for $\alpha4\beta1$. SA11, RRV and CRW-8, but not Wa or RV-5, were shown to infect $\alpha4$-K562 cells following $\alpha4\beta1$ binding (Fig. 3).

ND, Not done.

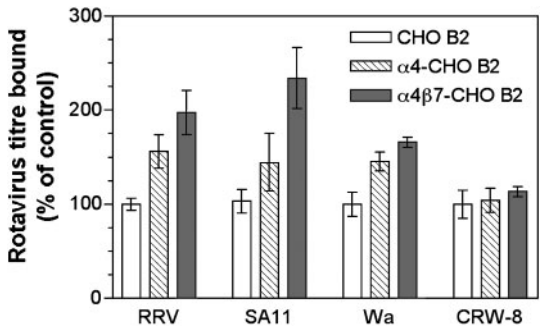

Fig. 1. SA11, RRV and Wa but not CRW-8 bound human $\alpha4\beta7$ and human $\alpha4$ combined with hamster $\beta1$. Rotavirus antigen in infected cells was detected with a rabbit antiserum to RRV. On the *y* axis, the titre of infectious virus bound to cells is expressed as a percentage of the titre bound to control CHO B2 cells. Results shown are representative of data obtained at an m.o.i. of 3·5–10 for all rotavirus strains tested. CHO cells bound $1{\cdot}1 \times 10^4$–$9{\cdot}1 \times 10^4$ f.f.u. ml^{-1} of virus, depending on the virus strain. Thus, 3–18 % of CHO cells bound virus and 0·22–1·8 % of input virus was bound.

P4C2 (Hewish *et al.*, 2000). This antibody maps to the second and third N-terminal repeats (R2 and R3) of $\alpha4$ that contain residues critical for ligand binding (Irie *et al.*, 1995, 1997; Kamata *et al.*, 1995). P4C2 also eliminated RRV and Wa binding to $\alpha4$-K562 cells, as virus titres bound in the presence of this antibody were indistinguishable from those bound to PBJ-K562 and $\alpha3$-K562 cells (Fig. 2). P4C2 similarly abrogated rotavirus binding to $\alpha4\beta7$. In contrast, the function-blocking mAb Fib27 that maps to aa 176–237 of $\beta7$ (Tidswell *et al.*, 1997) had no effect on virus binding to $\alpha4\beta7$. Similarly, function-blocking anti-$\beta1$ antibody 4B4 that inhibited SA11 binding to MA104 cells (Coulson, 1997) did not affect RRV binding to $\alpha4\beta1$. CRW-8 binding to CHO and K562 cells was independent of $\alpha4\beta1$ or $\alpha4\beta7$ and unaffected by the anti-$\alpha4$ or -$\beta7$ mAbs.

Peptides containing the integrin ligand sequences present in rotaviruses were used to further define the roles of these sequences in $\alpha4$ binding. Peptides used as negative controls were the same as those included in previous studies of integrin recognition of cellular ligands. Peptides inhibitory to rotavirus binding were SVVYGLR that binds $\alpha4\beta1$ and $\alpha4\beta7$, derived from osteopontin (Barry *et al.*, 2000; Green

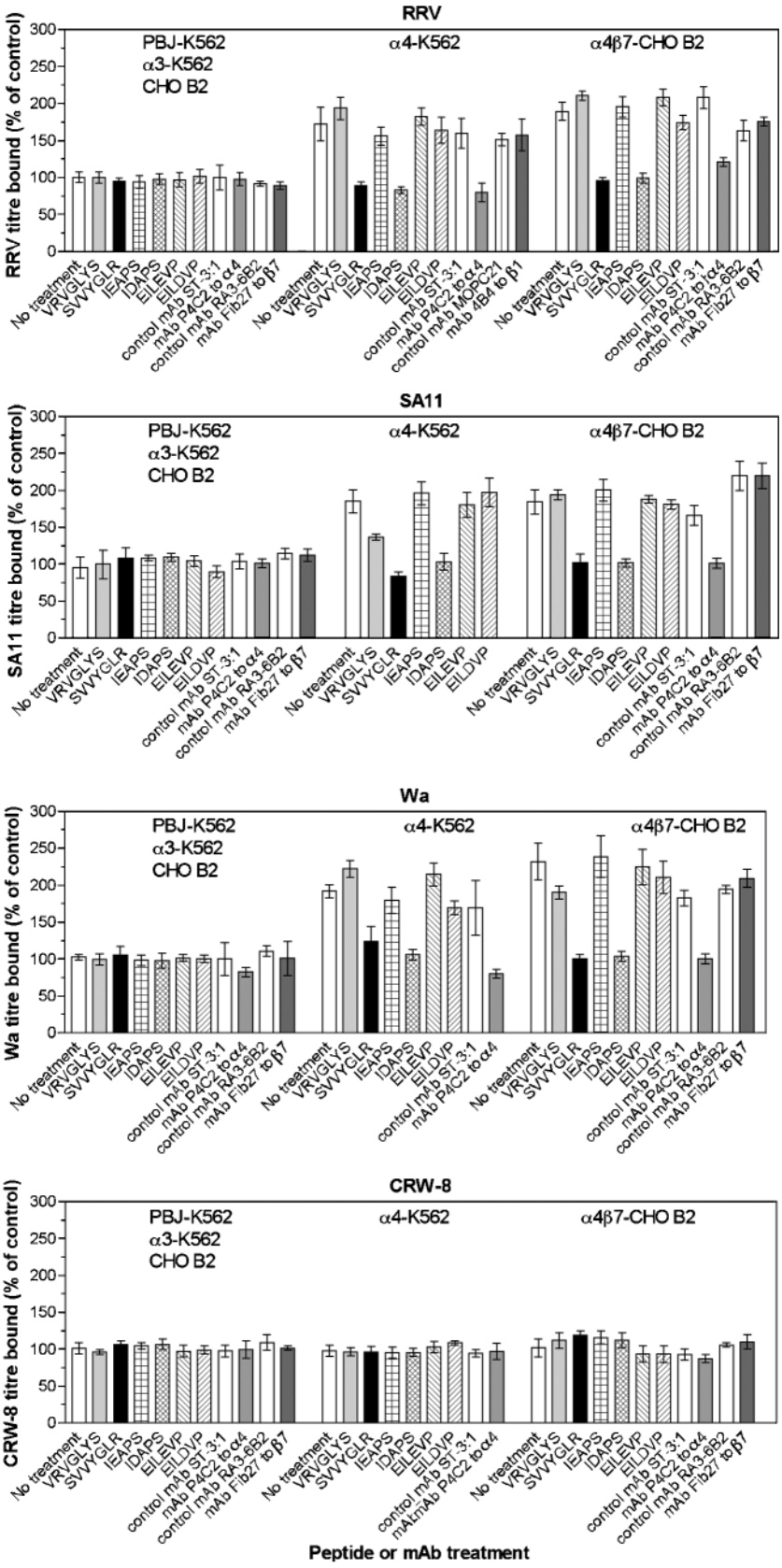

Fig. 2. Binding of RRV, SA11 and Wa to α4β1 and α4β7 was eliminated by anti-α4 mAb P4C2, and the peptides SVVYGLR and IDAPS, but not EILDVP. The cell lines tested are indicated above each set of bars. Where >1 cell line is listed, the data are representative of the results obtained with all the listed cell lines. On the *y* axis, the titre of infectious virus bound to cells is expressed as a percentage of the titre bound to control cells in the absence of any treatment. Cell-binding controls were PBJ-K562 for α4-K562 cells and CHO B2 for α4β7-CHO B2 cells. Studies in CHO B2 cell lines were performed at an m.o.i. of 10 with SA11, RRV and CRW-8 and an m.o.i. of 5 with Wa. Blockade of SA11 binding to α4-K562 cells by P4C2 was described previously (Hewish *et al.*, 2000).

et al., 2001), and IDAPS, constituting a region of fibronectin H1 that has an indirect role in $\alpha4\beta1$ binding. IDAPS peptide blockade of $\alpha4\beta1$ binding to fibronectin has been proposed to result from mimicry of the EILDVP sequence in fibronectin that directly binds $\alpha4\beta1$ and $\alpha4\beta7$ (Chan *et al.*, 1992; Komoriya *et al.*, 1991; Ruegg *et al.*, 1992; Sharma *et al.*, 1999; Wayner & Kovach, 1992). SVVYGLR and IDAPS eliminated SA11, RRV and Wa binding to recombinant, cell surface-expressed $\alpha4\beta1$ and $\alpha4\beta7$ (Fig. 2). However, peptide EILDVP, and control peptides VRVGLYS, IEAPS and EILEVP did not affect binding of these viruses to $\alpha4$ integrins. A low level of SA11 inhibition was seen with VRVGLYS. None of the peptides altered the background level of binding of CRW-8 to the transfected cells.

Infectivity and growth of some rotaviruses were mediated through $\alpha4\beta1$ and $\alpha4\beta7$

SA11 binding to $\alpha4\beta1$ resulted in increased SA11 yield (Hewish *et al.*, 2000). The abilities of other rotaviruses to infect and replicate via $\alpha4\beta1$ were determined. In the presence of $\alpha4\beta1$, the number of K562 cells infected by RRV and CRW-8 (detected by flow cytometry) increased from a mean $\pm$ SD of $45{\cdot}3 \pm 1{\cdot}9$ to $56{\cdot}9 \pm 2{\cdot}0$ % and from $0{\cdot}1 \pm 0{\cdot}0$ to $24{\cdot}7 \pm 0{\cdot}4$ %, respectively (Fig. 3a). RRV and CRW-8 yields increased four- to sixfold due to $\alpha4$ expression (Fig. 3b). The negligible proportion of $\alpha4$-K562 cells infected by Wa ($0{\cdot}2 \pm 0{\cdot}1$ %) was similar to that in K562 cells ($0{\cdot}1 \pm 0{\cdot}1$ %) and mock-infected cells ($0{\cdot}2 \pm 0{\cdot}1$ %), and no infectious Wa or RV-5 was produced by PBJ-K562, $\alpha3$-K562 or $\alpha4$-K562 cells at 24–72 h post-infection (p.i.) (data not shown). RRV and CRW-8 growth in $\alpha4$-K562 cells was inhibited by SVVYGLR and IDAPS (Fig. 3c), the same peptides that abolished virus binding to $\alpha4$ integrins (Fig. 2). Thus, although SA11, RRV, Wa and RV-5 all bound $\alpha4\beta1$, only SA11 and RRV were able to utilize this binding for infection and virus growth in K562 cells. Conversely, CRW-8 interacted with $\alpha4\beta1$ to greatly increase its infectivity, but did not use this integrin for initial cell binding.

Rotavirus usage of naturally expressed $\alpha4\beta1$ on RD cells (Londrigan *et al.*, 2000) was studied. P4C2 and fibronectin showed a dose-dependent blockade of RRV infectivity in RD cells, to $26 \pm 7{\cdot}9$ % at 10 μg ml^{-1} and $35 \pm 3{\cdot}9$ % at 160 μg ml^{-1}, respectively. P4C2 did not affect RRV (data not shown) or SA11 (Coulson *et al.*, 1997) infectivity in $\alpha4$ integrin-negative MA104 cells.

CHO B2 cells lack $\alpha5$ integrin expression, so they lost adhesion during prolonged incubation at 37 °C (Schreiner *et al.*, 1989). Therefore, rotavirus infectivity mediated by $\alpha4\beta7$ was determined using $\alpha4\beta7$-CHO K1 cells. The mean $\pm$ SD of the respective SA11 and RRV titres in f.f.u. ml^{-1} produced at an m.o.i. of 40 in $\alpha4\beta7$-CHO K1 cells (5800 ± 750 and 6500 ± 1000) were significantly higher than those in CHO K1 cells (2400 ± 750 and 3300 ± 850) at 16 h p.i. (*t*-test, $P < 0{\cdot}0001$). Wa and CRW-8 did not infect either cell line. Thus, binding of SA11 and RRV, but not Wa, to $\alpha4\beta7$ resulted in increased infectivity in CHO cells.

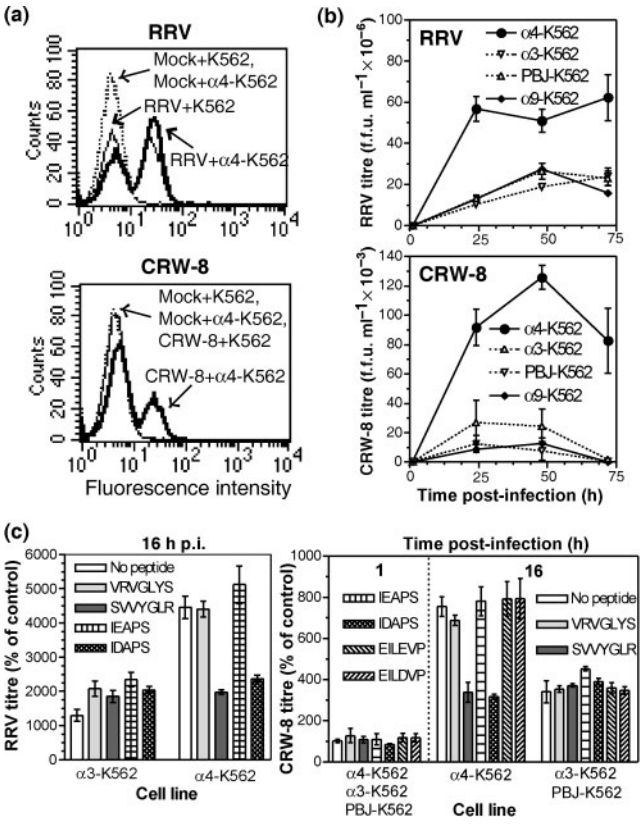

Fig. 3. Infectivity and growth of RRV and CRW-8 were mediated by recombinant, cell surface-expressed $\alpha4\beta1$. (a) Flow cytometric histograms show that RRV and CRW-8 were able to infect $\alpha4$-K562 cells using $\alpha4\beta1$. Histograms of $\alpha4$-K562 cells infected with RRV or CRW-8 (RRV + $\alpha4$-K562, CRW-8 + $\alpha4$-K562) are shown in bold lines, those of K562 cells infected with RRV or CRW-8 (RRV + K562, CRW-8 + K562) are shown in thin lines and those of mock-infected $\alpha4$-K562 and K562 cells are shown in thin dashed lines. (b) Titres of infectious RRV and CRW-8 produced in $\alpha4$-K562 cells were greater than those in $\alpha9$-K562, $\alpha3$-K562 and control cells. (c) Peptides SVVYGLR and IDAPS, but not EILDVP, inhibited RRV and CRW-8 growth in $\alpha4$-K562 cells. On the *y* axes, the titre of infectious virus bound is expressed as a percentage of the titre bound to PBJ-K562 cells at 1 h p.i. in the absence of peptide.

Rotaviruses did not utilize $\alpha9\beta1$ for cell attachment or entry

SA11, RRV and CRW-8 did not use recombinant $\alpha9\beta1$ for K562 cell infection (Fig. 3b) or replication (Fig. 4a, lower panel). SA11 (Fig. 4a, upper panel) and Wa (data not shown) did not bind $\alpha9\beta1$. Caco-2 cell expression of $\alpha9$ was detected by flow cytometry with anti-$\alpha9$ mAb Y9A2 (data not shown). This antibody did not affect SA11, RRV and Wa infection of Caco-2 cells, and peptide SVVYGLR did not affect SA11 infection of Caco-2 cells (Fig. 4b). Thus, $\alpha9\beta1$ was not a rotavirus receptor or entry co-factor.

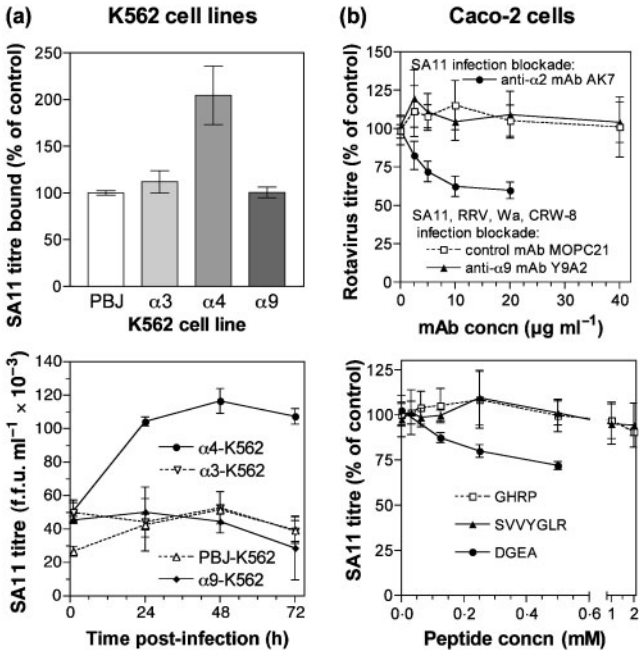

Fig. 4. SA11 rotavirus-cell binding, infectivity and replication were not mediated through α9β1. (a) SA11 binding (upper panel) and growth curves (lower panel) in α9-K562, α4-K562, α3-K562 and PBJ-K562 cells. In the *y* axis of the upper panel, the titre of infectious SA11 bound to cells is expressed as a percentage of the titre bound to PBJ-K562 cells. (b) Anti-α9 mAb Y9A2 (upper panel) and α9β1 ligand peptide SVVYGLR (lower panel) did not affect rotavirus infectivity in Caco-2 cells. Caco-2 cells were shown to express α9β1 by flow cytometry. At 40 mg ml^{-1}, Y9A2 detected α9β1 on 85 % of Caco-2 cells harvested from confluent monolayers, with a positive MFI of 1·41, consistent with a previous study (Basora *et al.*, 1998). Rotavirus infectious titres are expressed as a percentage of the titres in the absence of any treatment. Positive controls were anti-α2 antibody AK7 (upper panel), and the DGEA peptide that is inhibitory to rotavirus usage of α2β1 (lower panel).

Activation of β1 increased SA11 growth in α4-K562 cells

Activating antibodies induce integrins into high affinity states for ligand binding. Eosinophil resistance to α4β1-mediated detachment from VCAM-1 and MAdCAM-1 is increased by activating anti-β1 antibody 8A2 (Sriramarao *et al.*, 2000). To determine if β1 activation affects rotavirus–α4β1 interactions, SA11 replication was measured in α4-K562 cells that had been treated with 8A2 at the concentration (0·1–0·2 μg ml^{-1}) previously shown to increase K562 cell binding to fibronectin (Faull *et al.*, 1996) and SA11 binding to α2β1 (Graham *et al.*, 2003). Activation of β1 increased SA11 yield from α4-K562 cells by 50–70 % at 1 and 24 h p.i. (Fig. 5a). Thus, rotavirus infectivity was enhanced by α4β1 activation.

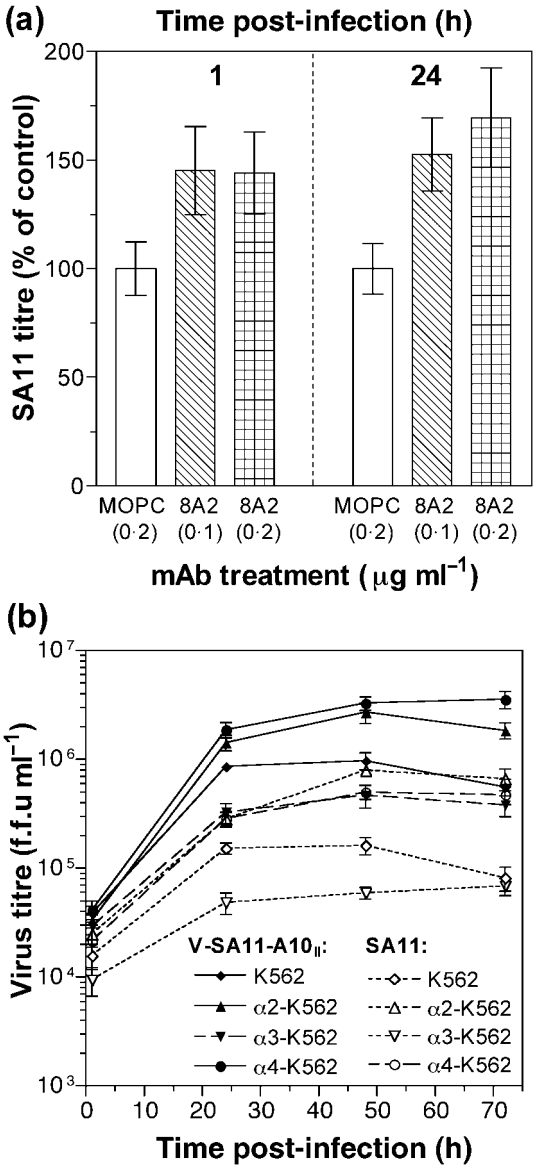

Fig. 5. Increased SA11 growth resulted from α4β1 activation (a) and mutation of VP7 LDVT sequence (b). (a) Effects of β1-activating (8A2) and control (MOPC21) mAbs on SA11 infectivity in α4-K562 cells. (b) Comparison of growth curves of V-SA11-A10$_{II}$ and SA11 in integrin-transfected and control K562 cells. See Legend to Fig. 4.

Mutation of LDVT in VP7 of SA11 did not affect α4β1 recognition

The minimal active site of the dominant α4β1-binding region in fibronectin is LDV, which is totally dependent on its Asp residue (Komoriya *et al.*, 1991). The role of VP7 LDVT sequence in rotavirus replication was directly analysed using an SA11 variant, V-SA11-A10$_{II}$, which has a single mutation in the Asp residue of the LDVT sequence (D238N) (Lazdins *et al.*, 1995). Similarly to SA11, V-SA11-A10$_{II}$

replicated to a higher titre in α4-K562 and α2-K562 cells than in control α3-K562 cells, so VP7 LDVT was not required for SA11 usage of α4β1 or α2β1 (Fig. 5b). V-SA11-$A10_{II}$ grew to a 10-fold higher titre than SA11 in these K562 cells. This might be directly related to the LNVT mutation, but the possibility that other viral genes are involved cannot be ruled out.

Domains and sequence of the α4-subunit required for rotavirus binding

The α4 domains crucial for rotavirus binding were located using swapping mutagenesis. Predicted protruding loops at or near α4β1 sites necessary for natural ligand binding were replaced with corresponding α5 regions (Higgins *et al.*, 2000; Irie *et al.*, 1997). Rotaviruses do not bind α5β1. Therefore, if the swapped α4 region is necessary for virus binding, swapping to the α5 region will prevent virus binding to α4. The region swapping will affect virus binding only if the exchanged regions are sufficiently distinct in sequence. The R3b and R3c domains are conservative in sequence between α4 and α5. The only point mutations in the putative ligand-binding region of α4 (aa 108–268) of 61 tested that abolish natural α4 ligand binding are non-conservative and located in R2 (Y120/G130), R3b (Y187) and R3c (G190) (Higgins *et al.*, 2000; Irie *et al.*, 1995, 1997). As shown in Fig. 6, α4 was detected at similar levels on all CHO cell lines transfected to express these swapping and point mutations in α4, using anti-α4 antibody P4G9 that maps to an epitope outside the swapped domains (Irie *et al.*, 1997; Kamata *et al.*, 1995). Binding of RRV, SA11 and Wa to α4β1 on CHO B2 cells was abolished by swapping R2 or R4, but was unaffected by swapping R1, R3a, R3b, R3c or R5 (Fig. 7). The point mutations eliminated Wa, RRV and SA11 binding to α4β1. CRW-8 did not bind α4β1 on any cell line tested, showing the specificity of the Wa, SA11 and RRV binding (Fig. 7). CS-1 peptide, VCAM-1 and MAdCAM-1 also required α4 R2 and R4, but not R1, R3a, R3b, R3c or R5 for binding (Higgins *et al.*, 2000; Irie *et al.*, 1997). Thus, the same α4 regions and amino acids are critical for α4 binding by both rotaviruses and these natural α4 ligands.

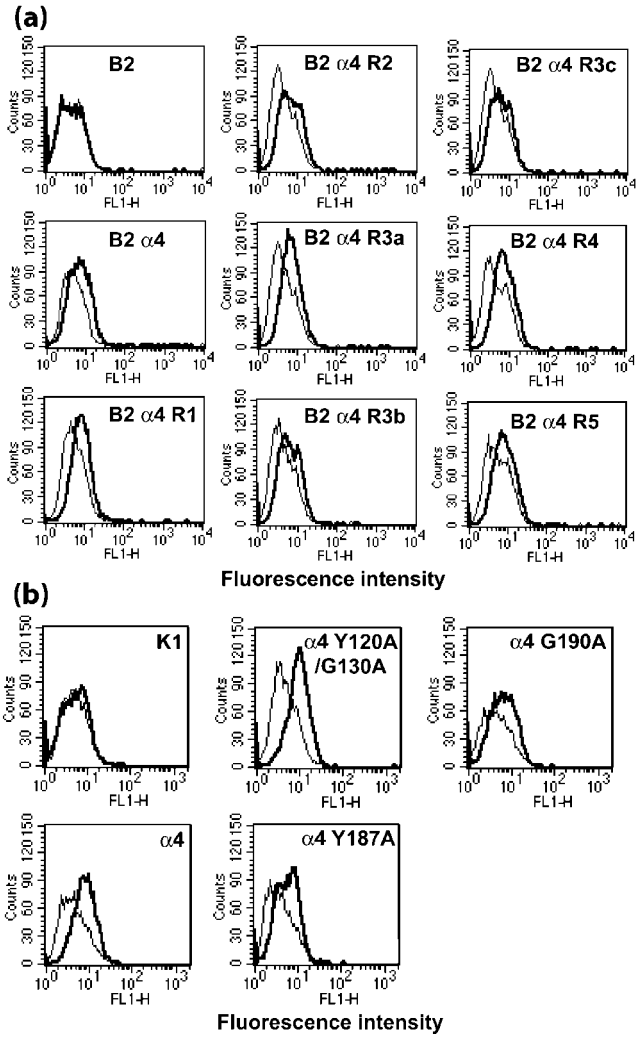

Fig. 6. Cell lines transfected with cDNA encoding mutated human α4 expressed α4 on their surface. (a) Domain swapping mutations of α4 expressed in CHO B2 cells that lack α5. (b) CHO K1 cell lines expressing α4 with point mutations. In each panel, the flow cytometric histogram of α4 expression (detected with P4G9 antibody at 10 mg ml^{-1}) is indicated with thick lines and that of the control (MOPC21 antibody at 10 mg ml^{-1}) is indicated with thin lines. The identity of the cell line depicted is indicated at the top of each panel.

DISCUSSION

In this study, the repertoire of integrins used by rotaviruses for cell-binding and post-binding interactions was extended to include α4β7, an intestinal homing receptor crucial for protective immune responses. It was shown for the first time that human rotaviruses bind α4β1 and α4β7, and rotaviruses require the same α4 residues for binding as natural α4 ligands. Three modes of rotavirus interactions with α4 integrins were identified. Firstly, SA11 (and RRV) uses α4β1 and α4β7 as receptors, resulting in K562 and CHO K1 cell infection. Secondly, Wa and RV-5 bind α4β1 but fail to infect K562 cells expressing α4β1. Thirdly, CRW-8 infectivity in K562 cells is greatly increased by α4β1 expression, but CRW-8 appears to use α4β1 as a co-entry factor rather than a receptor. The latter two modes have not been demonstrated previously.

A further novel finding is the likely involvement of the conserved VP5* YGL sequence in α4 recognition by rotaviruses. The peptide SVVYGLR inhibited α4β1 and α4β7 binding by rotaviruses and virus infectivity, and α4β1 binding by rotaviruses segregated with VP4 in reassortants. It is proposed that VP4 binds α4β1 using YGL. In the X-ray crystallographic structure of RRV VP5* containing aa 252–523 (VP5CT), most of the Tyr residue in the YGL sequence is exposed on the surface (Dormitzer *et al.*, 2004). The shorter

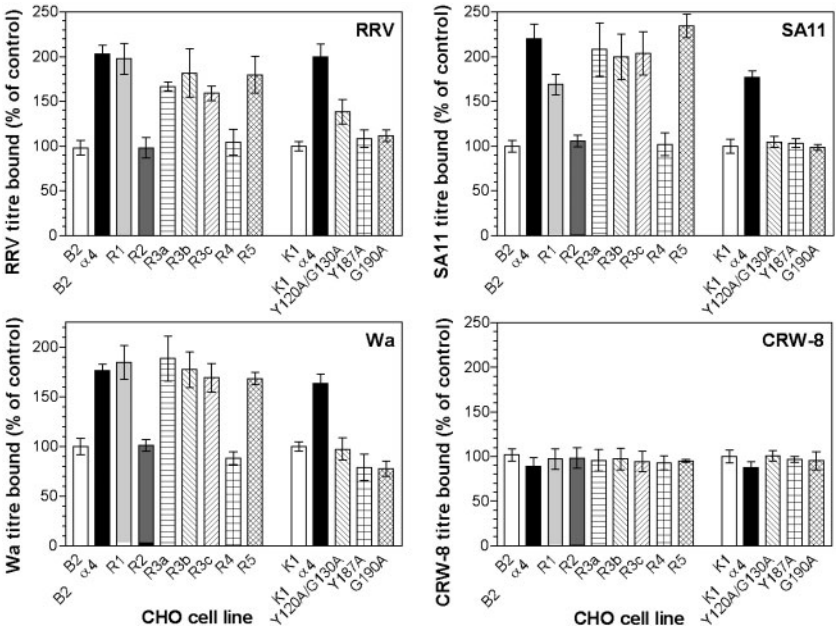

Fig. 7. Effect of domain swapping and point mutagenesis of α4 on RRV, SA11, Wa and CRW-8 binding to cell surface-expressed α4β1. On the *y* axis, the titre of infectious virus bound to cells is expressed as a percentage of the titre bound to control cells. Control cells were CHO B2 for α4 domain swapping mutations and CHO K1 for α4 point mutations. SA11, RRV and CRW-8 at an m.o.i. of 10, and Wa at an m.o.i. of 5 were used throughout.

sequence SVVYG is sufficient to mediate adhesion to α4 integrins (Gao *et al.*, 2004). Possibly, exposure of the Tyr and Gly residues in YGL may be sufficient for binding. The YGL sequence might become accessible through opening of a possible diglycine hinge in overlying β-sheets (Dormitzer *et al.*, 2004).

The VP5* IDA sequence was not necessary for α4 integrin binding by rotaviruses, consistent with the IDA location in a predicted heptad repeat region that may be inaccessible (Bremont *et al.*, 1992; Lopez *et al.*, 1991). Peptide IDAPS elimination of rotavirus binding to α4 integrins probably occurred via non-IDA viral sequence, similarly to IDAPS inhibition of fibronectin binding to α4 through EILDVP mimicry (Sharma *et al.*, 1999). SVVYGLR and EILDVP peptides compete for α4β1 binding, and have similar affinities for α4β1 (Barry *et al.*, 2000). As SVVYGLR, IDAPS and EILDVP peptides inhibit ligand binding through related α4 regions, the differential effects of these peptides on rotavirus binding and infection probably relate to sequence differences in parts of the peptides with portions of the α4-binding regions in rotavirus. The VP7 LDV sequence was not involved in α4 integrin binding, as the presence of LDVT, LDVT mutation and EILDVP blockade did not affect α4 interactions with rotaviruses. This lack of inhibition by EILDVP also suggests that the VP7 LDI is not necessary for α4 recognition by rotaviruses.

Anti-VP5* mAb 2G4, and anti-VP7 antibodies RV-4 : 2 and F45 : 2, inhibited rotavirus–α4β1 binding to a titre similar to their neutralization titres. Acute- and convalescent-phase sera from children with gastroenteritis also specifically inhibited Wa binding to α4β1 (K. L. Graham, F. E. Fleming, P. Halasz, Y. Takada and B. S. Coulson, unpublished results). These findings support the proposed roles for VP4 and VP7 in α4β1 recognition by rotaviruses.

Rotavirus usage of α4 integrins but not α9β1 was unexpected, as SVVYGLR is a common ligand for these integrins. Mutation of the SVVYGLR peptide showed that Leu and Tyr are important for α4 binding (Green *et al.*, 2001), whereas the Tyr appears to be critical for α9β1 binding (Yokosaki *et al.*, 1999). The first Val has a role in α4 binding, and mutation of the Arg increased activity against α4β1 but reduced activity against α4β7 (Green *et al.*, 2001). Non-conservative amino acid changes for the Val and Arg adjacent to YGL in VP5* could prevent α9β1 binding by rotaviruses.

It is at present unclear how rotavirus interactions with α4 fit with current models of rotavirus cell entry. SA11 and RRV binding to α4β1 and α4β7 may facilitate virus cell entry by endocytosis or direct membrane penetration (Lopez & Arias, 2004), as proposed previously for rotavirus usage of other integrins (Graham *et al.*, 2003). Human rotavirus binding to α4 might affect cellular functions that relate to antirotaviral responses, without facilitating infection. CRW-8 cell binding is independent of cellular protein synthesis (Jolly *et al.*, 2001), and OSU, another porcine rotavirus of the same P serotype as CRW-8, binds ganglioside GM3 (Rolsma *et al.*, 1998). Therefore, CRW-8 may bind GM3, facilitating usage of α4 integrins in a co-receptor role that results in cell entry. The identification of several possible modes of rotavirus interactions with α4 in this study will allow further dissection of the mechanisms by which different rotaviruses utilize these integrins.

The adjacent R2, R3b, R3c and R4 loops of α4 in the upper face of the predicted β-propeller structure of the integrin head were critical for rotavirus α4 recognition. However, loops R1 and R5 that are located distal from R2, R3b R3c and R4 on the upper or lower face, respectively (Higgins *et al.*, 2000; Irie *et al.*, 1997; Springer, 1997), were not involved in

rotavirus α4 binding. The α4 loops required for binding by rotaviruses, MAdCAM-1, fibronectin and VCAM-1 are, therefore, identical (Higgins *et al.*, 2000; Irie *et al.*, 1995, 1997). Judging from their predicted positions, these loops are highly exposed on the head of activated α4 and ideally placed to mediate binding to rotaviruses and natural ligands. The increased rotavirus infectivity after α4β1 activation supports this conclusion.

This binding of rotaviruses to α4 integrins through the same α4 domains as natural ligands strongly suggests that virus α4 binding is likely to affect the functions of these competing ligands. The blockade of rotavirus α4 binding by SVVYGLR reinforces this conclusion, as SVVYGLR completely inhibits α4β7 binding to MAdCAM-1 and α4β1 binding to fibronectin CS-1, and partially inhibits α4β1 binding to VCAM-1 (Green *et al.*, 2001). Rotavirus α4β7 binding might affect immune responses dependent on α4β7 recognition of MAdCAM-1 for intestinal homing, and modulate the protection afforded against reinfection by rotavirus-specific $\alpha4\beta7^{hi}$ B and T cells (Gonzalez *et al.*, 2003; Rose *et al.*, 1998; Rott *et al.*, 1997; Williams *et al.*, 1998; Youngman *et al.*, 2002). Potential immune modulation by rotavirus–integrin interactions is a topic for further study.

Lamina propria mesenchymal cells are the only resident gut cell known to express α4 (Choy *et al.*, 1990; Pender *et al.*, 2000). Possibly, α4β1 on these cells may bind rotaviruses. Rotavirus disease may be associated with viraemia (Blutt *et al.*, 2003), so virus in the circulation could contact immune cells expressing α4. Rotavirus recognition of activated α4β1 might facilitate virus carriage and infection. Lymphoid cells mediate rotavirus escape from the gut (Mossel & Ramig, 2003), which might involve virus α4 integrin binding. Rotavirus infection has been associated with encephalitis (Iturriza-Gomara *et al.*, 2002) and pancreatic islet autoantibody responses (Honeyman *et al.*, 2000). Rotavirus α4β7 binding might disseminate virus within the intestine and to the pancreas and brain where MAdCAM-1 is also expressed. These issues are important subjects for continuing research.

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REFERENCES

Barry, S. T., Ludbrook, S. B., Murrison, E. & Horgan, C. M. (2000). Analysis of the α4β1 integrin-osteopontin interaction. *Exp Cell Res* **258**, 342–351.

Basora, N., Desloges, N., Chang, Q., Bouatrouss, Y., Gosselin, J., Poisson, J., Sheppard, D. & Beaulieu, J. F. (1998). Expression of the α9β1 integrin in human colonic epithelial cells: resurgence of the fetal phenotype in a subset of colon cancers and adenocarcinoma cell lines. *Int J Cancer* **75**, 738–743.

Blutt, S. E., Kirkwood, C. D., Parreno, V., Warfield, K. L., Ciarlet, M., Estes, M. K., Bok, K., Bishop, R. F. & Conner, M. E. (2003). Rotavirus antigenaemia and viraemia: a common event? *Lancet* **362**, 1445–1449.

Bremont, M., Juste-Lesage, P., Chabanne-Vautherot, D., Charpilienne, A. & Cohen, J. (1992). Sequences of the four larger proteins of a porcine group C rotavirus and comparison with the equivalent group A rotavirus proteins. *Virology* **186**, 684–692.

Brown, K. A., Kriss, J. A., Moser, C. A., Wenner, W. J. & Offit, P. A. (2000). Circulating rotavirus-specific antibody-secreting cells (ASCs) predict the presence of rotavirus-specific ASCs in the human small intestinal lamina propria. *J Infect Dis* **182**, 1039–1043.

Caruso, M., Belloni, L., Sthandier, O., Amati, P. & Garcia, M. I. (2003). α4β1 integrin acts as a cell receptor for murine polyomavirus at the postattachment level. *J Virol* **77**, 3913–3921.

Chan, B. M., Elices, M. J., Murphy, E. & Hemler, M. E. (1992). Adhesion to vascular cell adhesion molecule 1 and fibronectin. Comparison of α4β1 (VLA-4) and α4β7 on the human B cell line JY. *J Biol Chem* **267**, 8366–8370.

Choy, M. Y., Richman, P. I., Horton, M. A. & MacDonald, T. T. (1990). Expression of the VLA family of integrins in human intestine. *J Pathol* **160**, 35–40.

Ciarlet, M., Ludert, J. E., Iturriza-Gomara, M., Liprandi, F., Gray, J. J., Desselberger, U. & Estes, M. K. (2002). Initial interaction of rotavirus strains with *N*-acetylneuraminic (sialic) acid residues on the cell surface correlates with VP4 genotype, not species of origin. *J Virol* **76**, 4087–4095.

Coulson, B. S. (1997). Effects of workshop monoclonal antibodies on rotavirus infection of cells. In *Leucocyte Typing VI*, pp. 391–393. Edited by T. Kishimoto, H. Kikutani, A. E. G. Kr. von dem Borne, S. M. Goyert, D. Y. Mason, M. Miyasaka, L. Moretta, K. Okumura, S. Shaw, T. A. Springer, K. Sugamura & H. Zola. New York: Garland Publishing, Inc.

Coulson, B. S., Grimwood, K., Hudson, I. L., Barnes, G. L. & Bishop, R. F. (1992). Role of coproantibody in clinical protection of children during reinfection with rotavirus. *J Clin Microbiol* **30**, 1678–1684.

Coulson, B. S., Londrigan, S. L. & Lee, D. J. (1997). Rotavirus contains integrin ligand sequences and a disintegrin-like domain that are implicated in virus entry into cells. *Proc Natl Acad Sci U S A* **94**, 5389–5394.

Dormitzer, P. R., Nason, E. B., Prasad, B. V. & Harrison, S. C. (2004). Structural rearrangements in the membrane penetration protein of a non-enveloped virus. *Nature* **430**, 1053–1058.

Eto, K., Puzon-McLaughlin, W., Sheppard, D., Sehara-Fujisawa, A., Zhang, X. P. & Takada, Y. (2000). RGD-independent binding of integrin α9β1 to the ADAM-12 and -15 disintegrin domains mediates cell–cell interaction. *J Biol Chem* **275**, 34922–34930.

Faull, R. J., Wang, J., Leavesley, D. I., Puzon, W., Russ, G. R., Vestweber, D. & Takada, Y. (1996). A novel activating anti-β1 integrin monoclonal antibody binds to the cysteine-rich repeats in the β1 chain. *J Biol Chem* **271**, 25099–25106.

Franco, M. A. & Greenberg, H. B. (1999). Immunity to rotavirus infection in mice. *J Infect Dis* **179**, S466–S469.

Gao, Y. A., Agnihotri, R., Vary, C. P. & Liaw, L. (2004). Expression and characterization of recombinant osteopontin peptides representing matrix metalloproteinase proteolytic fragments. *Matrix Biol* **23**, 457–466.

Gonzalez, A. M., Jaimes, M. C., Cajiao, I. & 8 other authors (2003). Rotavirus-specific B cells induced by recent infection in adults and children predominantly express the intestinal homing receptor α4β7. *Virology* **305**, 93–105.

Graham, K. L., Halasz, P., Tan, Y., Hewish, M. J., Takada, Y., Mackow, E. R., Robinson, M. K. & Coulson, B. S. (2003). Integrin-using rotaviruses bind α2β1 integrin α2 I domain via VP4 DGE sequence and recognize αXβ2 and αVβ3 by using VP7 during cell entry. *J Virol* **77**, 9969–9978.

Graham, K. L., Zeng, W., Takada, Y., Jackson, D. C. & Coulson, B. S. (2004). Effects on rotavirus cell binding and infection of monomeric and polymeric peptides containing α2β1 and αxβ2 integrin ligand sequences. *J Virol* **78**, 11786–11797.

Green, P. M., Ludbrook, S. B., Miller, D. D., Horgan, C. M. & Barry, S. T. (2001). Structural elements of the osteopontin SVVYGLR motif important for the interaction with α4 integrins. *FEBS Lett* **503**, 75–79.

Guerrero, C. A., Mendez, E., Zarate, S., Isa, P., Lopez, S. & Arias, C. F. (2000). Integrin αvβ3 mediates rotavirus cell entry. *Proc Natl Acad Sci U S A* **97**, 14644–14649.

Guerrero-Esteo, M., Ruiz-Velasco, N., Munoz, M. & Teixido, J. (1998). Role of two conserved glycine residues in the β-propeller domain of the integrin α4 subunit in VLA-4 conformation and function. *FEBS Lett* **429**, 123–128.

Hewish, M. J., Takada, Y. & Coulson, B. S. (2000). Integrins α2β1 and α4β1 can mediate SA11 rotavirus attachment and entry into cells. *J Virol* **74**, 228–236.

Higgins, J. M., Cernadas, M., Tan, K., Irie, A., Wang, J., Takada, Y. & Brenner, M. B. (2000). The role of α and β chains in ligand recognition by β7 integrins. *J Biol Chem* **275**, 25652–25664.

Honeyman, M. C., Coulson, B. S., Stone, N. L. & 7 other authors (2000). Association between rotavirus infection and pancreatic islet autoimmunity in children at risk of developing type 1 diabetes. *Diabetes* **49**, 1319–1324.

Hoshino, Y., Jones, R. W. & Kapikian, A. Z. (1998). Serotypic characterization of outer capsid spike protein VP4 of vervet monkey rotavirus SA11 strain. *Arch Virol* **143**, 1233–1244.

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673–687.

Irie, A., Kamata, T., Puzon-McLaughlin, W. & Takada, Y. (1995). Critical amino acid residues for ligand binding are clustered in a predicted β-turn of the third N-terminal repeat in the integrin α4 and α5 subunits. *EMBO J* **14**, 5550–5556.

Irie, A., Kamata, T. & Takada, Y. (1997). Multiple loop structures critical for ligand binding of the integrin α4 subunit in the upper face of the β-propeller mode 1. *Proc Natl Acad Sci U S A* **94**, 7198–7203.

Iturriza-Gomara, M., Auchterlonie, I. A., Zaw, W., Molyneaux, P., Desselberger, U. & Gray, J. (2002). Rotavirus gastroenteritis and central nervous system (CNS) infection: characterization of the VP7 and VP4 genes of rotavirus strains isolated from paired fecal and cerebrospinal fluid samples from a child with CNS disease. *J Clin Microbiol* **40**, 4797–4799.

Jolly, C. L., Beisner, B. M., Ozser, E. & Holmes, I. H. (2001). Non-lytic extraction and characterisation of receptors for multiple strains of rotavirus. *Arch Virol* **146**, 1307–1323.

Kamata, T., Puzon, W. & Takada, Y. (1995). Identification of putative ligand-binding sites of the integrin α4β1 (VLA-4, CD49d/CD29). *Biochem J* **305**, 945–951.

Kirkwood, C., Masendycz, P. J. & Coulson, B. S. (1993). Characteristics and location of cross-reactive and serotype-specific neutralization sites on VP7 of human G type 9 rotaviruses. *Virology* **196**, 79–88.

Kirkwood, C. D., Bishop, R. F. & Coulson, B. S. (1998). Attachment and growth of human rotaviruses RV-3 and S12/85 in Caco-2 cells depend on VP4. *J Virol* **72**, 9348–9352.

Komoriya, A., Green, L. J., Mervic, M., Yamada, S. S., Yamada, K. M. & Humphries, M. J. (1991). The minimal essential sequence for a major cell type-specific adhesion site (CS1) within the alternatively spliced type III connecting segment domain of fibronectin is leucine-aspartic acid-valine. *J Biol Chem* **266**, 15075–15079.

Lazdins, I., Coulson, B. S., Kirkwood, C., Dyall-Smith, M., Masendycz, P. J., Sonza, S. & Holmes, I. H. (1995). Rotavirus antigenicity is affected by the genetic context and glycosylation of VP7. *Virology* **209**, 80–89.

Londrigan, S. L., Hewish, M. J., Thomson, M. J., Sanders, G. M., Mustafa, H. & Coulson, B. S. (2000). Growth of rotaviruses in continuous human and monkey cell lines that vary in their expression of integrins. *J Gen Virol* **81**, 2203–2213.

Londrigan, S. L., Graham, K. L., Takada, Y., Halasz, P. & Coulson, B. S. (2003). Monkey rotavirus binding to α2β1 integrin requires the α2 I domain and is facilitated by the homologous β1 subunit. *J Virol* **77**, 9486–9501.

Lopez, S. & Arias, C. F. (2004). Multistep entry of rotavirus into cells: a Versaillesque dance. *Trends Microbiol* **12**, 271–278.

Lopez, S., Lopez, I., Romero, P., Mendez, E., Soberon, X. & Arias, C. F. (1991). Rotavirus YM gene 4: analysis of its deduced amino acid sequence and prediction of the secondary structure of the VP4 protein. *J Virol* **65**, 3738–3745.

Mittelbrunn, M., Molina, A., Escribese, M. M., Yanez-Mo, M., Escudero, E., Ursa, A., Tejedor, R., Mampaso, F. & Sanchez-Madrid, F. (2004). VLA-4 integrin concentrates at the peripheral supramolecular activation complex of the immune synapse and drives T helper 1 responses. *Proc Natl Acad Sci U S A* **101**, 11058–11063.

Mossel, E. C. & Ramig, R. F. (2003). A lymphatic mechanism of rotavirus extraintestinal spread in the neonatal mouse. *J Virol* **77**, 12352–12356.

Munoz, M., Serrador, J., Nieto, M., Luque, A., Sanchez-Madrid, F. & Teixido, J. (1997). A novel region of the α4 integrin subunit with a modulatory role in VLA-4-mediated cell adhesion to fibronectin. *Biochem J* **327**, 727–733.

Nagesha, H. S. & Holmes, I. H. (1991). VP4 relationships between porcine and other rotavirus serotypes. *Arch Virol* **116**, 107–118.

Nagesha, H. S., Brown, L. E. & Holmes, I. H. (1989). Neutralizing monoclonal antibodies against three serotypes of porcine rotavirus. *J Virol* **63**, 3545–3549.

Pender, S. L., Salmela, M. T., Monteleone, G., Schnapp, D., McKenzie, C., Spencer, J., Fong, S., Saarialho-Kere, U. & MacDonald, T. T. (2000). Ligation of α4β1 integrin on human intestinal mucosal mesenchymal cells selectively up-regulates membrane type-1 matrix metalloproteinase and confers a migratory phenotype. *Am J Pathol* **157**, 1955–1962.

Powell, D. W., Mifflin, R. C., Valentich, J. D., Crowe, S. E., Saada, J. I. & West, A. B. (1999). Myofibroblasts. II. Intestinal subepithelial myofibroblasts. *Am J Physiol Cell Physiol* **277**, C183–C201.

Rolsma, M. D., Kuhlenschmidt, T. B., Gelberg, H. B. & Kuhlenschmidt, M. S. (1998). Structure and function of a ganglioside receptor for porcine rotavirus. *J Virol* **72**, 9079–9091.

Rose, J. R., Williams, M. B., Rott, L. S., Butcher, E. C. & Greenberg, H. B. (1998). Expression of the mucosal homing receptor α4β7 correlates with the ability of $CD8^+$ memory T cells to clear rotavirus infection. *J Virol* **72**, 726–730.

Rott, L. S., Rose, J. R., Bass, D., Williams, M. B., Greenberg, H. B. & Butcher, E. C. (1997). Expression of mucosal homing receptor α4β7 by circulating $CD4^+$ cells with memory for intestinal rotavirus. *J Clin Invest* **100**, 1204–1208.

Ruegg, C., Postigo, A. A., Sikorski, E. E., Butcher, E. C., Pytela, R. & Erle, D. J. (1992). Role of integrin α4β7/α4βP in lymphocyte adherence to fibronectin and VCAM-1 and in homotypic cell clustering. *J Cell Biol* **117**, 179–189.

Ruiz-Velasco, N., Guerrero-Esteo, M., Briskin, M. J. & Teixido, J. (2000). The α4 integrin subunit Tyr^{187} has a key role in $\alpha 4\beta 7$-dependent cell adhesion. *J Biol Chem* **275**, 7052–7059.

Schreiner, C. L., Bauer, J. S., Danilov, Y. N., Hussein, S., Sczekan, M. M. & Juliano, R. L. (1989). Isolation and characterization of Chinese hamster ovary cell variants deficient in the expression of fibronectin receptor. *J Cell Biol* **109**, 3157–3167.

Sharma, A., Askari, J. A., Humphries, M. J., Jones, E. Y. & Stuart, D. I. (1999). Crystal structure of a heparin- and integrin-binding segment of human fibronectin. *EMBO J* **18**, 1468–1479.

Springer, T. A. (1997). Folding of the N-terminal, ligand-binding region of integrin α-subunits into a β-propeller domain. *Proc Natl Acad Sci U S A* **94**, 65–72.

Sriramarao, P., DiScipio, R. G., Cobb, R. R., Cybulsky, M., Stachnick, G., Castaneda, D., Elices, M. & Broide, D. H. (2000). VCAM-1 is more effective than MAdCAM-1 in supporting eosinophil rolling under conditions of shear flow. *Blood* **95**, 592–601.

Tidswell, M., Pachynski, R., Wu, S. W. & 10 other authors (1997). Structure-function analysis of the integrin $\beta 7$ subunit: identification of domains involved in adhesion to MAdCAM-1. *J Immunol* **159**, 1497–1505.

Triantafilou, K., Takada, Y. & Triantafilou, M. (2001). Mechanisms of integrin-mediated virus attachment and internalization process. *Crit Rev Immunol* **21**, 311–322.

Wayner, E. A. & Kovach, N. L. (1992). Activation-dependent recognition by hematopoietic cells of the LDV sequence in the V region of fibronectin. *J Cell Biol* **116**, 489–497.

Williams, M. B., Rose, J. R., Rott, L. S., Franco, M. A., Greenberg, H. B. & Butcher, E. C. (1998). The memory B cell subset responsible for the secretory IgA response and protective humoral immunity to rotavirus expresses the intestinal homing receptor, $\alpha 4\beta 7$. *J Immunol* **161**, 4227–4235.

Xiao, T., Takagi, J., Coller, B. S., Wang, J. H. & Springer, T. A. (2004). Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. *Nature* **432**, 59–67.

Yokosaki, Y., Matsuura, N., Sasaki, T. & 7 other authors (1999). The integrin $\alpha 9\beta 1$ binds to a novel recognition sequence (SVVYGLR) in the thrombin-cleaved amino-terminal fragment of osteopontin. *J Biol Chem* **274**, 36328–36334.

Youngman, K. R., Franco, M. A., Kuklin, N. A., Rott, L. S., Butcher, E. C. & Greenberg, H. B. (2002). Correlation of tissue distribution, developmental phenotype, and intestinal homing receptor expression of antigen-specific B cells during the murine anti-rotavirus immune response. *J Immunol* **168**, 2173–2181.

Yuan, L., Ward, L. A., Rosen, B. I., To, T. L. & Saif, L. J. (1996). Systematic and intestinal antibody-secreting cell responses and correlates of protective immunity to human rotavirus in a gnotobiotic pig model of disease. *J Virol* **70**, 3075–3083.