Rotavirus spike protein VP5* binds α2β1 integrin on the cell surface and competes with virus for cell binding and infectivity

Kate L. Graham,1 Yoshikazu Takada2 and Barbara S. Coulson1

1Department of Microbiology and Immunology, The University of Melbourne, Victoria 3010, Australia
2The University of California, Davis, UC Davis Medical Center, 4645 2nd Avenue, Sacramento, CA 95817, USA

Rotaviruses recognize several cell-surface molecules, including the α2β1 integrin, and the processes of rotavirus cell attachment and entry appear to be multifactorial. The VP5* subunit of the rotavirus spike protein VP4 contains the α2β1 ligand sequence Asp–Gly–Glu at residues 308–310. Binding to α2β1 and infectivity of monkey rotavirus strain RRV and human rotavirus strain Wa, but not porcine rotavirus strain CRW-8, are inhibited by peptides containing Asp–Gly–Glu. Asp308 and Gly309 are necessary for the binding of RRV VP5* (aa 248–474) to expressed α2 integrin subunit. Here, the ability of RRV VP5* to bind cells and affect rotavirus–integrin interactions was determined. Interestingly, VP5* bound to cells at 4 and 37 °C, both via α2β1 and independently of this integrin. Prior VP5* binding at 37 °C eliminated RRV binding to cellular α2β1 and reduced RRV and Wa infectivity in MA104 cells by 38–46 %. VP5* binding did not affect the infectivity of CRW-8. VP5* binding at 4 °C did not affect permissive-cell infection by RRV, indicating an energy requirement for VP5* competition with virus for infectivity. Mutagenesis of VP5* Asp308 and Gly309 eliminated VP5* binding to α2β1 and the VP5* inhibition of rotavirus cell binding and infection, but not α2β1-independent cell binding by VP5*. These studies show for the first time that expressed VP5* binds cell-surface α2β1 using Asp308 and Gly309 and inhibits the infection of homologous and heterologous rotaviruses that use α2β1 as a receptor.

INTRODUCTION

Rotaviruses are members of the family Reoviridae and are major gastrointestinal pathogens of humans and animals. The virions are formed by three concentric protein layers, with the outermost layer comprising VP7 and protruding VP4 spikes (Yeager et al., 1981; Estes et al., 1991; Kirkwood et al., 1994; Crawford et al., 1994). In group A rotaviruses, VP4 and VP7 dictate the P and G serotypes, respectively. VP4 is the major viral cell-attachment protein, whereas VP7 has a lesser role (Bass et al., 1991; Crawford et al., 1994; Kirkwood et al., 1998; Ludert et al., 1996). Proteolytic cleavage of VP4 by trypsin at residue 247 results in greatly increased infectivity and produces two subunits, VP5* (aa 248–776) and VP8* (aa 1–247) that remain associated with the virion (Espejo et al., 1981; Estes et al., 1981; Gilbert & Greenberg, 1998). Trypsin treatment does not appear to alter virus–cell binding, but increases the rate of cell entry by an unknown mechanism (Clark et al., 1981; Kaljot et al., 1988). However, it is known that trypsin-treated, but not untreated, virions can induce cellular fusion from without (Falconer et al., 1995). Also, expressed VP5* permeabilizes membranes (Denisova et al., 1999; Dowling et al., 2000; Golantsova et al., 2004). Trypsin cleavage of VP4 results in dimeric VP4 spikes with a well-ordered structure (Crawford et al., 2001), suggesting the induction of a conformational change in VP4. Crystal structures of most of the portions of VP8* and VP5* that project from the virion show that VP4 also undergoes a second rearrangement that may facilitate VP4–membrane interactions and results in a stable trimer (Dormitzer et al., 2002, 2004). At high pH, VP4 spikes on the virion undergo conformational change to a trilobed structure (Pesavento et al., 2005).

Rotaviruses recognize several cell-surface molecules, and the processes of rotavirus cell attachment and entry appear to be multifactorial. Binding of a minority of animal rotaviruses to terminal sialic acids has been demonstrated, although this interaction is not essential (Ludert et al., 2002; Mendez et al., 1993). Terminal sialic acid usage relates to P serotype (Ciarlet et al., 2002b), and VP8* of the neuraminidase-sensitive rotavirus strain RRV contains a sialoside-binding region involved in cell binding and haemagglutination (Dormitzer et al., 2002; Fiore et al., 1991). Other rotaviruses, including some human strains, may recognize subterminal sialic acids that are not removed by neuraminidase treatment, or other sugars, on glycoproteins or glycolipids including gangliosides.
Several human and monkey rotaviruses bind recombinant, cell-surface-expressed \(\alpha 2\beta 1\) and \(\alpha 4\beta 7\) integrins and require the same \(\alpha 4\) subunit regions for binding as natural \(\alpha 4\) ligands. Rotaviruses may recognize \(\alpha 4\) integrins on cells of the immune system, possibly facilitating virus spread or host immune response modulation (Graham et al., 2003; Halasz et al., 2005; Hewish et al., 2000). The heat-shock cognate protein 70 (Hsc70) has also been proposed to form a component of a rotavirus receptor complex that is recognized through aa 642–659 of VP5* (Zárate et al., 2003).

Infection of permissive cells by many human and animal rotaviruses depends, to a significant extent, on VP4 recognition of \(\alpha 2\beta 1\) integrin and VP7 interactions with integrins \(\alpha x\beta 2\) and \(\alpha y\beta 3\) (Ciarlet et al., 2002a; Coulson et al., 1997; Graham et al., 2003, 2004; Guerrero et al., 2000). These rotaviruses are classified as integrin-using strains and include monkey virus strains RRV and SA11 and human strain Wa. Integrin usage relates to \(\alpha\) serotype independently of terminal sialic acid usage (Graham et al., 2003). Almost all group A rotaviruses have the Asp–Gly–Glu (DGE) sequence in VP5* at aa 308–310, a motif that has been implicated in \(\alpha 2\beta 1\) recognition by type I collagen, and the Gly–Pro–Arg (GPR) sequence in VP7 that is a ligand in fibrinogen for \(\alpha x\beta 2\) (Coulson et al., 1997). Monomeric and polymeric peptides containing the DGE and/or GPR sequences inhibit the infectivity of integrin-using rotaviruses by 30–90% (Coulson et al., 1997; Graham et al., 2004; Zárate et al., 2000a). Integrin-using rotaviruses have been reported to use \(\alpha x\beta 2\) and \(\alpha y\beta 3\) at a post-binding stage to facilitate infection (Graham et al., 2003; Guerrero et al., 2000).

Most evidence indicates that integrin-using human and animal rotaviruses bind to cell-surface \(\alpha 2\beta 1\). Infectious SA11, RRV and Wa binding to recombinant \(\alpha 2\beta 1\) on the K562 cell surface was specifically inhibited by DG-containing peptides and a function-blocking antibody to the \(\alpha 2\) I domain (Graham et al., 2003, 2004; Hewish et al., 2000). SA11 and RRV precipitated two cell-surface proteins with characteristics of \(\alpha 2\) and \(\beta 1\) integrin subunits and bound recombiant human \(\alpha 2\beta 1\) on Chinese hamster ovary cells to a greater extent than human \(\alpha 2\) combined with hamster \(\beta 1\). This binding was inhibited by antibody to the \(\alpha 2\) I domain, but not anti-\(\alpha 2\) antibodies that mapped outside the I domain, and was eliminated by I domain deletion (Londrigan et al., 2003). VP5* (aa 248–474, representing the N-terminal half of VP5*), expressed as a glutathione S-transferase (GST) fusion protein, retained the ability to bind a conformation-dependent neutralizing antibody and bound expressed \(\alpha 2\) I domain. This I domain binding depended on the presence of the D308 and G309 residues in the DGE sequence of VP5*, as alanine mutagenesis of these residues in VP5* did not affect neutralizing-antibody recognition, but did abolish VP5* binding to the \(\alpha 2\) I domain (Graham et al., 2003). In contrast to the above findings, binding of naturally occurring, integrin-using rotaviruses to cellular \(\alpha 2\beta 1\) was not detected by two other groups (Ciarlet et al., 2002a; Zárate et al., 2000a, b). One reason for this was their use of normally adherent MA104 cells as cell suspensions during virus–cell binding assays (Graham et al., 2003). However, recent studies by one of these groups using adherent MA104 cells still failed to demonstrate \(\alpha 2\beta 1\) binding by RRV (Zárate et al., 2003, 2004).

To examine the mechanism of rotavirus binding to cellular \(\alpha 2\beta 1\) further and resolve this difference, we aimed here to analyse directly the importance of VP5* (aa 248–474) containing the DGE sequence in rotavirus cell binding and infection mediated by \(\alpha 2\beta 1\). These studies provide the first direct experimental evidence that purified recombinant VP5* binds cell-surface \(\alpha 2\beta 1\) using DGE and inhibits the infection of homologous and heterologous rotaviruses that use \(\alpha 2\beta 1\) as a receptor.

**METHODS**

**Cell lines, viruses and antibodies.** The origins and maintenance of MA104 and K562 cells and the derivation of the K562 cells transfected with cDNA encoding empty vector (PB1-K562), \(\alpha 2\) (\(\alpha 2\)-K562), \(\alpha 3\) (\(\alpha 3\)-K562) and \(\alpha 4\) (\(\alpha 4\)-K562) used in this study have been described previously (Coulson et al., 1997; Graham et al., 2003; Hewish et al., 2000). By flow cytometry, MA104 cells express moderate levels of surface \(\alpha 2\beta 1\) (Coulson et al., 1997; Londrigan et al., 2000). Monitoring of the surface expression of \(\alpha 2\beta 1\), \(\alpha 3\beta 1\) and \(\alpha 4\beta 1\) on K562 cell lines was carried out by flow cytometry as described previously (Graham et al., 2003; Hewish et al., 2000). The origins, cultivation in MA104 cells and characterization of Rhesus monkey rotavirus P5B[3], G3 strain RRV; human rotavirus P1A[8], G1 strain Wa and porcine rotavirus P9[7], G3 strain CRW-8 have been described previously (Coulson & Kirkwood, 1991; Coulson et al., 1985, 1986). Rabbit antiserum to purified GST and RRV were each produced and obtained as described previously (Londrigan et al., 2003; Warner et al., 2001).

**Production and characterization of purified recombinant VP5* proteins.** The RRV VP4 plasmid pBS/VP4 was provided by Dr E. Mackow (Department of Medicine and Department of Molecular Genetics and Microbiology, Stony Brook University, NY, USA). Soluble RRV VP5* containing aa 248–474 and the D308A, G309A mutant of this VP5* generated by site-directed mutagenesis were produced as purified GST fusion proteins GST–VP5* and GST–VP5*D308A/G309A, respectively, by *Escherichia coli* expression as described previously (Graham et al., 2003). Most of the GST–VP5* and GST–VP5*D308A/G309A protein produced was insoluble, as reported previously (Dormitzer et al., 2004), but under the expression conditions used, a fraction that co-purified with the heat-shock bacterial chaperonin GroEL was soluble, as determined previously (Graham et al., 2003). By enzyme immunoassay (EIA), the soluble GST–VP5* and GST–VP5*D308A/G309A produced for the studies here were bound by anti-VP5* monoclonal antibody 2G4 (indicating preservation of this conformational epitope), and GST–VP5* but not GST–VP5*D308A/G309A bound to the \(\alpha 2\) integrin I domain fusion protein, as described previously (Graham et al., 2003). The soluble protein obtained therefore was functional for 2G4 recognition and \(\alpha 2\) integrin I domain binding. Plasmid insert identity was verified by DNA sequencing. Fusion protein identity was confirmed by Western blotting using anti-GST monoclonal antibody CH-1, as described previously (Graham et al., 2003).
Flow cytometric assay of recombinant VP5* binding to cells. The ability of GST–VP5*, GST–VP5*D308A/G309A and GST to bind PBJ-K562, z2-K562, z3-K562 and z4-K562 cells was determined by flow cytometry as described previously (Warner et al., 2001). In brief, equimolar amounts of GST–VP5*, GST–VP5*D308A/G309A or GST (0.1–10 μg) were incubated with 5 x 10⁵ washed cells for 45 min at 4 °C unless stated otherwise. Cell numbers were quantified carefully to ensure that results were comparable between cell lines. All the following steps were carried out at 4 °C. Cell-bound protein was detected with rabbit anti serum to GST diluted 1:500. Similarly diluted, normal rabbit serum negative for rotavirus antibodies by ELISA and with a neutralization titre against SA11 of ≈ 1:100 was used as a negative control. Bound rabbit antibodies were detected using fluorescein isothiocyanate-conjugated sheep anti-rabbit F(Ab')₂ (Chemicon) and cell lines were analysed by flow cytometry, as described previously (Graham et al., 2003). The relative level of recombinant protein bound to cells was quantified by calculation of the relative linear median fluorescence intensity (RLMFI) from the flow cytometric histograms, as described previously (Graham et al., 2003). An RLMFI value of ≥1:20 was considered to indicate cell binding by the recombinant protein, as has been determined previously for monoclonal antibody binding (Graham et al., 2003).

Indirect immunofluorescence assays of virus–cell binding and infectivity and recombinant VP5* inhibition of virus–cell binding and infectivity. Measurement of the binding of infectious rotavirus to cells was determined using 5 x 10⁵ cells and infectivity assays were carried out using 1 x 10⁸ cells, as described previously (Coulson et al., 1997; Graham et al., 2003; Hewish et al., 2000; Londrigan et al., 2000). Assays were carried out using clarified virus–cell harvests at an m.o.i. of 3:5 (virus–cell binding) or 0:02 (infectivity). The determination of these optimum m.o.i. values, and demonstration that the titres of purified virus and clarified virus–cell harvests bound to cellular integrins are indistinguishable, have been described previously (Coulson et al., 1997; Graham et al., 2003, 2005). Titres are given as fluorescent cell-forming units (f.f.u.) ml⁻¹.

Briefly, for binding assays, trypsin-activated virus was adsorbed to cells at 4 °C for 1 h. Bound virus was harvested by two cycles of freezing and thawing in the presence of 1 μg porcine trypsin ml⁻¹ and activated with 10 μg trypsin ml⁻¹ at 37 °C for 10–20 min, depending on the virus strain. In conjunction with vigorous vortex mixing, these trypsin digestions reduced levels of virion-associated protein and virion aggregation. Cell-bound virus titres were determined by incubation of MA104 cells with activated virus at 37 °C for 1 h, followed by indirect immunofluorescent staining of infected cells after 15 h. In experiments analysing recombinant protein inhibition of virus–cell binding and infectivity, cells were treated with GST–VP5*, GST–VP5*D308A/G309A or GST at 37 °C for 1 h prior to virus addition, unless otherwise stated. Assays were then completed as described above. None of the proteins caused aggregation of PBJ-K562 or integrin-transfected K562 cells. Cell viability (measured by trypan blue exclusion) and the microscopic appearance of MA104 and K562 cell lines were unaltered by protein treatment.

On graphs, results were expressed as a percentage of the virus titre in the absence of any treatment and given as mean ± SD of at least three experiments.

RESULTS

RRV GST–VP5* (aa 248–474) binds K562 cells by both a2β1-dependent and -independent mechanisms

Previously, it was shown by EIA that RRV GST–VP5* binds recombinant α2 integrin I domain protein (Graham et al., 2003). The ability of GST–VP5* to bind cell-surface-expressed α2β1, α3β1 or α4β1 was examined at 4 and 37 °C by flow cytometry, using K562 cells transfected with the empty vector (PBJ-K562) or expressing recombinant human α2β1 (z2-K562), α3β1 (z3-K562) or α4β1 (z4-K562). The only endogenous β1 integrin expressed by K562 cells is α2β1. GST–VP5* bound to PBJ-K562, z2-K562, z3-K562 and z4-K562 cell lines at both 4 °C (Fig. 1a) and 37 °C (Fig. 1b). However, z2-K562 cells supported substantially more GST–VP5* binding than PBJ-K562, z3-K562 and z4-K562 cells at both 4 and 37 °C (Fig. 1a–d). GST–VP5* binding to z2-K562 and z3-K562 cells was concentration-dependent over a 100-fold range (0:1–10 μg GST–VP5*) and was saturated at 5 μg GST–VP5* per 5 x 10⁵ cells (Fig. 1c and d). RLMFI values calculated from the experiments using GST–VP5* indicated that z2-K562 cells supported 1.3–2.2-fold more GST–VP5* binding than PBJ-K562, z3-K562 and z4-K562 cells (Table 1). Overall, at 4 and 37 °C, GST–VP5* bound to K562 cells using α2β1 and also via an additional mechanism that was independent of α2β1, α3β1 or α4β1.

RRV GST–VP5*D308A/G309A only binds to K562 cells independently of α2β1

RRV GST–VP5* binding to recombinant α2 integrin I domain protein has been demonstrated to depend on the presence of D308 and/or G309 in VP5*, as RRV GST–VP5*D308A/G309A did not bind the α2 I domain (Graham et al., 2003). The ability of RRV GST–VP5*D308A/G309A to bind K562 cells by α2β1-dependent and α2β1-independent means was examined. As shown in Fig. 2 and Table 1, GST–VP5*D308A/G309A binding to z2-K562 cells was detected (RLMFI = 1:4) at a lower level than GST–VP5* binding to z2-K562 cells (RLMFI = 3:1). GST–VP5*D308A/G309A and GST–VP5* bound z3-K562 cells indistinguishably, with identical RLMFI values of 1:4, at levels indistinguishable from VP5*D308A/G309A binding to z2-K562 cells (RLMFI = 1:4). These results showed that mutation of the DG sequence in VP5* abolished VP5* binding to cell-surface-expressed α2β1, but that VP5* binding to cells independently of α2β1 remained detectable.

Binding of RRV GST–VP5*, but not RRV GST–VP5*D308A/G309A, to cell-surface-expressed α2β1 prevents RRV binding

The ability of cell-bound RRV GST–VP5* and RRV GST–VP5*D308A/G309A to inhibit infectious RRV binding to cellular α2β1 was examined (Fig. 3). In the absence of cellular treatment with recombinant protein, the RRV titre bound to z2-K562 cells was increased 1.9-fold over that bound to PBJ-K562 and z3-K562 cells, as shown previously (Graham et al., 2003). Cellular treatment with RRV GST–VP5* at 37 °C eliminated infectious RRV binding to α2β1 on z2-K562 cells, as the RRV titre bound was indistinguishable from that bound to PBJ-K562 and z3-K562 cells. However, RRV GST–VP5*D308A/G309A and GST had no effect on the titre of RRV bound to α2β1 on z2-K562 cells and did not alter the background level of RRV binding to PBJ-K562 and
a2-K562 cells. Thus, RRV binding to cellular a2/b1 was prevented by prior cellular treatment with homologous GST–VP5* at 37°C and this inhibition was completely dependent on VP5* D308 and/or G309.

**Binding of RRV GST–VP5*, but not RRV GST–VP5*D308A/G309A, to cell-surface-expressed a2/b1 inhibits RRV and Wa infection of MA104 cells**

As RRV GST–VP5*, but not RRV GST–VP5*D308A/G309A, was able to bind cell-surface a2/b1 on K562 cells and so prevent RRV binding to the same integrin, the abilities of these proteins to bind permissive MA104 cells and compete with rotavirus infectivity were examined. Both GST–VP5* and GST–VP5*D308A/G309A bound to MA104 cells, showing RLMFI values of 5.2±0.5 and 4.0±0.4, respectively. These RLMFI values are consistent with the results described above, which demonstrated that GST–VP5* binds K562 cells both via a2/b1 and independently of this integrin, whereas GST–VP5*D308A/G309A binds K562 cells independently of a2/b1 only. The higher RLMFI value shown by GST–VP5* probably reflects its additional ability to bind a2/b1. Application of RRV GST–VP5* to MA104 cells at 4°C had no effect on the infectivity of RRV added subsequently (Fig. 4a). However, cellular treatment with RRV GST–VP5* at 37°C inhibited RRV infectivity in a dose-dependent fashion with a mean ± SD of 38±4% at 50 µg ml⁻¹. This infectivity blockade was abrogated by the D308A and G309A mutations in VP5*, as RRV GST–VP5*D308A/G309A did not inhibit RRV infectivity at any concentration tested (Fig. 4b). Importantly, RRV GST–VP5* similarly inhibited
infection by the heterologous human rotavirus strain Wa, with a mean of 46 ± 3 % at 50 μg l⁻¹, whereas RRV GST–VP5*D308A/G309A did not inhibit Wa infectivity (Fig. 4c). Neither RRV GST–VP5* nor RRV GST–VP5*D308A/G309A had any effect on infection by CRW-8 rotavirus (Fig. 4d), which does not use α2β1 for cell binding or growth (Graham et al., 2003, 2004; Hewish et al., 2000). Thus, RRV GST–VP5* bound to cells at 37 °C but not at 4 °C inhibited RRV and Wa, but not CRW-8, rotavirus infectivity in MA104 cells. This blockade required the presence of VP5* D308 and/or G309 and so was dependent on VP5* binding to α2β1.

### Table 1. Relative levels of GST–VP5* and GST–VP5*D308A/G309A binding to K562 cell lines

<table>
<thead>
<tr>
<th>Rotavirus GST fusion protein†</th>
<th>Level of rotavirus protein binding to given cell line (mean RLMFI value)‡</th>
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<tr>
<td></td>
<td>PBJ-K562</td>
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<tr>
<td>VP5* (5 μg; Expt 1)</td>
<td>6·4</td>
</tr>
<tr>
<td>VP5* (5 μg, 37 °C; Expt 1)</td>
<td>4·0</td>
</tr>
<tr>
<td>VP5* (5 μg; Expt 2)</td>
<td>5·0</td>
</tr>
<tr>
<td>VP5* (1 μg; Expt 2)</td>
<td>3·2</td>
</tr>
<tr>
<td>VP5* (0·1 μg; Expt 2)</td>
<td>1·4</td>
</tr>
<tr>
<td>VP5* (1 μg; Expt 3)</td>
<td>ND</td>
</tr>
<tr>
<td>VP5*D308A/G309A (1 μg; Expt 3)</td>
<td>ND</td>
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</table>

†Binding was determined at 4 °C unless otherwise stated, with the given protein quantity per 5 × 10⁶ cells. The experiment number is indicated. Representative histograms from experiments 1, 2 and 3 are illustrated in Figs 1(a) and (b) and 2, respectively.

‡The RLMFI value was calculated as the ratio of test median fluorescence intensity to control median fluorescence intensity. Control values were determined as the mean of the three controls (tested in duplicate) for the given cell line. These controls were: GST fusion protein binding detected with negative-control antiserum, GST binding detected with antiserum to GST and GST binding detected with control antiserum. The RLMFI value of duplicate test samples varied by <10 % from the mean RLMFI value. An RLMFI value of ≥1·2 was taken as a positive result, indicating cellular binding by the test protein. ND, Not done.

**Fig. 2.** Flow cytometric histograms of GST–VP5*, GST–VP5*D308A/G309A and GST bound to α2-K562 (a) and α3-K562 (b) cells show that GST–VP5*D308A/G309A binding to K562 cells was independent of α2β1. Proteins were added at equimolar levels, containing 1 μg VP5* or VP5*D308A/G309A. Completely overlapping histograms in (b) are bracketed. Controls (dotted lines) were GST–VP5* or GST–VP5*D308A/G309A bound to each K562 cell line and detected with negative-control rabbit serum and GST bound to each cell line and detected with anti-GST or negative-control serum. Graphs show data representative of at least two experiments.

**Fig. 3.** Binding of recombinant RRV GST–VP5* to recombinant α2β1 on the K562 cell surface at 37 °C prevents infectious RRV binding and this blockade depends on the presence of D308 and/or G309 in GST–VP5*. Prior to RRV binding, PBJ-K562, α2-K562 and α3-K562 cells were treated with 50 μg recombinant proteins GST, GST–VP5* or GST–VP5*D308A/G309A ml⁻¹ or were mock treated. The mean titre of infectious RRV bound to each cell line is expressed as a percentage of the titre bound to PBJ-K562 cells in the absence of GST fusion protein, which gave a mean ± SD of 8·0 × 10⁴ ± 6·4 × 10³ f.f.u. ml⁻¹.
DISCUSSION

In this study, the mechanisms of rotavirus cell attachment involving $\alpha_2\beta_1$ integrin were elucidated further. The direct relevance to rotavirus cell attachment and infectivity of the previously described binding of RRV spike protein VP5* to $\alpha_2$ subunit I domain protein (Graham et al., 2003) was established. It was shown that binding of cellular $\alpha_2\beta_1$ by infectious rotavirus was inhibited by VP5* (aa 248–474), which bound to cells in a DGE-dependent manner. This cell-bound VP5* resulted in a reduction in infectivity by both homologous and heterologous rotaviruses that use $\alpha_2\beta_1$ as a cellular receptor.

**Rotavirus VP5* binds cellular $\alpha_2\beta_1$ via D308 and G309 and competes with infectious virus for cell binding and infectivity**

RRV VP5* bound K562 cells at 4 and 37 °C, both via $\alpha_2\beta_1$ and independently of $\alpha_2\beta_1$, $\alpha_3\beta_1$ and $\alpha_4\beta_1$ (Fig. 1). These data extend previous findings with a similar expressed GST–VP5* construct that bound MA104 cells at 4 °C, as binding of this construct was partially inhibited by an anti-$\alpha_2$ antibody (Zárate et al., 2003). The requirement for the DGE sequence to mediate VP5* binding to cellular $\alpha_2\beta_1$ was demonstrated by the inability of GST–VP5*D308A/G309A to bind $\alpha_2\beta_1$ (Fig. 2). As GST–VP5*D308A/G309A did show $\alpha_2\beta_1$-independent binding to K562 cells (Fig. 2), mutation of the DGE sequence did not affect $\alpha_2\beta_1$-independent binding functions. This demonstrated the specificity of the effect of DGE mutation in RRV VP5* on $\alpha_2\beta_1$ binding.

RRV VP5* (aa 248–474) permeabilizes membranes and directs the formation of transient size-selective pores without membrane lysis. These functions require the hydrophobic and basic domains, but are independent of $\alpha_2\beta_1$ recognition and have been proposed to mediate rotavirus entry into cells (Denisova et al., 1999; Dowling et al., 2000; Golantsova et al., 2004). The $\alpha_2\beta_1$-independent cell binding by GST–VP5* and GST–VP5*D308A/G309A (Figs 1 and 2) may have been mediated by these domains. Other VP5* domains may also be responsible for integrin-independent cell binding, such as the MA104 cell-binding domains identified in CRW-8 VP5* using a gene-targeted phage display library (Jolly et al., 2001b). Hsc70 interactions could not account for the integrin-independent binding of VP5*, as the VP5* amino-terminus that binds Hsc70 was not present in the VP5* used in our studies.

GST–VP5* almost completely blocked binding of RRV to $\alpha_2\beta_1$ and dose-dependently inhibited MA104 cell infection by RRV and Wa but not CRW-8. These inhibitory functions of VP5* depended on the presence of the DGE sequence and...
so were specific for α2/β1. The reduction of heterologous rotavirus infection by RRV GST–VP5* depended on the ability of the infecting virus to utilize α2/β1, rather than the terminal sialic acid dependence or VP7 serotype of the virus (Ciarlet et al., 2002b; Nagesha & Holmes, 1991). Approximately 40% of rotaviruses in the pool failed to enter cells productively when competed with VP5*. This is similar to the levels of blockade of rotavirus cell binding and infectivity by antibodies to α2 and monomeric DGEA peptides (Graham et al., 2004) and is consistent with VP5* blockade of α2/β1 binding being a major mechanism by which VP5* inhibits virus–cell interactions.

The blockade of RRV cell binding and infection by GST–VP5* depended entirely upon the presence of VP5* D308 and G309. Thus, recombinant GST–VP5* shares with infectious RRV a DGE-dependence for cell binding. Taken in conjunction with GST–VP5* binding to the α2 I domain, which was also eliminated by the D308A and G309A mutations (Graham et al., 2003), this is clear evidence that VP5* of infectious RRV binds cellular α2/β1 via the α2 I domain in an interaction dependent upon the presence of VP5* residues D308 and G309. Infection by RRV and Wa rotaviruses was blocked to a similar degree by RRV VP5* and depended similarly on the presence of VP5* D308 and G309. This is the first time that any competition with Wa for infectivity by a protein from RRV has been demonstrated. Wa blocks infection by the RRV nar3 mutant, but nar3 does not affect Wa infectivity (Méndez et al., 1999). Wa binds recombinant α2/β1 on K562 cells and Wa infectivity is substantially reduced by DGE-containing peptides (Graham et al., 2003, 2004). The new findings here are a crucial component of the accumulating evidence that Wa also binds to cellular α2/β1 integrin via the α2 I domain utilizing VP5* residues D308 and G309. It is also possible that GST–VP5* may inhibit infectious virus binding or infection through the peripheral membrane domain, the hydrophobic domain or an undefined cell-binding domain, if these functions are dependent on the presence of D308 and G309 or prior VP5* binding to α2/β1.

Apparently conflicting findings on the ability of rotaviruses to bind α2/β1 are reconciled by the demonstration that VP5* bound to cells at 37°C, but not 4°C, inhibits virus infectivity

The blockade of virus binding and infection by RRV GST–VP5* shown in Figs 3 and 4 contrasts with earlier reports that RRV binding or infection in MA104 cells is unaffected by recombinant RRV GST–VP5*. RRV GST–VP5* was reported to inhibit RRV infectivity only when MA104 cells were neuraminidase-treated (Zárate et al., 2000b). As the cell-binding partner(s) for VP5* and virus were not identified, the role of α2/β1 was not investigated. These earlier studies were performed using MA104 cells treated with VP5* at 4°C (Zárate et al., 2000a, b, 2003, 2004). In agreement with these reports, it was found here (Fig. 3) that treatment of MA104 cell monolayers with GST–VP5* at 4°C had no effect on RRV infection. However, we demonstrated that treatment with GST–VP5* at 37°C resulted in dose-dependent inhibition of RRV and Wa infection (Fig. 4). RRV binding to α2/β1 on α2-KS62 cells was also almost completely blocked by GST–VP5* at 37°C (Fig. 3). This GST–VP5* bound α2/β1 on α2-KS62 cells at 4°C by flow cytometry (Fig. 1) and binding of a similar construct to MA104 cells at 4°C was partially dependent on α2/β1 (Zárate et al., 2003); thus, the inability of GST–VP5* to block infection when added at 4°C was not due to a lack of GST–VP5* binding to cellular α2/β1 at this temperature.

It is clear from the above that rotavirus cell binding and infection should be measured using cells in their natural state of adhesion and at physiologically relevant temperatures when possible, and that results of experiments conducted under other conditions should be interpreted with caution. The inability of RRV VP5* at 4°C to compete with RRV binding that was reported previously was interpreted to mean that RRV binds to the cell mainly through VP8*, as RRV VP8* at 4°C was found to reduce RRV cell binding (Zárate et al., 2003). However, the demonstration (Fig. 3) that RRV VP5* competes at 37°C with RRV for α2/β1 binding is evidence that RRV binds to the cell through VP5* recognition of α2/β1. To explain the inability of RRV VP5* at 4°C to compete with RRV infectivity, it was proposed that RRV can attach to the cell via VP8* and then efficiently displace already bound recombinant VP5* (Zárate et al., 2000b). However, it was shown in Figs 3 and 4 that VP5* effectively competes with RRV for cell binding and infection at 37°C, so VP5* is not displaced by RRV under these conditions. In fact, the reverse is the case. In addition, VP5* competition with RRV is for binding to α2/β1 (Fig. 3), definitively establishing cellular α2/β1 as a VP5* ligand that is important for rotavirus cell binding and infectivity. The conclusion that trypsin activation is necessary for RRV nar3 mutant cell attachment via VP5* also needs to be re-evaluated, as RRV GST–VP5* was reacted with cells at 4°C in that study (Zárate et al., 2004).

Implications of the temperature dependence on the ability of VP5* to compete with rotavirus infectivity

The lack of GST–VP5* competition with rotavirus infectivity at 4°C shows that interaction between GST–VP5* and α2/β1, which results in blockade of virus infection, requires energy and/or active cellular processes. A likely scenario is that VP5* bound to cellular α2/β1 undergoes conformational change between 4 and 37°C to a structure presenting the DGE sequence analogously to virions primed to engage α2/β1. Virions used in cell-binding studies are trypsin-activated at 37°C, which primes virus for entry by triggering a rearrangement stabilizing the VP4 spikes as upright dimers (Crawford et al., 2001). A further, unknown event during entry has been proposed to trigger a second structural rearrangement in VP5* to produce the trimeric form seen in a crystal structure of VP5*. This second rearrangement could involve a 180° rotation of the VP5* antigen domain, including the DGE sequence (Dormitzer et al., 2004).
GST–VP5* bound to α2β1 may take up the primed conformation, or a conformation involved in the second rearrangement, at 37 °C but not at 4 °C. Also, α2β1 undergoes conformational changes and lateral movement in the cell membrane at 37 °C, including activation, that may facilitate stable VP5* binding in the correct conformation for inhibition of virus–cell binding and infection (Luque et al., 1996). Supporting this, incubation of cells with anti-α2 antibodies at 37 °C but not at 4 °C results in antibody blockade of RRV cell binding, and activation of α2β1 at 37 °C increases infectious SA11 rotavirus binding to α2β1 (Graham et al., 2003).

Our results show that virion VP5* binds cellular α2β1, an important receptor for rotavirus infection, and that VP5* additionally binds cells independently of α2β1. Rotavirus cell attachment and entry also involve virus recognition of several other cellular components. Further analysis of these processes is important for an understanding of rotavirus tropism and pathogenesis.

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Rotavirus VP5* binds cell-surface α2β1 integrin


