The capsid protein of infectious bursal disease virus contains a functional α4β1 integrin ligand motif

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Infectious bursal disease virus (IBDV), a member of the dsRNA Birnaviridae family, is an important immunosuppressive avian pathogen. We have identified a strictly conserved amino acid triplet matching the consensus sequence used by fibronectin to bind the α4β1 integrin within the protruding domain of the IBDV capsid polypeptide. We show that a single point mutation on this triplet abolishes the cell-binding activity of IBDV-derived subviral particles (SVP), and abrogates the recovering of infectious IBDV by reverse genetics without affecting the overall SVP architecture. Additionally, we demonstrate that the presence of the α4β1 heterodimer is a critical determinant for the susceptibility of murine BALB/c 3T3 cells to IBDV binding and infectivity. Our data suggests that the IBDV might also use the α4β1 integrin as a specific binding receptor in avian cells.

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Introduction

Infectious bursal disease virus (IBDV) is the best characterized member of the Birnaviridae family that groups naked viruses with a bipartite double-stranded (dsRNA) RNA genome (Delmas et al., 2004). IBDV is responsible for an immunosuppressive disease that affects young domestic chickens (Gallus gallus), and causes major economic losses to the poultry industry world-wide (van den Berg et al., 2000).

The knowledge about IBDV structural and molecular biology has experienced a great progress over the last few years, thus the crystal structure of all structural polypeptides has been completely or partially solved (Casañas et al., 2008; Coulibaly et al., 2005; Feldman et al., 2006; Garriga et al., 2007, 2006; Lee et al., 2006; Pan et al., 2007), and the virus assembly pathway has been extensively characterized (Chevalier et al., 2005; Luque, 2007; Oña et al., 2004; Saugar et al., 2005). However, our understanding about fundamental aspects of the virus replication cycle, e.g. the entry and egress mechanisms is, as yet, scarce.

Viruses are obligate intracellular parasites, thus the first requirement to initiate a successful infection consists on the recognition of a suitable receptor(s) on the surface of target cells. This recognition sets off a series of events, generally involving an elaborated interplay between different cellular and viral components, allowing the virus to reach the appropriate cellular compartment for virus replication (Smith and Helenius, 2004). Indeed, the presence and distribution of specific virus receptor(s) molecules are in many cases critical determinants for virus host-range, tropism, and pathogenesis.

The IBDV capsid (65–70 nm in diameter) is a single shell built by 260 trimers of the VP2 polypeptide arranged in an icosahedral lattice with T=13l symmetry (Bottcher et al., 1997; Coulibaly et al., 2005; Saugar et al., 2005). VP2 is produced by proteolytic maturation of a precursor polypeptide, known as pVP2 (Birghan et al., 2000; Feldman et al., 2006). VP2 is the only component of the virus capsid, and thus responsible to the interaction with host cell receptors. Although the tropism of IBDV for lymphocytic cell populations found at the Fabricius bursa has been extensively characterized (Becht, 1980; Kibenge et al., 1988), the identification of specific cell binding receptor(s) has remained elusive.

The VP2 crystal structure, independently solved by three different groups (Coulibaly et al., 2005; Garriga et al., 2006; Lee et al., 2006), revealed the presence of three distinct domains designated base (B), shell (S), and projection (P) (Fig. 1A). Domain P, formed by a β-barrel with a jelly roll topology, is well exposed to the solvent, thus being a likely candidate to interacting with the host cell binding receptor(s). This hypothesis is supported by the presence within this domain of several neutralizing epitopes (Fahey et al., 1989), and amino-acid residues directly involved both in virulence and tissue-culture adaptation (Brandt et al., 2001; van Loon et al., 2002). Additionally, the P domain shows a high degree of structural homology with projection domains of reovirus capsid polypeptides directly involved...
Similarly to many other icosahedral viruses (Boulanger, 1999), some members of the dsRNA Reoviridae family use integrins as binding receptors and/or essential entry molecules (Graham et al., 2005, 2006; López and Arias, 2004; Maginnis et al., 2006; Tan et al., 2001). Integrins form a superfamily of adhesion receptors involved in a wide variety of matrix–cell and cell–cell interactions. Members of this superfamily are heterodimers formed by two glycosylated transmembrane proteins termed α and β, respectively. Both the structural similarity of the IBDV VP2 P domain to the corresponding projection domains of reovirus capsid polypeptides (Coulibaly et al., 2005), and the glycosylated nature of the integrins suggested that IBDV might also employ an integrin(s) during its cell entry process. Integrins mediate cell adhesion to extracellular matrix, cell membrane, and virus capsid polypeptides through the recognition of short linear ligand sequence motifs. Many integrin-binding motifs have been extensively characterized (Hynes, 1999; Komoriya et al., 1991; Plow et al., 2000; Staatz et al., 1991), facilitating the search for putative integrin-binding motifs within the VP2 sequence. This search resulted in the identification of the Ile-Asp-Ala (IDA) sequence within the VP2 P domain. That motif matches the XDY triplet (X=G, L, I, E; Y=V, A) used by the α4β1 integrin in binding to fibronectin (Fn) (Mould and Humphries, 1991). This finding prompted us to perform a series of experiments aimed at characterizing the roles of both the VP2 IDA motif and the α4β1 integrin on the binding of IBDV to susceptible cells.

The results described in this report show that, with the virus strain and cell lines used for the present study, the VP2 IDA motif plays a critical role in the binding of IBDV-derived SVP and virus particles to IBDV-susceptible cells, and that a single point mutation on this motif completely abrogates SVP cell binding and virus infectivity. Additionally, we demonstrate that transformation with a recombinant expression plasmid harboring the α4 subunit gene is sufficient to turn naturally α4-lacking murine BALB/c 3T3 fibroblasts from resistant to IBDV attachment and infection to permissive for both phenomena.

**Results**

Identification of a putative integrin-ligand motif within the IBDV capsid polypeptide

The amino acid sequence of the VP2 polypeptide from the IBDV Soroa strain was scanned for integrin-binding sequences. This led to the identification of the Ile-Asp-Ala (IDA) residues 234–236 (Fig. 1B) matching the XDY (X=G, L, I, E; Y=V, A) amino acid triplets used by the α4β1 integrin in binding to Fn, and in particular to the IDA motif found in the Fn H1 fragment (Mould and Humphries, 1991). The identified VP2 IDA sequence is located within a flexible loop, connecting the C and D β-strands, at the base of the P domain (Fig. 1A).

A multiple alignment performed with over 100 VP2 sequences from antigenic variant, classical virulent, very virulent, as well as tissue culture-adapted IBDV serotype 1 and serotype 2 isolates, indicated that the IDA triplet is strictly conserved in all members of the Avibirnavirus genus. Fig. 1B, showing a multiple alignment of VP2 sequences from representative IBDV strains, summarizes this finding.

**Binding of IBDV-derived SVP to DF-1 cells**

In order to assess the feasibility of using IBDV-derived SVP as a model to analyze the role of the IDA motif on cell attachment, it was critical to determine whether they were able to bind to IBDV-susceptible cells. IBDV and SVP were produced, purified, and analyzed by EM as previously described (Castón et al., 2001a). DF-1 monolayers, grown on coverslips, were incubated for 1 h at 4 °C with a suspension of either purified virus or SVP. Thereafter, cells were processed for CLSM using an anti-VP2 specific antiserum. As shown in
Fig. 2A, cells incubated with SVP show an intense membrane staining pattern, similar to that observed after incubation of cell monolayers with purified virus, thus demonstrating that SVP efficiently bind to the surface of DF-1 cells.

SVP efficiently block IBDV infection

Results described above suggested that both IBDV and SVP might bind to the same cell receptor(s) on the membrane of DF-1 cells. To test this hypothesis, the ability of SVP to block IBDV infection was tested. DF-1 monolayers were incubated for 30 min at 37 °C with suspensions containing a constant amount of purified IBDV and increasing SVP concentrations. After incubation, cultures were maintained at 39 °C during 16 h. Accumulation of the virus-encoded VP3 polypeptide and viral RNA was determined by Western blot and qRT-PCR, respectively. As shown in Figs. 2B and C, the presence of SVP at concentrations ≥ 1 μg/ml causes a major reduction on the accumulation of both IBDV polypeptides and RNAs. These results strongly suggest that virions and SVP compete for the same receptor(s) on the cell surface, and support the use of SVP as a tool to characterizing IBDV entry mechanism.

Effects of single point mutations of the VP2 IDA sequence on SVP cell binding

Mutations on the D residue present in the Fn H1 fragment IDA motif completely abolish the ability of the fragment to interact with the α4β1 integrin (Mould and Humphries, 1991). Hence, it was important to

Table 1

| Oligonucleotide primers used for VP2 mutagenesis and RT-PCR assays |
|-----------------|-----------------|
| **Mutation**    | **Sense primer (5′–3′)** | **Anti-sense primer** |
| I234A           | CTCAGCCAACGCTGATGCCATCACAA | GAGGCTTCTGATGGCATCAGCGTTGGCTGA |
| D234A           | AGCCAACATTGCTGCCATCACGTGATGGCAGCAATGTTG | GTATGGCAGCAATGTTG |
| A236G           | AACATTGATGGCATCACAAGC | GCTTGTAGCCATCAGCTGTTT |
| **Gene** | **Sense primer (5′–3′)** | **Anti-sense primer** |
| VP2 (IBDV)      | TACCAACCAGGTGGGGTAACAGCTGGGTTATCTCGTTTGTTGG | GAGGCTTCTGATGGCATCAGCGTTGGCTGA |
| GAPDH (Gallus gallus) | ATGGTGAAAGTCGGAGTCAGG | GACAGTGCCCTTGAAGTGTC |
| GAPDH (Mus musculus) | TGACGTGCCGCCTGGAGAAGTTG | AGTGTAGCCCAAGATGCCCTCCAG |

4 Accession number AAD30136.
5 Accession number AF047874.
6 Accession number NM008084.
determine the effect of single point mutations of the VP2 IDA triplet on the ability of SVP to bind DF-1 cells. Accordingly, three mutant VP2 recombinant genes harboring the following single amino acid substitutions were generated: i) I234A (ADA-SVP); ii) D235A (IAA-SVP); and iii) A236G (IDG-SVP) (Table 1). All three mutant polypeptides, expressed in H5 cells using rBVs, gave rise to standard amounts of SVP. SVP resulting from the expression of mutant VP2 genes were purified, negatively-stained, and observed by EM. As shown in Fig. 3A, the mutations introduced on the VP2 sequence do not cause a microscopically detectable effect on the morphology or the size of the resulting SVP.

To characterize the effect of the described mutations on SVP cell binding activity, DF-1 monolayers were incubated with suspensions of purified SVP. After incubation, cultures were processed for CLSM analysis using anti-VP2 antibodies. The results of this analysis, shown in Fig. 3B, demonstrate that whilst mutant ADA- and IDG-SVP retain to some extent the ability to attach to DF-1 cells, IAA-SVP are apparently unable to bind to the cell surface. In order to confirm these results, a quantitative analysis was performed using images collected from three independent experiments simultaneously performed with all SVP under analysis. The results of this analysis (Fig. 3C) confirmed the initial observations. Reductions of 85 and 59% with respect to control WT-SVP were observed with ADA- and IDG-SVP, respectively. As expected, a higher drop on cell binding activity (≥95%) was documented with IAA-SVP.

These results led us to hypothesize that IAA-SVP might have also lost the ability to compete IBDV infectivity. To assess this possibility, a series of SVP/IBDV competition experiments was performed. DF-1 cell monolayers were incubated for 30 min at 37 °C with suspensions...
containing a constant amount of purified IBDV and increasing concentrations of either WT- or IAA-SVP. After incubation, monolayers were maintained at 39 °C during 16 h. After this period, cultures were harvested, and the accumulation of the virus-encoded VP3 polypeptide and viral RNA determined by Western blot and qRT-PCR, respectively.

As shown in Figs. 4 and B, IAA-SVP have a much lesser impact on IBDV infectivity than that observed with WT-SVP (Fig. 2), thus indicating that, in addition to the reduction on the binding capacity, the D235A substitution strongly reduces the ability of SVP to block IBDV infection. The observation that non-binding IAA-SVP retain to some extent their ability to block IBDV infection was not unexpected. We have previously shown that SVP and IBDV particles form regularly shaped aggregates (Castón et al., 2001a). X-ray analysis revealed that this phenomenon is due to the establishment of extensive contacts between the VP2 P domains of neighboring particles (Garriga et al., 2006). Hence, it seems likely that, when present at high concentrations, SVP might interact with infectious virus particles, thus preventing the contact of the latter with specific cell receptor molecules, and consequently reducing their infectivity.

The results obtained correlate with the definition of Fn H1 IDA motif, where mutations affecting the central aspartate residue cause the loss of the Fn H1 adhesive activity (Mould and Humphries, 1991), and further support the hypothesis that the VP2 IDA sequence is directly implicated in the binding of SVP and virus particles to DF-1 cells.

**Structural characterization of IAA-SVPs**

The results described above strongly suggested that the D235A substitution might be responsible for a drastic alteration on the ability of the resulting SVP to interact with a cell surface receptor. However, the available information did not rule out the possibility that this

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**Fig. 5.** Three-dimensional reconstructions of WT IDA- and mutant IAA-SVP. (A, B) Surface-shaded representations of the outer surfaces of WT- (A) and IAA-SVP (B) viewed along a five-fold axis of icosahedral symmetry, at 9.1 Å resolution. Insets show representative cryo-micrographs of WT- (left) and IAA-SVP (right). The scale bar represents 200 Å. (C) Structural homology of WT- and IAA-SVP assessed by the FSC resolution curve between both maps. The resolutions where the correlations drop below 0.5 and 0.3 are indicated. For the 0.3 threshold, both maps are identical at 9.1 Å (for the 0.5 threshold, the value is 10.5 Å). (D) SVP 50-Å-thick slab of WT- (left) and IDA-SVP (right) three dimensional reconstructions. The scale bar represents 50 Å.
mutation might affect either the stability and/or the overall architecture of the SVP. To gather information about SVP stability, suspensions containing 100 μg/ml of either WT- or IAA-SVPs were prepared using DMEM, and collected after a 2 h incubation period at 4 °C, from DF-1 cultures. SVP suspensions were subjected to a sucrose-gradient-based purification process as previously described. Samples from fractions collected from the corresponding gradients were subjected to SDS-PAGE and Western blot using anti-VP2 serum. The results of this analysis did not evidenced significant differences on the stability of wild type and mutant SVPs (data not shown).

In order to explore the effect of the D235A substitution on the structure of the mutant SVP, three-dimensional density maps of WT- and IAA-SVP capsids were generated using images from cryomicrographs (Figs. 5A, B, insets). The final SVP density maps were calculated to a 7.2 Å and 9.3 Å resolution, respectively (Figs. 5A, B). At this resolution, numerous rod- and sheet-like densities are apparent (Figs. 5A, B, and D), and secondary structural elements were identified by docking the closely related 2.6 Å VP2 X-ray map (PDB entry 2GSY) (Garriga et al., 2006) into both density maps (Supplemental information, Fig. S1). The molecular architecture of IAA-SVP is essentially identical to that of previously described WT-SVP (Bottcher et al., 1997; Castón et al., 2001a; Coulibaly et al., 2005) at ∼9 Å resolution, as assessed by FSC calculated between them (Fig. 5C).

Altogether, the results of the structural analysis rule out the possibility that the non-binding phenotype observed with IAA-SVPs might be due to a conspicuous alteration on SVP architecture.

### Table 2

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<th>Virus titers obtained by reverse genetics</th>
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<td>Virus titers (PFU/ml)</td>
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<td>First round</td>
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<td>Third round</td>
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<tr>
<td><strong>pT7-SA-Rz</strong></td>
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<tr>
<td>+</td>
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<tr>
<td>1.3 ± 0.5 × 10²</td>
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<tr>
<td>9.0 ± 1.3 × 10⁴</td>
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<tr>
<td>6.0 ± 1.5 × 10⁷</td>
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<td><strong>pT7-SB-Rz</strong></td>
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<td>+</td>
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<tr>
<td><strong>pT7-SA(D235A)-Rz</strong></td>
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<td><strong>pT7-SB-Rz</strong></td>
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**Fig. 6.** The binding of IBDV and SVP to BALB/c 3T3 cells is dependent upon the expression of the α4 integrin subunit. (A and B) [α4−] and [α4+] BALB/c 3T3 cultures were incubated with (A) suspensions of purified virus or (B) purified WT- and IAA-SVP, respectively. After incubation, cells were processed for CLSM using specific anti-VP2 antibodies. The green signal (Alexa 488) corresponds to VP2. The blue signal (ToPro-3) corresponds nuclear staining. (C and D) Quantification of the relative intensity of fluorescence signals. Data are represented as percentages with respect to values obtained in cultures of [α4+] BALB/c 3T3 cells (C) or in cultures incubated with WT-SVP (D).
VP2 D235A substitution abolishes IBDV infectivity

Results described so far demonstrate that the VP2 IDA triplet is critical for the binding of SVP to the membrane of IBDV-susceptible DF-1 cells. Hence, it was important to assess the relevance of this tripeptide on IBDV infectivity. This analysis was carried out using a reverse genetics approach using plasmids pT7-SA-Rz and pT7-SB-Rz containing the cDNA sequences corresponding to IBDV segments A and B, respectively. A third plasmid, pT7-SA/D235A-Rz, harboring a mutant version of segment A cDNA containing the D235A substitution was generated as described above.

QM7 cells were transfected with a mixture of plasmids pT7-SA-Rz and pT7-SB-Rz, and pT7-SA/D235A-Rz and pT7-SB-Rz, respectively. After transfection, cultures were infected with VT7LacO1. At 72 h p.i., cultures were harvested, and used to assess the presence of infectious IBDV.

The results obtained, presented in Table 2, demonstrate that the D235A substitution abolishes the production of an infectious IBDV progeny. Western blot analysis indicated that failure to recovering infectious IBDV from cells transfected with plasmids pT7-SA/D235A-Rz and pT7-SB-Rz was not due to problems in the expression of the polyprotein containing the D235A substitution (data not shown).

This finding, in agreement with the observations described above, evidences the critical importance of the VP2 D235 residue on IBDV infectivity.
Expression of the α4β1 integrin is necessary for SVP attachment to murine BALB/c 3T3 cells

The results described above suggested that the α4β1 integrin might act as a binding receptor for IBDV. Our initial strategy to assess this possibility was based on the silencing of α4 and/or β1 subunit gene expression. However, repeated attempts to blocking the expression of these genes using different RNA oligonucleotide sets failed (data not shown). Consequently, we decided to explore an alternative approach based on the use of a previously described BALB/c 3T3 cell system. Murine BALB/c 3T3 fibroblasts do not naturally express the α4 subunit, thus their cell membrane lacks the α4β1 integrin (Zeller et al., 1998). Caruso et al. (2003) generated a couple of BALB/c 3T3-derived cell lines, termed [α4+] and [α4−] BALB/c 3T3, respectively. The only documented difference between these two cell lines was the expression of the α4 subunit, and the consequent presence of the α4β1 heterodimer on the membrane of [α4+] cells.

Before initiating our experiments, the expression of the α4 and β1 integrins at the surface of [α4+] and [α4−] BALB/c 3T3 cells was analyzed by flow cytometry. The β1 subunit was abundantly expressed on both [α4+] and [α4−] cells. As expected, the expression of the α4 subunit was exclusively detected in [α4+] BALB/c 3T3 cells (data not shown).

The ability of IBDV particles as well as WT- and IAA-SVP to bind to [α4+] and [α4−] BALB/c 3T3 cells was analyzed by CLSM. Cell monolayers were incubated with suspensions containing purified virus, WT-, and IAA-SVP, respectively. After incubation, cells were processed for CLSM analysis using anti-VP2 antiserum. As shown in Figs. 6A, B, IBDV virions and WT-SVP efficiently attach to [α4+] BALB/c 3T3 cells but do not to [α4−] BALB/c 3T3 cells. Interestingly, IAA-SVP do not bind neither [α4+] nor [α4−] BALB/c 3T3 cells (Figs. 6A, B). Quantitative analyses of CLSM images (Figs. 6C, D) confirmed the information gathered by the direct microscopic observation.

As described above, IBDV uses the Hsp90 polypeptide as a post-binding receptor (Lin et al., 2007). It has been recently shown that depletion of the Hsp90 polypeptide from murine B lymphocytes results in a major reduction on the surface expression of the α4β1 integrin (Liu and Li, 2008). Accordingly, it seemed feasible that α4β1 expression might enhance Hsp90 surface expression. This might facilitate the interaction of SVP with Hsp90 instead of with the α4β1 integrin under study. However, as determined by flow cytometry analyses, both cell lines, [α4+] and [α4−] BALB/c 3T3, exhibit negligible Hsp90 surface levels (Supplemental data Fig. S2), thus ruling out such possibility.

The results of this series of experiments show that the attachment of both IBDV and SVP to BALB/c 3T3 cells is strictly dependent upon the presence of the α4β1 integrin at the cell membrane.

α4β1 integrin expression is an essential factor for the susceptibility of BALB/c 3T3 cells to IBDV infection

Results described above demonstrate that the α4β1 heterodimer is essential for IBDV attachment to BALB/c 3T3 cells. However, at this point we could not rule out that IBDV binding to α4β1-expressing cells might be unrelated with the ability of the virus to enter the cells and initiate a productive infection. To investigate this possibility [α4+] and [α4−] BALB/c 3T3 monolayers were incubated for 1 h at 37 °C with purified IBDV. At 24, 48, and 72 h p.i., cells were harvested and used for qRT-PCR, Western blot, and virus titration analyses. The results obtained clearly evidenced that incubation of [α4+] BALB/c 3T3 cells leads to accumulation of IBDV-specific RNAs (Fig. 7A), and the IBDV VP3 polypeptide (Figs. 7B and C). Additionally, virus titrations showed that [α4+] BALB/c 3T3 support the production of an infectious progeny (Fig. 7D). The low IBDV titers obtained were expected due to the fact that the virus had not been previously adapted to growing in this cell line. As expected from previous results, [α4−] BALB/c 3T3 were completely resistant to IBDV infection as evidenced by their resistance to accumulate IBDV-specific RNAs and polypeptides, and to produce infectious virus (Figs. 7A–D).

Taken together, these results demonstrate that the presence of the α4β1 heterodimer allows the initiation of a productive IBDV replication cycle, thus demonstrating that the α4β1 integrin acts as a bona-fide receptor for IBDV in [α4+] BALB/c 3T3 cells.

Inhibition of IBDV infectivity by function-blocking anti-α4 and -β1 mAbs

To further confirm the role of the α4β1 heterodimer on the ability of transfected [α4+] BALB/c 3T3 cells to support IBDV infection, the effect of anti-α4β1 function-blocking mAbs was analyzed. [α4+] BALB/c 3T3 cultures were incubated for 1 h at 4 °C with mAbs directed against the α4 or the β1 polypeptide, or a combination of both. As controls for this experiment, cultures were also incubated with anti-αβ1 integrin mAb as well as with irrelevant mAbs of the same isotype to those specific for α4 and β1 subunits. Cultures were then incubated for 1 h at 4 °C with 1 PFU/cell of purified IBDV and maintained for 20 h at 37 °C. Infectivity was calculated by counting the number of VP3-positive cells versus the total cell count. Infectivity reductions of 53 ± 11% and 52 ± 13% were observed in cells pre-treated with anti-α4 and -β1 mAbs, respectively (Fig. 8). The combination of the anti-α4 and -β1 mAbs do not further increase the blocking activity observed with single mAb treatments. As expected, pretreatments with irrelevant control mAbs do not significantly affect IBDV infectivity.

Reductions on IBDV infectivity, nearing 50%, obtained with the anti-α4 and -β1 mAbs used in our study are similar to those previously described with other function-blocking mAbs directed against integrins involved in the attachment and/or entry of different naked icosahedral viruses (Coulson et al., 1997; Guerrero et al., 2000; Hewish et al., 2000; Lendriogian et al., 2000). Together with data described above, these results demonstrate that the α4β1 integrin is involved in the entry of IBDV into BALB/c 3T3 cells.

Discussion

IBDV SVP are produced as a result of the expression of the mature form of the VP2 protein (Castón et al., 2001a). This recombinant polypeptide self-assembles into all-pentamer particles (23 nm in diameter) formed by 20 VP2 trimers exhibiting an icosahedral T = 1 symmetry (Castón et al., 2001a; Saugar et al., 2005). The SVP atomic structure has been solved by X-ray crystallography (Coulibaly et al., 2005; Garriga et al., 2006; Lee et al., 2006). SVP VP2 trimers are symmetric (Castón et al., 2001a; Saugar et al., 2005). The SVP atomic structure has been solved by X-ray crystallography (Coulibaly et al., 2005; Garriga et al., 2006; Lee et al., 2006). SVP VP2 trimers are symmetric (Castón et al., 2001a; Saugar et al., 2005). The SVP atomic structure has been solved by X-ray crystallography (Coulibaly et al., 2005; Garriga et al., 2006; Lee et al., 2006). SVP VP2 trimers are symmetric (Castón et al., 2001a; Saugar et al., 2005). The SVP atomic structure has been solved by X-ray crystallography (Coulibaly et al., 2005; Garriga et al., 2006; Lee et al., 2006). SVP VP2 trimers are symmetric (Castón et al., 2001a; Saugar et al., 2005). The SVP atomic structure has been solved by X-ray crystallography (Coulibaly et al., 2005; Garriga et al., 2006; Lee et al., 2006). SVP VP2 trimers are symmetric (Castón et al., 2001a; Saugar et al., 2005). The SVP atomic structure has been solved by X-ray crystallography (Coulibaly et al., 2005; Garriga et al., 2006; Lee et al., 2006). SVP VP2 trimers are symmetric (Castón et al., 2001a; Saugar et al., 2005). The SVP atomic structure has been solved by X-ray crystallography (Coulibaly et al., 2005; Garriga et al., 2006; Lee et al., 2006). SVP VP2 trimers are symmetric (Castón et al., 2001a; Saugar et al., 2005).
The role of the VP2 IDA sequence motif on IBDV cell binding and replication

A large number of viruses use members of the integrin superfamily as specific cell receptors at the attachment or post-attachment level (Stewart and Nemerow, 2007). The interaction of integrins to their ligands occurs through the recognition of short linear sequence motifs on their ligands. The availability of a number of well-characterized integrin-ligand motifs facilitated our search for putative integrin ligand motifs on the VP2 primary sequence. As described above, this search resulted in the identification of the IDA motif, matching the previously described consensus sequence of amino acid triplets employed by Fn to bind the α4β1 integrin (Mould and Humphries, 1991). Additionally, the detected VP2 IDA triplet is placed within a flexible loop connecting two long β-sheets, thus fulfilling an important structural feature common to integrin ligands: the location of the critical acidic (D or E) residue within a flexible loop (Michishita et al., 1993; Ruoslahti, 1996).

The strict conservation of VP2 IDA triplet across the Avibirnavirus genus suggested that this tripeptide might play a critical role on virus replication. Furthermore, the putative specificity of the IDA sequence for the α4β1 heterodimer, a highly abundant integrin in immature lymphocytes (Rose et al., 2002), the main IBDV cell target (Becht, 1980; Kibenge et al., 1988), led to the hypothesis that IBDV might use this integrin during its entry mechanism. Although RT-PCR analyses showed the presence of RNA transcripts corresponding to both α4 and β1 integrin subunit genes in DF-1 cells (data not shown), the lack of commercially available antibodies specifically recognizing the avian α4 subunit precluded the possibility of directly assessing the presence of the α4β1 heterodimer on the membrane of the avian cells. The generation of a set of antibodies directed to the extracellular moiety of the α4 subunit, currently ongoing in our laboratory, will provide an essential tool for the future characterization of its expression in chicken cells, and to further analyze its role on IBDV infection both in vivo and in vitro.

Results gathered with SVP harboring single point mutations on the IDA triplet demonstrate that this sequence, and in particular the central aspartate residue, is essential for: i) SVP binding to the cell membrane; and ii) the ability of SVP to hinder the attachment of IBDV virions to the cell membrane, and block virus infection. These results strongly resemble those obtained with soluble peptides used to characterize the specificity of α4β1-binding sequences within the Fn H1 (Mould and Humphries, 1991). The structural analysis performed on mutant IAA-SVP indicates that the documented non-binding phenotype of mutant IAA-SVP is a direct consequence of the functional inactivation of the α4β1 binding motif. Furthermore, the results of the reverse genetic experiments demonstrate that the D235A mutation completely arrests virus viability. It seems plausible that the lethality of the D235A mutation might be a direct consequence of the inability of the VP2 D235A-bearing IBDV particles to attach to the membrane of target cells.

Noteworthy, a comparison of VP2 primary sequences from representative members of the different birnavirus genus (Avibirnavirus, Acquabirnavirus, and Entomobirnavirus) showed a strict conservation of the IDA D residue across the family (Supplemental information Fig. S3), thus suggesting that it might also play a critical role in the life cycle of other birnaviruses.

The role of the α4β1 integrin on IBDV cell binding and replication

Murine BALB/c 3T3 cells do not naturally express the α4 subunit, thus being devoid of the α4β1 integrin (Zeller et al., 1998). Two stably transfected BALB/c 3T3-derived cell lines, exclusively differing on the expression of the α4 subunit, were previously generated by Caruso et al. (Caruso et al., 2003) as a convenient tool analyzing the role of the α4β1 integrin during the entry process of murine polyomavirus. We borrowed these cell lines to determine whether the expression of the α4β1 integrin might be related with the ability of IBDV to bind to the cell membrane. The analysis of the results obtained with this cell system clearly demonstrate that the presence of the α4β1 heterodimer is a critical factor for the binding of both IBDV and IBDV-derived SVP to the cell surface. The specificity of these results was further confirmed by the non-binding phenotype exhibited by mutant IAA-SVP lacking an active α4β1-binding motif.

According to results obtained with BALB/c 3T3 cells, in addition to facilitating the binding to the cell membrane, the α4β1 heterodimer acts as a key factor for IBDV infection. This shows that, in this cell system, the IBDV–α4β1 interaction is essential in triggering the access of the virus to a cellular compartment suitable for virus replication, and thus allowing the production of an infective virus progeny. Hence, demonstrating that the murine α4β1 integrin acts as bona-fide binding receptor for IBDV.

IBDV isolates have been successfully adapted to replicate in established mammalian cells, i.e. Vero and BSC-1 cells (Lombardo et al., 2000; Yip et al., 2007). Tissue culture-adapted IBDV strains are able to bind to a specific cell receptor(s), enter the cell, and carry out a productive replication process, thus giving rise to an infective virus progeny. It was previously thought that tissue culture adaptation was strictly related to the ability of the adapted viruses to bind to a receptor(s) different from that present in natural IBDV cell targets. However, it has been recently shown that VP2 polypeptides from the non-adapted very virulent HK46 isolate and the tissue culture-adapted D78 IBDV strain share the same binding receptor on the membrane of Vero cells (Yip et al., 2007). This finding shows that the restriction to tissue culture growth exhibited by, at least, some non-adapted viruses takes place at a post-binding step (Lombardo et al., 2000; Yip et al., 2007). In view of the results presented here, it seems feasible that the α4β1 integrin might also act as a binding receptor in bursal lymphoid cells. However, further studies will be required to determine whether this is the case.

Although specific information regarding the IBDV entry mechanism is scarce, available data suggests that IBDV enters susceptible cells using a receptor-mediated endocytic pathway (Galloux et al., 2007; Lin et al., 2007). The interaction between IBDV particles and the α4β1 integrin might afford virus–cell binding. This step could be followed by the interaction with the CH5090e protein and, probably, other cellular polypeptides required for successful virus entry.

Materials and methods

Viruses, cells, and antibodies

The IBDV Soroa strain, a virulent serotype 1 virus, was propagated and titrated in QM7 quail muscle cells (ATCC, CRL-1962) as previously described (Lombardo et al., 1999). BALB/c 3T3-derived cell lines [α4 +] and [α4−] BALB/c 3T3, kindly provided by Dr. Paolo Amati, were grown in DMEM containing 10% FCS and supplemented with puromycin at 1.3 μg/ml. [α4+] BALB/c 3T3 cells are stably transfected with pRKS-α4, a plasmid expression vector carrying the cDNA of the murine α4 integrin subunit. [α4−] BALB/c 3T3 cells are stably transfected with the empty version of the pRKS plasmid (Caruso et al., 2003). Construction of recombinant baculoviruses (rBVs) FB/VB2-452, FB/VB-452[1234A], FB/VB2-452(D235A) and FB/VB-452 (A236G) is described below. rBV stocks were grown and titrated in S. frugiperda H5 insect cells (Invitrogen) as described before (Maraver et al., 2003). H5 insect cells were infected with the appropriate rBVs, and used to produce and purify SVP using sucrose gradients as described previously (Castón et al., 2001b). The recombinant vaccinia virus (rVV) VT7LacO1 was grown and titrated as previously described (Ward et al., 1995). H5 cells were grown at 28 °C in TC100 medium.
containing 10% inactivated fetal calf serum (FCS). QM7 and DF-1 cells (ATCC, CRL-12203) were grown at 37 °C and 39 °C, respectively, in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% inactivated fetal calf serum (FCS). [α4+] and [α4−] BALB/c 3T3 cells were grown at 37 °C in DMEM containing 10% FCS and supplemented with 1.3 μg/ml of puromycin.

Western blot and confocal laser scanning microscopy (CLSM) analyses were carried out using anti-VP2 and -VP3 specific sera as previously described (Fernandez-Arias et al., 1998). The monoclonal antibody (mAb) mAbC3, specific for the 14K rVV structural polypeptide (Rodriguez et al., 1985), was used to detect the presence of contaminant rVV in reverse genetic assays.

Function-blocking antibodies directed to mouse integrin subunits α4 (CD49d, clone R1-2) and its isotype control mAb (A95-1) were purchased from BD PharMingen. mAbs directed to mouse integrin subunit β1 (CD29, clone HM1/1-1) and its control isotype mAb (eBio299Arm) were purchased from ebioscience. mAbs to α/viβ6 integrin (mAb 2077Z) was purchased from Chemicon. The mAb anti-β-actin (AC-15), purchased from Sigma, was used to control the total protein content of cell extracts under study.

**IBDV titration**

Monolayers of DF-1 cells grown in 6-well tissue culture plates were infected with serial dilutions of infected cell supernatants. Following virus adsorption for 60 min at 37 °C, the inoculums were removed. Infected cells were washed twice with DMEM without serum and incubated with fresh DMEM containing 2% of FCS at 39 °C in a 5% CO2 atmosphere. At 72 h.p.i., cells were fixed in acetone-methanol (1:1), washed, and incubated for 60 min with anti-VP2 antibodies, followed by incubation with horseradish-peroxidase-conjugated polyclonal goat anti-rabbit antibody. Thereafter, plates were washed with phosphate buffer saline (PBS), and horseradish-peroxidase-conjugated polyclonal goat anti-rabbit antibody (GE Healthcare) added. The mixture was incubated for 45 min at 20 °C. After washing with PBS, antibody-labeled cells were developed using 3, 3′-diaminobenzidine (Sigma) substrate solution, foci of stained cells were counted, and virus titers calculated.

**Reverse genetic analysis**

DNA fragments containing cDNA versions corresponding to the complete positive strand RNAs corresponding to the IBDV Soroa strain segments A and B, fused to the T7 bacteriophage promoter (in 5′ position with respect of the cDNA) and a cDNA corresponding to the hepatitis delta virus ribozyme (in 3′ position with respect of the cDNA) sequences were generated by in vitro gene synthesis (Gene-script Co.), and inserted into the multiple cloning site of the pUC57 cloning plasmid (GenBank/EMBL accession number Y14837). These plasmids were named pT7-SA-Rz, containing the cDNA corresponding to segment A, and pT7-SB-Rz, containing the cDNA corresponding to segment B, respectively.

Plasmid pT7-SA-Rz was used as the template for the introduction of selected point mutations, using a PCR-based protocol, on the VP2 coding region. The core-coding region of VP2 was amplified using primers (Table 1) containing the desired nucleotide changes to generate the D235A mutation. Primary PCR products containing the engineered mutations were assembled by PCR amplification with the flanking primers 5′-TCACCGTCTTACGTATAC and 5′-TCAGGATTTGGAATGTTAGTATTGTGTTGAAATGACCG. The resulting DNA fragments were purified, digested with BamHI and NdeI, and ligated to pT7-SA-Rz previously digested with the same enzymes. The resulting plasmid, pT7-SA(D235A)-Rz, was amplified, and analyzed by nucleotide sequencing to determine the correctness of its sequence.

QM7 cells seeded in 60-mm dishes (1.5 × 10⁶ cells/dish) were transfected with a combination of either pT7-SA-Rz and pT7-SB-Rz or pT7-SA(D235A)-Rz and pT7-SB-Rz, respectively. Transfections were carried out with 2.5 μg of each plasmid using lipofectamine (Gibco BRL). At 6 h after transfection, cultures were infected with 1 PFU/cell of VT7LacO1, an rVV inducibly expressing the T7 RNA polymerase (Ward et al., 1995). After infection, cultures were maintained at 37 °C in DMEM containing 10% FCS supplemented with the inducer isopropyl β-D-thiogalactoside (IPTG) (1 mM final concentration). At 72 h.p.i., cultures were harvested, and subjected to three freeze–thawing cycles. After removing cell debris by low speed centrifugation (10,000 × g for 5 min at 4 °C), supernatants were recovered and passed through 0.1 μm filters (MillexVV, Millipore) to eliminate contaminant rVV particles, and used to infect fresh QM7 cell monolayers. Infections were performed in quadruplicate with undiluted and 10⁻¹ through 10⁻⁵ serial dilutions of the initial stocks. Two sets of infected cells were used to determine the IBDV titer at 72 h.p.i. Another set was harvested at 72 h.p.i., and the corresponding extracts used to detect the presence of IBDV VP1 and VP2 polypeptides by Western blot using specific antibodies. The absence of contaminant infecting rVV particles was also monitored by Western blot, using the mAbBC3. The fourth cell set was also harvested at 72 h.p.i., and used to collect cells originally infected with the undiluted stocks. These samples were subjected to three freeze–thawing cycles, and used to repeat the procedure described above. The process was repeated once more, thus allowing the analysis of the initial virus stock and two subsequent virus amplification rounds.

**Construction of rBVs**

A DNA fragment corresponding to the region encoding the 452 N-terminal residues of the IBDV polyprotein was generated by PCR synthesis using pFastBac/POLY (Martinez-Torrecuadrada et al., 2000). PCR reactions were carried out with Vent DNA polymerase (New England Biolabs) using primers 5′-CCGCGATCATGCAACACCTGCTGACGATCAAACCC and 5′-CCGCGAAGCTTACCTTATGGCCCGGATTATGTCTTTGGAACCC. The resulting DNA fragment was purified, digested with BglII and HindIII and cloned into the pFastBac1 plasmid (Invitrogen) previously digested with BglII and HindIII. The resulting plasmid vector, pFB/VP2-452, was used as the template for the introduction of selected point mutations using a PCR-based protocol. The core-coding region of VP2-452 was amplified using primers containing the desired changes (Table 1). Primary PCR products containing the engineered mutations were assembled by amplification with the flanking primers 5′-TCACCGTCTTACGTATAC and 5′-TCAGGATTTGGAATGTTAGTATTGTGTTGAAATGACCG. The resulting DNA fragments were purified, digested with BamHI and NdeI, and ligated to pFB/VP2-452 previously treated with the same enzymes, giving rise to plasmids pFB/VP2-452(D234A), pFB/VP2-452(D235A), and pFB/VP2-452(A236G).

**Protein sequence analysis**

Searches for integrin binding consensus sequences within the IBDV VP2 protein, and sequence alignments were carried out using the CLUSTAL W multiple-sequence alignment software (Larkin et al., 2007). Birnavirus VP2 sequences were retrieved from UniProt (http://www.pir.uniprot.org/).

**SVP blockade of virus infectivity**

DF-1 and transfected BALB/c 3T3 cells were grown into 24-well plates with DMEM supplemented with 5% FCS for 16 h. Thereafter, the medium was removed, and cells incubated with suspensions containing a constant amount of 5 × 10⁶ PFU/ml of purified IBDV and increasing SVP concentrations (0.01–100 μg/ml). SVP/virus suspensions were prepared in DMEM. Monolayers were covered with 500 μl of the corresponding SVP/virus suspension, and maintained at 37 °C for 30 min. Thereafter, cultures were washed, supplemented with fresh medium, and maintained for 16 h at 39 °C (DF-1 cells) or 32 h at 32 °C.
37 °C (transfected BALB/c 3T3 cells). Finally, cultures were harvested and divided into two aliquots used to determine the accumulation of both virus-encoded proteins and viral RNA, respectively.

**Western blot analysis**

Cell extracts were added to Laemmli’s sample buffer to a 1× final concentration, and heated (100°C, 2 min). Electrophoreses were performed in 11% polyacrylamide gels, followed by electrobloinding onto polyvinyldene difluoride membranes. Membranes were cut into two halves. The lower half was used to evaluate the accumulation of the IBV-encoded VP3 polypeptide, and the upper one to assess the accumulation of both virus-encoded proteins and viral RNA, respectively.

**Conventional electron microscopy analysis**

2–5 μl aliquots of SVP-containing samples were placed on top of copper grids covered with collodion and carbon that had previously been made hydrophilic by glow discharge. Grids were then incubated at 20 °C for 2 min. Adsorbed particles were negatively stained for 30 s with a 2% solution of uranyl acetate. Micrographs were recorded with a JEOL 1200 EXII electron microscope operating at 100 kV at a nominal magnification of 40,000×.

**Cryo-electron microscopy (cryo-EM)**

SVP suspensions (5 μl drops) were applied to one side of a holey carbon film, blotted, and plunged into a liquid ethane bath following standard procedures (Castón et al., 2001b; Saugar et al., 2005). Micrographs were recorded under minimal exposure conditions. Imaged specimens received exposures of 6–10 e−/nm² at a nominal magnification of 50,000× on a Tecnai G2 electron microscope operating at 200 kV and equipped with a field emission gun.

**SVP image analysis**

General image processing operations were performed using Bsoft (http://www.niams.nih.gov/rcn/labbran/lbr/software/bsoft/), Xmipp (http://xmipp.cnb.csic.es/), and Spider (http://www.wadsworth.org/spider_doc/spider_docs/) programs. For the selected micrographs analyzed, defocus values were determined with the Bshow program (http://www.niams.nih.gov/rcn/labbran/lbr/software/bsoft/bshow/bshow.html), ranged from 0.6 to 3 μm (first zeros of the contrast transfer function [cft] at spacing of 11 to 28 Å). A Zeiss PhotoScan TD scanner was used to digitalize micrographs at 7 μm/pixel (1.4 Å at the specimen), and micrographs were binned to give 14 or 21 μm/pixel during the initial refinements. The X3d program (Conway et al., 1993) was used to manually extract particle images, including 26,386 for wild type IDA (WT-SVP) and 19,433 for mutant IAA-SVP. Initial estimates of the origin and orientation angles were determined for a base set of particles, using polar Fourier transform procedures (Baker and Cheng, 1996), taking previous maps, appropriately scaled at a 28-Å resolution, as a starting model (Saugar et al., 2005). A new density map was calculated and used for all subsequent orientation and origin refinements. Phases were ctf-corrected by flipping them in the required lobes of the ctf with Bsoft bctf routine. Enhancement of the high-resolution Fourier amplitudes was carried out with the Spider program as previously described (40). Three-dimensional density (3D) maps were calculated using Fourier–Bessel methods (Crowther, 1971). The final reconstructions combined 23,754 and 17,505 images for WT- and IAA-SVP, respectively. The resolution was assessed by FSC calculated between independent half-data-set maps, applying a correlation limit of 0.5 (or 0.3). The resolutions calculated for WT- and IAA-SVP were of 7.8 Å (or 7.2 Å) and 10.5 Å (or 9.3 Å), respectively. The structural identity of both density maps was assessed by FSC calculated between them. Density maps and atomic models were visualized with UCSF Chimera software (http://www.cgl.ucsf.edu/chimera/).

**RNA isolation and cDNA synthesis**

Total RNA was extracted from frozen cells (1 × 10⁷) using Trizol (Invitrogen) following the manufacturer’s recommendations. DNase digestion of contaminating DNA in the RNA samples was carried out with the RNase-Free DNase Set (Qiagen). Final RNA purification was performed using the RNasy Mini Kit (Qiagen) according to standard protocols. Total RNA (1 μg) was reverse transcribed in a reaction mixture of 20 μl containing 1× PCR Buffer II (Applied Biosystem), 5 mM MgCl₂, 1 mM dNTP, 20 U RNase inhibitor, 50 U MuLV reverse transcriptase (Applied Biosystems), 5 μM random primers ( Gibco) and DEPC treated dH₂O.

**Real-time quantitative RTPCR (qRTPCR) assays**

Transcript levels were determined by qRTPCR using a 7300 Real-Time PCR System (Applied Biosystems) and SYBR Green dye (Applied Biosystems). Reactions were performed in a final volume of 20 μl containing 10 μl of 2× Power SYBR Green PCR Master Mix (including AmpTaq Gold DNA Polymerase-LD, dNTPs and SYBR Green dye), 250 nM forward and reverse specific primers (Table 1) and a 1:10 dilution of cDNA. After enzyme activation at 95 °C for 10 min, amplifications were carried out using a two-step PCR procedure with 40 cycles of 15 s at 95 °C for denaturation and 1 min at 60 °C for annealing/extension. Gene specific primers were designed using the Oligo Explorer 1.2 software (Gene Link) based on sequences information deposited at the National Center of Biotechnology Information. Primer sequences used in the qRTPCR analyses are described in Table 1.

Non-template controls were included for each primer pair, and each PCR reaction was completed in triplicate. Data were analyzed using the 7300 SDS software 1.3 (Applied Biosystems). Dissociation curves for each amplicon were then analyzed to verify the specificity of each amplification reaction; the dissociation curve was obtained by heating the amplicon from 60 °C to 95 °C. For IBDV-SVP competition experiments specific RNA contents were calculated using the standard curve method from triplicate data obtained with the Gallus gallus glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, as internal control, and control cultures, infected with IBDV in the absence of SVP, as reference control samples. For experiments carried out in [α4+] and [α4−] transfected BALB/c 3T3 cells, specific RNA contents were calculated using data obtained with the Mus musculus GAPDH gene, as internal control, and control uninfected cultures. Results are means of triplicate data of one representative experiment, and are represented as the percentage with respect to the reference sample representing a 100% infection value.

**Confocal laser scanning microscopy (CLSM) analysis**

For binding experiments, purified IBDV or SVP were used. DF-1 or [α4+] and [α4−] transfected BALB/c 3T3 cells seeded onto glass coverslips were incubated with suspensions containing either SVP (50 μg/ml) or IBDV virions (10 PFU/cell) at 4 °C for 30 min. Afterwards, cells were methanol-fixed, incubated with rabbit anti-VP2 specific serum, followed by incubation with goat anti-rabbit Ig coupled to Alexa 488 (green). Cell nuclei were stained with ToPro-3. Fluorescent signals detected by CLSM were visualized by epifluorescence using a Zeiss Axiovert 200 microscope equipped with a Bio-Rad Radiance 2100 confocal system. Images were captured using the Laser Sharp software package (Bio-Rad). Relative quantification of fluorescence was performed using LaserPix 4.0 software (ImagePro Plus.
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Appendix A. Supplementary data


References


