More recent swine vesicular disease virus isolates retain binding to coxsackie–adenovirus receptor, but have lost the ability to bind human decay-accelerating factor (CD55)

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INTRODUCTION

Swine vesicular disease virus (SVDV) is a member of the genus Enterovirus within the family Picornaviridae. It causes a highly contagious disease of pigs that spreads rapidly by direct contact among infected animals and by environmental contamination (Dekker et al., 1995). Whilst it is not a severe disease, because of the similarity of the lesions that it causes with those produced by foot-and-mouth disease, routine surveillance for SVDV is maintained in European countries (reviewed by Escribano-Romero et al., 2000). The disease was first described in Italy in 1966 (Nardelli et al., 1968) and, since then, numerous outbreaks have occurred throughout Europe and Asia.

SVDV is a porcine variant of the human pathogen coxsackie B virus serotype 5 (CVB5) (Brown et al., 1973; Graves, 1973; Inoue et al., 1993; Knowles & McCauley, 1997; Seechurn et al., 1990; Zhang et al., 1993). The antigenic and molecular relationships between these two viruses suggest that CVB5 crossed the species barrier from humans to pigs at some time between 1945 and 1965, when it was first identified as a porcine pathogen, and has since continued to adapt to the new host (Zhang et al., 1999).

The progenitor virus CVB5 uses the coxsackie–adenovirus receptor (CAR) as primary receptor and decay-accelerating factor (DAF; CD55) as co-receptor (Bergelson et al., 1997; Martino et al., 2000; Shafren et al., 1995; Spiller et al., 2000).
Several other human enteroviruses, including CVB serotypes 1 and 3 and serotypes of echovirus, enterovirus 70 and coxsackievirus A21, have also been reported to bind human DAF (Bergelson et al., 1994; Karnauchw et al., 1996; Shafren et al., 1997; Ward et al., 1994). Although these viruses belong to the same family, different viruses bind to different sites on DAF, suggesting independent evolution of DAF binding (Bergelson et al., 1994, 1995; Clarkson et al., 1995; Karnauchw et al., 1998; Shafren et al., 1995, 1997). It was therefore of interest to investigate whether SVDV had retained receptor-binding properties after crossing to pigs.

Sequences for many different SVDV strains isolated over the last 35–40 years are now available and sequence comparisons allow a unique insight into the selective pressures required for maintaining consensus sequences in the capsid of this virus. SVDV has been subdivided into four groups, A–D, based on sequence and antigenic-epitope divergence from CVB5 (Brocchi et al., 1997). Receptor usage by SVDV is poorly defined. A single report found that mAbs against human DAF or human CAR each decreased the capacity of SVDV strain UK 27/72 to infect human HeLa cells (Martino et al., 2000). UK 27/72 is a member of group B and is representative of SVDV isolates from early outbreaks of disease that are highly similar to CVB5, whilst isolates obtained in the early 1990s belong to groups C and D and have far less similarity to CVB5. No data currently exist regarding receptor usage of the isolates from more recent outbreaks.

Here, we compare the ability of progenitor CVB5 and SVDV isolates from early and recent outbreaks to utilize DAF and CAR for binding and infection of cells. The ability of specific antibodies raised against recombinant DAF and CAR, as well as pre-incubation of the virus with these recombinant proteins, to block infection of permissive cells was used to assess receptor usage. The role of human DAF in human and porcine virus infection was also assessed through radiolabelled virus binding and enhancement of infection following expression of human DAF on pig cells. The inability of these viruses to bind to pig DAF was also confirmed.

**METHODS**

**Cells, viruses and virus propagation.** IB-RS-2 pig kidney cells (de Castro, 1964) were obtained through the reference laboratory for SVD (CISA, Valdeolmos, Madrid, Spain). Buffalo green monkey (BGM), HeLa (ATCC CCL-2) and Chinese hamster ovary (CHO) (ATCC CCL-61) cells were obtained through the ATCC (Manassas, VA, USA). IB-RS-2, BGM and HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, 100 μg streptomycin ml⁻¹ and 5% fetal calf serum. CHO cells were grown in RPMI 1640 medium supplemented with the same antibiotics and 10% fetal calf serum. IB-RS-2 cells were also stably transfected with human DAF in the eukaryotic expression vector pBR2DEF1x (Harris et al., 2000) by using Lipofectamine (Invitrogen) for transfection, followed by selection in 100 μg hygromycin ml⁻¹ (Invitrogen). Cell-surface expression of proteins was confirmed by flow cytometry using specific mAbs recognizing human DAF (MBC1), pig DAF (PD3) or human CAR (BRAD3O, which also recognizes pig CAR equally) and phycocyanin-conjugated rabbit anti-mouse IgG (Dako), secondary antibody. Rabbit polyclonal antisera were also raised against soluble recombinant pig DAF, human DAF or human CAR in-house and the IgG fraction of the antiserum was isolated by purification on a protein A-Sepharose column (Amersham Biosciences). We have previously shown that rabbit polyclonal anti-human CAR recognizes pig CAR, due to the high similarity between pig and human CAR (Spiller et al., 2002).

The SVDV isolates used in this work were: UK 27/72 (UK’72; GenBank accession no. X54521) as reference isolate, provided by the Institute of Animal Health (IAH, Pirbright, UK); IT/1/66 (It’66; GenBank accession no. Y14464), from the first SVDV outbreak in 1966; isolates R1072 (Borrego et al., 2002) and R1120 (GenBank accession no. Y11474), from two independent outbreaks in the early 1990s, provided by E. Brocchi, IZSLE, Brescia, Italy; and the SPA/1/93 isolate (SPA/93; GenBank accession no. AF114383) was provided by A. Tenorio, Enterovirus Laboratory, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain. Low passage number is crucial to maintaining the integrity of virus receptor usage from the initial isolate and was kept to a minimum: R1120 and R1072 at three to four passages on IB-RS-2 cells, SPA/93 and UK/72 at seven to eight passages on IB-RS-2 cells. It’66 at eight to ten passages on IB-RS-2 cells and CVB5 (not including passage history at ATCC) at four to five passages on BGM cells.

**Binding assays.** Viruses were labelled metabolically with 0.42 mCi (155 MBq) [³⁵S]cysteine/methionine (ICN) as described previously (Spiller et al., 2000).Following removal of cell debris by centrifugation (3000 g), labelled viruses in the supernatant were separated from unincorporated radiolabel by centrifugation (125 000 g) through a 30% sucrose cushion in PBS (pH 7.4). Pelleted viruses were resuspended in serum-free medium and particulate material was removed by centrifugation at 16 000 g for 10 min at 4°C and stored frozen at −70°C in aliquots until used. A 50 μl aliquot of each virus preparation (10⁶ TCID₅₀ ml⁻¹), containing the following amounts of incorporated [³⁵S] label, was used for each sample: SVDV It’66 (80 349 c.p.m.), SVDV UK’72 (52 726 c.p.m.), SVDV R1072 (47 790 c.p.m.), SVDV R1120 (59 829 c.p.m.), SVDV SPA/93 (41 747 c.p.m.) and CVB5 (45 494 c.p.m.). For each sample (repeated in triplicate), 10⁶ EDTA-disaggregated cells were pelleted and resuspended in 50 μl serum-free medium containing radiolabelled virus and incubated on ice for 2 h. Unbound virus was removed by three 0.25 ml washes (1000 g, 5 min) in ice-cold, serum-free cell medium and bound virus was quantified by scintillation counting. Statistical analysis was performed by using one-way ANOVA followed by a Tukey test post hoc (GraphPad Software).

**Virus-infection assays.** Duplicate 10× dilutions of virus stocks were incubated with confluent monolayers of HeLa, IB-RS-2 or transfected cell lines grown in 96-well plates, in serum-free DMEM supplemented with antibiotics, for 48–72 h in a CO₂ incubator at 37°C until development of cytopathic effect (CPE) was observed. Virus titre was defined as the reciprocal of the highest virus dilution able to produce detectable CPE in this assay.

**Antibody-blocking assays.** IB-RS-2 or HeLa cells were seeded into 96-well plates as for the virus-infection assays described above. For antibody blocking, 30 min prior to addition of a 10-fold dilution series of each virus, cells were incubated with 50 μg ml⁻¹ of rabbit polyclonal anti-human DAF, anti-pig DAF or anti-CAR IgG (which cross-reacts with both species; Spiller et al., 2002). Rabbit IgG was dialysed into PBS and filter-sterilized prior to addition to
cells. Virus infections with stocks of known virus titrations performed in the presence of anti-DAF or anti-CAR were compared with those performed in the absence of antibody.

**Blocking experiments with soluble receptors.** IB-RS-2 cells were seeded into 96-well plates and allowed to grow to 90% confluence. Prior to addition to cells, a 10-fold dilution series was made from each virus stock in serum-free medium and an equal volume of serum-free medium or serum-free medium containing 5 μM sterile, recombinant human (h) DAF-Fc or hCAR-Fc fusion proteins was added and incubated on ice for 30 min. Details of the construction, characterization and purification of soluble recombinant DAF or CAR fused with the Fc region of human IgG are provided elsewhere (Yanagawa et al., 2003, 2004). Infections were observed for CPE daily, but were fixed with 1% formaldehyde and stained with 0.1% crystal violet in PBS to observe residual cells at 72 h post-infection (p.i.).

**RESULTS**

Blocking CVB5 or SVDV infection of human HeLa cells with specific antibodies

It has previously been reported that SVDV strain UK’72 infected HeLa cells and pre-incubation of cells with mAbs against both human DAF and human CAR decreased infection by 75% (Martino et al., 2000). We were able to confirm this observation for isolate UK’72 (Table 1); however, we were only able to extend these studies to the other isolate from an early outbreak, It’66, as incubation of the isolates from more recent outbreaks with HeLa cells did not result in CPE or lysis of the cells. Blocking DAF on the HeLa cells with polyclonal anti-human DAF reduced the infectivity for each permissive virus by between 2 and 3 logs. On the other hand, blocking CAR with polyclonal anti-human CAR was far more efficient at inhibiting the infection of HeLa cells with these viruses, routinely decreasing their infectivity by at least 5 logs (Table 1).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Human HeLa cells</th>
<th>Pig IB-RS-2 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+anti-human DAF</td>
<td>+anti-human CAR</td>
</tr>
<tr>
<td>CVB5</td>
<td>1 \times 10^5</td>
<td>&gt; 1 \times 10^5</td>
</tr>
<tr>
<td>It’66</td>
<td>1 \times 10^5</td>
<td>&gt; 1 \times 10^5</td>
</tr>
<tr>
<td>UK’72</td>
<td>1 \times 10^5</td>
<td>1 \times 10^5</td>
</tr>
<tr>
<td>R1072</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>R1120</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SPA’93</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Blocking CVB5 or SVDV infection of pig IB-RS-2 cells with specific antibodies**

In order to assess the receptor usage of the recent SVDV isolates, these studies were performed on pig cells. All SVDV strains and CVB5 were able to productively infect the pig kidney epithelial cell line IB-RS-2 and flow-cytometry analysis using mAbs confirmed that these cells expressed both pig CAR and pig DAF (Fig. 1). We found that pre-incubation of the cells with rabbit polyclonal anti-human CAR antibody, prior to incubation with virus, resulted in a complete block to infection by all viruses (Table 1). We have previously shown that this antibody cross-reacts with pig CAR and is capable of blocking other CVB serotypes from infecting these cells (Spiller et al., 2002). Pre-incubation of these cells with rabbit polyclonal anti-pig DAF antibody did not result in any decrease in the ability of CVB5 or the SVDV strains to infect the pig cells (Table 1).

**Blocking CVB5 or SVDV infection of IB-RS-2 cells with soluble DAF and CAR**

We have previously reported that pre-incubation with 0.3 μM soluble recombinant hDAF-Fc was optimal for blocking CVB3 infection of permissive cells (Yanagawa et al., 2003). Pre-incubation of CVB5 with 0.3 μM soluble recombinant hDAF-Fc inhibited infection with CVB5 by 100-fold at 24 h p.i. (data not shown), but decreased to 10-fold by 72 h p.i. However, SVDV infection of IB-RS-2 cells was not inhibited by hDAF-Fc at any time during the infection (Table 2). Pre-incubation of CVB5 or all SVDV strains with 0.3 μM hCAR-Fc resulted in complete inhibition of infection. We further examined the ability of hCAR-Fc to block some of these viruses at lower concentrations (Table 2). At a concentration of 10 nM, hCAR-Fc was able to inhibit infection of CVB5, It’66 and SPA’93 completely, but only reduced the infectivity of UK’72 by 100-fold. The most recent isolate, SPA’93, was found to be
the most sensitive to inhibition by hCAR-Fc, as it was inhibited completely by 2 nM hCAR-Fc and inhibited by 1000-fold in the presence of 0·4 and 0·08 nM hCAR-Fc, whereas 2 nM hCAR-Fc only inhibited CVB5 and UK’72 weakly.

Virus binding to and infection of IB-RS-2 cells transfected with human DAF

In a final attempt to address the interaction of human DAF with the recent SVDV isolates, we transfected the permissive pig-cell line IB-RS-2 with human DAF to assess the effect on virus binding and infection. Stable transfectants were selected and uniformly expressed approximately 2·5 times more human DAF than those observed on HeLa cells (Fig. 1). Binding of the anti-DAF mAb to IB-RS-2 cells transfected with empty expression vector was identical to that of the isotype control, confirming that this antibody does not recognize any cross-reactive epitopes on these cells (data not shown). Although human DAF expression was found to increase the titre of CVB5 by fivefold on pig cells, this represents a single dilution in the series and, given the qualitative nature of this assay, it is probably not significant. However, human DAF expression was found to increase the relative titre of isolates It’66 and UK’72 by 25-fold in pig cells (Table 3), whereas no increase was observed for isolates R1072 and R1120. Whilst a fivefold increase was noted for SPA’93, for the reasons noted for CVB5 above, this is probably not significant.

We further tested the binding of radiolabelled CVB5 and SVDV isolates to these cells to assess whether the increased infection corresponded to increased virus binding (Table 3). With regard to binding to human DAF-expressing pig cells, 8·6-fold more CVB5 bound, compared with control cells. It’66 also showed a statistically significant ($P<0·05$) binding to these cells (1·28-fold) compared with cells lacking human DAF. The rest of the SVDV isolates did not bind significantly to human DAF-expressing pig cells, compared with control cells. However, UK’72 binding to these cells showed a trend towards significance, especially

![Flow-cytometry analysis of DAF and CAR expression on human HeLa cells (a), pig epithelial-cell line IB-RS-2 stably transfected with human DAF cDNA (b) and CHO cells stably transfected with empty expression vector (control) or cDNA encoding either human or pig DAF (c). Means ± SD for three separate samples are given to the right of the representative histogram. Expression was detected by using specific mAbs for pig DAF (PD3), human DAF (MBC1), human CAR (BRAD30) or pig CAR (BRAD30). No binding of MBC1 was observed to IB-RS-2 cells transfected with empty expression-vector control and expression of human DAF did not alter the expression levels of pig DAF or pig CAR (data not shown).](image)

**Fig. 1.** Flow-cytometry analysis of DAF and CAR expression on human HeLa cells (a), pig epithelial-cell line IB-RS-2 stably transfected with human DAF cDNA (b) and CHO cells stably transfected with empty expression vector (control) or cDNA encoding either human or pig DAF (c). Means ± SD for three separate samples are given to the right of the representative histogram. Expression was detected by using specific mAbs for pig DAF (PD3), human DAF (MBC1), human CAR (BRAD30) or pig CAR (BRAD30). No binding of MBC1 was observed to IB-RS-2 cells transfected with empty expression-vector control and expression of human DAF did not alter the expression levels of pig DAF or pig CAR (data not shown).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>hDAF-Fc (2·5 μM)</th>
<th>hCAR-Fc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2·5 μM</td>
<td>10 nM</td>
</tr>
<tr>
<td>CVB5</td>
<td>↓ 10</td>
<td>&gt; ↓ $10^4$</td>
</tr>
<tr>
<td>It’66</td>
<td>No change</td>
<td>&gt; ↓ $10^2$</td>
</tr>
<tr>
<td>UK’72</td>
<td>No change</td>
<td>&gt; ↓ $10^3$</td>
</tr>
<tr>
<td>R1072</td>
<td>No change</td>
<td>&gt; ↓ $10^4$</td>
</tr>
<tr>
<td>R1120</td>
<td>No change</td>
<td>&gt; ↓ $10^4$</td>
</tr>
<tr>
<td>SPA’93</td>
<td>No change</td>
<td>&gt; ↓ $10^7$</td>
</tr>
</tbody>
</table>

Table 2. Decrease in titre of CVB5 and SVDV isolates on IB-RS-2 following pre-incubation with different concentrations of soluble hDAF-Fc and hCAR-Fc

Log decrease in viral titre is shown. Where fold decrease includes ‘>’, this indicates that the viral infection was blocked completely and the value represents the maximum TCID$_{50}$ of the virus used for those conditions, determined in parallel. Final concentration of soluble hDAF-Fc or hCAR-Fc is given. ND, Not determined.
when you consider that binding of R1072 and SPA’93 to IB-RS-2–human DAF cells appeared to decrease relative to binding to control IB-RS-2 cells (Table 3).

**Virus-binding CHO cells transfected with DAF and CAR**

The same trends in virus binding noted above were observed if radiolabelled virus binding was compared between CHO cells and CHO cells expressing human DAF (Table 4): CHO–human DAF cells bound large amounts of CVB5 and lower amounts of It’66 and UK’72, but did not bind the other SVDV isolates. Although data in Tables 3 and 4 are shown relative to control-cell binding, it is important to note that virus bound significantly better to IB-RS-2 cells than to untransfected CHO cells. CPE was also only observed in CHO cells expressing pig CAR, following incubation with SVDV or CVB5 (data not shown). Furthermore, we also found no increase in binding of radiolabelled CVB5 or any SVDV isolate to CHO cells expressing the pig homologue of DAF, compared with control CHO cells (Table 4), even though these cells expressed 135-fold more pig DAF than is found physiologically on pig cells (Table 1).

**DISCUSSION**

Here, we describe differences in receptor usage between isolates of SVDV from early and recent outbreaks of disease. A single report has previously examined the role of human DAF and human CAR in infection of human HeLa cells by UK’72, an isolate from an early outbreak (Martino et al., 2000). We found that incubation of SVDV isolates from early outbreaks and CVB5 with HeLa cells resulted in CPE and cell lysis; however, no CPE was observed following incubation of HeLa cells with SVDV isolates from more recent outbreaks. Whilst we found that these latter isolates utilized CAR as a receptor (Table 1) and that CAR was expressed on HeLa cells (Fig. 1), more detailed investigations of entry, replication and virus production would be required to examine the mechanism behind this phenomenon. We were able to confirm that HeLa cell infection by isolate UK’72 was dependent on both human DAF and human CAR; we also found similar inhibition of HeLa cell infection by anti-DAF and anti-CAR antibodies of the SVDV isolate from the earliest outbreak, It’66 (Table 1).

Given that pigs are the natural host for SVDV, we then expanded our investigations of SVDV infection to the permissive pig-cell line IB-RS-2. The ability of CAR to mediate infection of all SVDV isolates was confirmed through complete inhibition of viral infection by pre-incubation of pig cells with antiserum recognizing pig CAR, or pre-incubation of SVDV isolates with recombinant

### Table 3. Effect of human DAF expression on radiolabelled virus binding to and virus titration in pig cells

For relative virus-titration studies, cells were inoculated with $10^6$ TCID$_{50}$ virus and infection was allowed to proceed for 72 h. Mean and coefficient of variation for three repeats are shown, with statistical significance indicated in parentheses. NS, Not significant.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>IB-RS-2 control</th>
<th>IB-RS-2+human DAF</th>
<th>Relative increase in virus titration</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVB5</td>
<td>100±24</td>
<td>858±97 ($P&lt;0.001$)</td>
<td>↑ fivefold</td>
</tr>
<tr>
<td>It’66</td>
<td>100±12</td>
<td>128±7-7 ($P&lt;0.005$)</td>
<td>↑ 25-fold</td>
</tr>
<tr>
<td>UK’72</td>
<td>100±19</td>
<td>121±9-8 (NS)</td>
<td>↑ 25-fold</td>
</tr>
<tr>
<td>R1072</td>
<td>100±20</td>
<td>88±6-4 (NS)</td>
<td>No change</td>
</tr>
<tr>
<td>R1120</td>
<td>100±16</td>
<td>99±17 (NS)</td>
<td>No change</td>
</tr>
<tr>
<td>SPA’93</td>
<td>100±30</td>
<td>92±13 (NS)</td>
<td>↑ fivefold</td>
</tr>
</tbody>
</table>

### Table 4. Radiolabelled virus binding to transfected CHO cells as a percentage of control

Means ± SD for three samples are given. NT, Not tested.

<table>
<thead>
<tr>
<th>Virus</th>
<th>CHO control</th>
<th>CHO pig DAF</th>
<th>CHO human DAF</th>
<th>CHO pig CAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVB5</td>
<td>100±12-6</td>
<td>115±22-4</td>
<td>4267±75 ($P&lt;0.01$)</td>
<td>824±25 ($P&lt;0.01$)</td>
</tr>
<tr>
<td>It’66</td>
<td>100±3-1</td>
<td>105±10-9</td>
<td>131±9-3 ($P&lt;0.05$)</td>
<td>238±11-9 ($P&lt;0.01$)</td>
</tr>
<tr>
<td>UK’72</td>
<td>100±11-2</td>
<td>118±17-0</td>
<td>127±8-8 ($P&lt;0.05$)</td>
<td>457±48-3 ($P&lt;0.01$)</td>
</tr>
<tr>
<td>R1072</td>
<td>100±2-8</td>
<td>99±8-4</td>
<td>90±10</td>
<td>NT</td>
</tr>
<tr>
<td>R1120</td>
<td>100±4-3</td>
<td>107±14-9</td>
<td>80±9-9</td>
<td>NT</td>
</tr>
<tr>
<td>SPA’93</td>
<td>100±7-6</td>
<td>101±13-5</td>
<td>75±5-7</td>
<td>797±12 ($P&lt;0.01$)</td>
</tr>
</tbody>
</table>
human CAR (Table 1 and 2). We found the SVDV isolate UK’72 to be the least sensitive to inhibition by recombinant human CAR, whereas our isolate from a much more recent outbreak, SPA’93, was the most sensitive (Table 2). Even though IB-RS-2 cells expressed pig DAF (Fig. 1), pre-incubation with rabbit polyclonal anti-pig DAF did not inhibit infection (Table 1). Furthermore, no binding of any radiolabelled SVDV isolate to CHO cells expressing pig DAF (expressed 135-fold times greater than levels on IB-RS-2 cells) was observed above binding to CHO control cells. However, it is unlikely that human DAF-utilizing viruses would evolve to use the pig DAF homologue, due to large structural differences between these proteins. Pig DAF is comprised of only three complement-control (CCP) domains, whereas human, rat and mouse DAF contain four CCP domains (Pérez de la Lastra et al., 2000). Sequence comparison amongst these proteins indicates that it is the homologue of the fourth DAF CCP domain that is missing and, as a result, pig DAF regulates human complement activation poorly and does not regulate pig complement at all (Pérez de la Lastra et al., 2000).

We still wished to assess whether the more recent SVDV isolates retained the ability to bind and utilize human DAF as a receptor. Whilst we found that pre-incubation of recombinant human DAF with CVB3 resulted in a reduced infection of pig cells, recombinant human DAF was unable to inhibit infection of any of the SVDV isolates. Although this inhibition cannot be due to competition between soluble and surface receptors, it is probably caused by steric hindrance, given the close proximity between DAF- and CAR-binding sites on the capsid, similar to our previous report showing that hDAF-Fc inhibited CVB3 infection of mice (Yanagawa et al., 2003). Transfection of the pig cells with human DAF was found to increase the titre of early SVDV isolates, but no significant effect was noted for infection of these cells by the isolates from more recent outbreaks (Table 3). Similar results were observed for the binding of radiolabelled CVB5 and SVDV isolates to IB-RS-2 cells expressing human DAF relative to binding to control cells: a big increase in binding for CVB5, a small increase in binding for SVDV isolates from early outbreaks and no increase in binding for SVDV isolates from more recent outbreaks. We conclude, therefore, that as SVDV has evolved in its new host, the capsid structures responsible for human DAF binding have been lost, due to the absence of this co-receptor or any close pig homologues.

Many recent studies have focused on identifying these capsid structures and it is possible that comparison of the capsid sequences between CVB5, SVDV and these other viruses may help to identify essential and non-essential residues in these areas (Fig. 2). These comparisons are greatly enhanced by the recent X-ray crystallography

![Fig. 2. Similarity between sequences of SVDV isolates, CVB5, CVB3 and echovirus 7 surrounding the receptor-contact points. Included are sequences from the VP2 ‘puff’ region (2129–2178), the VP3 ‘knob’ region (3058–3068) and the VP1 ‘canyon’ region (1091–1093, 1146–1154, 1199–1219 and 1258–1259). Single-underlined amino acids represent regions of contact between echovirus 7 and soluble DAF as determined by He et al. (2002). Double-underlined amino acids represent regions of contact between CVB3 and soluble CAR as determined by He et al. (2001). Regions corresponding to identical amino acid sequence comparing SVDV with CVB5 are represented by a dash, whilst regions of identity between CVB5 or SVDV and residues found to be involved in receptor contact for CVB3 and echovirus 7 are shown in bold. DAF-binding residues in VP2 as identified by Stuart et al. (2002) are also shown (single-underlined) for completeness.](image-url)
solution of the UK’72 isolate by Fry et al. (2003) and of the SPA’93 isolate by Verdaguer et al. (2003). Whilst the structures of SVDV complexed with CAR or DAF have not been solved directly, conclusions can be drawn by comparing the cryo-electron microscopy analysis of CVB3–CAR and echovirus 7–DAF complexes reported by He et al. (2001, 2002). The capsid sequences surrounding the larger parts of these footprints have been aligned for CVB3, echovirus 7, CVB5, early SVDV isolates and recent SVDV isolates in Fig. 2, which shows significant similarity between all of the viruses. Several changes to capsid sequence are shown within and surrounding these footprints by comparing CVB5 with SVDV and comparing SVDV isolates from early and more recent outbreaks; however, a more detailed comparison of sequences for the entire capsid-coding region, without emphasis on the receptor-binding sites, can be found in the paper by Verdaguer et al. (2003). The CVB3 capsid sequences identified as binding to CAR were found primarily in the VP1 capsid protein, with some contribution of the hypervariable ‘puff’ region in VP2. The echovirus 7 sequences identified as binding to DAF have also been mapped to the VP2 ‘puff’ region (region A), although two other, smaller, DAF-binding regions (B and C) have been found in VP3, which include the hypervariable VP3 ‘knob’ region. Although the initial sequence comparison found that recent SVDV isolates have more DAF-binding contact residues conserved with CVB5 than the early SVDV isolates, there are many changes in the intervening capsid sequence that could result in the loss of DAF binding. Alignment of the echovirus 7 DAF-binding sequence IKV in the VP3 ‘knob’ region was not exact; however, a GKV sequence is located at almost the same position in early SVDV isolates and this sequence is altered in recent SVDV isolates to GKE, which could also be responsible for the loss of DAF binding. Four sequence alterations that were conserved between all SVDV isolates were not found in CVB5: T2151I, S2161T and I2177V in the VP2 ‘puff’ region, and Q1091Y in the CAR-binding region of VP1. There were also four sequence alterations that were conserved between all SVDV isolates that had lost DAF binding; they also identified a contribution of the VP2 ‘puff’ region, which has been included in Fig. 2. However, for the most part, the DAF-binding regions of both echovirus 7 and 11 represent the areas of greatest sequence divergence when compared with SVDV and CVB, reinforcing the hypothesis that DAF probably binds to a different area of the CVB and early SVDV isolate capsids compared with echoviruses. Whether the loss of DAF binding or possible increase in CAR binding is due to any one, or a combination of, the above identified capsid sequence alterations will require extensive studies utilizing site-directed mutagenesis of infectious cDNA constructs.

Finally, Fry et al. (2003) speculated that the changes between early and recent SVDV capsid sequence could be responsible for adapting the virus to using pig CAR and pig DAF. We show here that SVDV has progressively lost the ability to bind human DAF and has not gained the ability to bind pig DAF. This raises the question of whether SVDV has acquired the use of another co-receptor. We have recently reported that heparan sulphate mediates the attachment of the SVDV isolate SPA’93 to host cells (Escribano-Romero et al., 2004). It is interesting to note that the location of the heparan sulphate-binding site on the capsid (Escribano-Romero et al., 2004; Verdaguer et al., 2003) is in close proximity to the SVDV capsid region proposed by Fry et al. (2003) to bind human DAF. Therefore, it is possible that the capsid alterations responsible for the loss of DAF binding may correspond to capsid changes in the more recent SVDV isolates required for increased heparan sulphate binding.

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