

Coxsackie B viruses that use human DAF as a receptor infect pig cells via pig CAR and do not use pig DAF

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Coxsackie B viruses (CVB) are enteroviruses belonging to the family *Picornaviridae*. Serotypes 1, 3 and 5 of CVB bind to the human membrane complement regulator decay-accelerating factor (DAF) and the coxsackievirus/adenovirus receptor (CAR), using either or both as receptors. These viruses are known to infect pig cell lines, but the receptor(s) involved has not been identified. We have recently characterized the pig homologue of DAF and here explore the interactions of human DAF-binding CVB with pig homologues of DAF and CAR. CVB infection of three pig cell lines resulted in cytolysis, which could not be blocked by anti-pig DAF antibodies. CVB bound to CHO cells transfected with human DAF, but not pig DAF. Modification of pig DAF by incorporation of the fourth short consensus repeat of human DAF did not confer CVB-binding capacity. CVB did bind CHO cells expressing pig or human CAR, and pre-incubation of pig cells with anti-CAR antibody blocked CVB infection.

Introduction

Viruses initiate infection by attaching to cell surface receptor molecules, and expression of specific receptors is an important determinant of virus host range and tissue tropism. Coxsackie B viruses (CVB) belong to the genus *Enterovirus* within the family *Picornaviridae* and have been associated with a myriad of pathological sequelae, including aseptic meningitis, non-specific febrile illness and myocarditis (Morens & Pallansch, 1995). Prototype strains of CVB serotypes 1–6 have been found to compete with each other for binding to HeLa cells, but did not interfere with cell binding by other enterovirus types, suggesting that CVB1–6 share a common receptor (Lonberg-Holm *et al.*, 1976). Subsequent investigations identified a single mAb, RmcB, that recognized the putative CVB receptor and blocked virus binding and infection by CVB reference strains (Hsu *et al.*, 1988). The putative CVB receptor has now been cloned and characterized and it has also been found to act as an attachment molecule for adenovirus fibre proteins (Bergelson *et al.*, 1997b; Carson *et al.*, 1997; Roelvink *et al.*, 1998; Tomko *et al.*, 1997). In addition, CVB

serotypes 1, 3 and 5 have been found to bind to the complement regulator decay-accelerating factor (DAF; CD55) on the surface of permissive cells (Bergelson *et al.*, 1994).

The ability of CVB serotypes 1, 3 and 5 to infect pig kidney cells *in vitro* has already been established (Knowles *et al.*, 1979) and a recent report indicates that CVB5 is related more closely to the pathogenic swine vesicular disease virus (SVDV) than to the other CVB serotypes (Zhang *et al.*, 1999). The homology of SVDV to CVB5 suggests strongly that the former is a pig-adapted version of the latter that crossed the species barrier some time between 1945 and 1965 (Zhang *et al.*, 1999). SVDV retained the ability to infect HeLa cells and infection was blocked by anti-human DAF antibodies (Martino *et al.*, 2000). These data suggested a role for DAF in the infection of pig cells with SVDV and raised the possibility that the closely related CBV may bind pig DAF.

The ability of viruses to cross species between humans and pigs is of increasing concern due to interest in using pigs as a source of transplant tissue (Weiss, 1999; Weiss *et al.*, 1999). Although these concerns have focused predominantly on the transmission of pig retroviruses to humans, the possibility of human virus infection and destruction of transplanted pig organs should not be ignored. It is therefore important to establish the capacity of human viruses to bind and infect pig

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cells and to identify the receptors involved in cell entry. These receptors may be useful for therapeutic intervention or gene manipulation in the future.

Here, we investigate the ability of CVB serotypes 1, 3 and 5 to bind pig DAF and CAR analogues. Initially, the capacity of CVB to infect three pig cell lines was confirmed. We then assessed the ability of anti-pig DAF and CAR antibodies to block CVB infection. CHO cells, which do not bind CVB without the addition of CVB receptors, were used as target cells following transfection with human and pig DAF or CAR. Since pig DAF has only three short consensus repeats (SCRs), compared with four SCRs in human DAF, we also tested the binding of CVB to pig DAF following the addition of a fourth SCR from human DAF.

Methods

■ **Virus strains.** Defined CVB of different serotypes were the kind gift of Brian Megson (Public Health Laboratory Service, Colindale, London, UK). The viruses were propagated in rhabdomyosarcoma (RD) cells [obtained from the European Collection of Animal Cell Cultures (ECACC), Porton Down, UK]. RD cells were grown in Dulbecco's modified essential medium (DMEM) containing glutamine and penicillin/streptomycin and supplemented with 10% FCS (all from Gibco BRL). Virus infections and propagation were performed in the absence of FCS.

■ **Antibodies.** A mouse mAb recognizing DAF, MBC1, and the anti-pig DAF mAb PD3 were generated in-house. The hybridoma cell line, OX23, secreting mouse anti-human factor H mAb was obtained from the ECACC and was used as the isotype-matched control for the mouse mAbs. Rabbit polyclonal antisera were raised against soluble recombinant pig DAF and human CAR (described below) in-house. Rabbit polyclonal antiserum raised against soluble human herpesvirus 8 (HHV-8) ORF4, also raised in-house, provided a control antibody for the rabbit polyclonal antiserum. Phycoerythrin (PE)-conjugated goat anti-mouse Ig secondary antibody was purchased from DAKO and PE-conjugated goat anti-rabbit Ig secondary antibody was purchased from Sigma. Horseradish peroxidase-conjugated goat anti-rabbit Ig secondary antibody was purchased from Bio-Rad.

■ **Sequence data.** All amino acid sequences used for alignments and primer design were identical to those available at the NIH National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>). Accession numbers for each protein are as follows: AF228059 (pig DAF), M15799 (human DAF), AF109646 (pig CAR), Y11929 (mouse CAR) and BC003684 (human CAR). The boundaries for each SCR in pig DAF were defined by sequence similarity to human DAF.

■ **Cell lines.** A pig kidney cell line, ESK-4, and human cell lines HeLa and RD were obtained from the ECACC and the pig cell lines IB-RS-2 (kidney) and ST (testis) were obtained from the ATCC. All cells were propagated in DMEM containing 10% FCS (Gibco BRL). CHO cells obtained from ECACC were transfected with the empty eukaryotic transfection vector pDR2EF1 α or with the vector containing cDNA encoding pig or human DAF or CAR using lipofectamine (Gibco BRL) according to the manufacturer's instructions. Stable clones were generated by selection and propagation in the presence of 100 μ g/ml hygromycin B. Cell surface expression for CHO-transfected cells was measured by flow cytometry by using a Becton-Dickinson FACScalibur.

■ **Enterovirus capsid detection assay.** Pig and human cell lines were seeded into 24-well plates (Gibco BRL) at a density of 5×10^5 cells per well and allowed to grow overnight. The following morning, the cell layers were washed twice with serum-free DMEM and infected with virus stocks diluted to m.o.i. of 50, 10, 1 or 0.1. Cells were incubated with virus for 30 min at room temperature and then for 30 min at 37 °C, unbound virus was removed by two washes of serum-free DMEM, cells were overlaid with 1 ml DMEM per well and the infection was allowed to proceed for 24 h at 37 °C in a CO₂ incubator. All infections were performed in triplicate and experiments were repeated at least once. At 24 h post-infection, the cell layers were washed twice with PBS and then incubated at -70 °C for 15 min with an acetone-methanol (1:1) fixative. Fixed cell sheets were then washed three times with PBS and three times with PBS with 1% BSA and virus capsid protein was then detected by using an anti-enterovirus mAb (DAKO) at room temperature for 1 h with constant rocking. After three washes with PBS, bound anti-virus antibody was detected with β -galactosidase-conjugated goat anti-mouse Ig (Harlan Sera-lab), following the manufacturer's instructions. Cells containing enterovirus capsids were observed as blue cells under the microscope and the number of blue cells compared with unstained cells in 10 microscope fields was counted for each well.

■ **Virus binding studies.** CVB serotypes were metabolically labelled by propagation in RD cells maintained overnight in cysteine-/methionine-free medium containing 0.5 MBq ³⁵S-labelled cysteine/methionine (Amersham Pharmacia). Cell debris was removed by low-speed centrifugation (5 min at 1300 *g*) and the virus was separated from unincorporated radiolabel by centrifugation through a 30% sucrose cushion in 1 M NaCl, 20 mM Tris-acetate (pH 7.5) with 0.1% BSA (125 000 *g* in an SW28 rotor, overnight at 4 °C). Pelleted virus was resuspended in serum-free medium, particulate material was removed by centrifugation (1300 *g*, 10 min) and the concentration of virus was determined by TCID₅₀ on RD cells. The virus was stored in aliquots at -20 °C until used.

Fifty μ l of each radiolabelled virus (diluted 10-fold from stocks with DMEM) was counted to determine the input radioactivity for CVB1 (54 000 c.p.m./50 μ l), CVB3 (39 000 c.p.m./50 μ l) and CVB5 (64 000 c.p.m./50 μ l). TCID₅₀ values for all viruses used were approximately 10⁸ per ml. The binding of each virus was tested for each CHO-transfected cell line by pelleting 10⁷ EDTA-disaggregated cells (1000 *g*, 5 min) in ice-cold, FCS-free cell medium and then resuspending the cell pellet in 50 μ l of radiolabelled virus. After a 2 h incubation on ice, unbound virus was removed by three 1 ml washes in ice-cold, serum-free cell medium and bound virus was quantified by scintillation counting. Each condition was performed in triplicate and repeated at least twice to confirm the results.

■ **Blocking of CVB lysis of pig cells.** Rabbit IgG was purified from antiserum raised against human CAR, pig DAF or HHV-8 ORF4 by using a Protein A-Sepharose column (Amersham Pharmacia), following the manufacturer's instructions, and dialysed into PBS. Pig or HeLa cells (10 000 per well) were subcultured into 96-well tissue culture plates (Gibco BRL) and cultured overnight at 37 °C. Antibodies were added to DMEM at a final concentration of 10 μ g/ml, sterile-filtered and then used to replace the medium on the cells to be blocked 30 min prior to addition of virus. Cells incubated with medium without added antibody served as the mock control and cells incubated with anti-HHV-8 ORF4 served as an irrelevant antibody control. Fivefold dilutions of each CVB were then overlaid onto the cells and incubated at 37 °C for 3 days. The remaining viable cells were visualized by staining with 0.1% crystal violet, 0.1% formaldehyde in PBS followed by extensive washing in running water. Successful blocking of infection was observed as a viable blue cell layer at 3 days post-infection.

■ **PCR amplification of pig and human CAR cDNA.** RNA extracted from HeLa, IB-RS-2, ESK-4 and ST cell lines was subjected to reverse transcription followed by PCR according to the supplier's instructions. Total cellular RNA was extracted using UltraSpec (Biotecx Laboratories) according to the manufacturer's instructions.

Primers used were as follows (5'–3'): HCAR F1 (CACTAGTATGCGCTCCTGCTGT), CAR B802 (TCCATTCTTCTCTGCGCTTTTACGAC), CAR F88 (GAAAAAGCCAAAGGGGA), CAR B1098 (CTATACTATAGACCC) and OVERLAP R (CCCTTTGGCTTTTCAATCATCTCTTCA).

CAR primers F88, B802 and B1098 were all designed based on areas of high similarity between human and murine CAR sequences. Primer CAR B802 includes a stop codon at the 3' end for truncated expression of the cDNA product.

A PCR product encoding full-length human CAR cDNA was obtained from reverse-transcribed HeLa mRNA by using primers HCAR F1 and CAR B1098. This was used for expression of human CAR. No product was observed when reverse-transcribed mRNA from pig cell lines was used with these primers. Only primers CAR F88 and CAR B802 gave a PCR product from pig mRNA; the product encoded the pig homologue of human CAR, but was missing the signal sequence and ended 15 nt after the transmembrane region. Since the sequence for pig CAR was not available at the time, a recombinant pig CAR was constructed that had a human CAR signal sequence and was truncated 15 nt after the transmembrane region by the insertion of an in-frame stop codon by overlapping PCR mutagenesis. All products were ligated into pCR 2.1-TOPO by using the TOPO TA cloning system (Invitrogen) and then subcloned into the eukaryotic expression vector pDR2ΔEF1α (Harris *et al.*, 2000). Recombinant soluble human CAR coupled to human IgG1 Fc was constructed by PCR with the primers HCAR F1 and HCAR B618 (5' GGCGGCCGCTTTATTTGAAGGAGGG 3'), which truncates the predicted protein just above the putative transmembrane region and adds a *NotI* restriction site at the 3' end of the cDNA. These primers allow the insertion of the PCR product into the expression vector Signal pIgplus (R&D Systems), which adds (when in-frame and in the absence of premature stop codons) cDNA encoding the hinge and Fc regions of human IgG1 to the 3' end of the cDNA. Fidelity and orientation of all cDNA and PCR products were confirmed by sequencing.

■ **Human and pig DAF expression constructs.** Eukaryotic expression vectors based on pDR2ΔEF1α and containing human DAF and pig DAF were already available in-house (Harris *et al.*, 2000; Perez de la Lastra *et al.*, 2000). Pig DAF contains three only SCRs compared to four SCRs for human DAF; a fusion construct was therefore created to insert the fourth SCR of human DAF between the pig DAF SCR3 and the Ser-/Thr-rich region and transmembrane anchor by overlapping PCR mutagenesis. Primers used for the fusion were (5'–3'): PDAF S1 (GGTCTAGAGCGGTGAGGCGCCTAATGG), PDAF END (GGTGATCCAGTGTTACACATGCACGTCC), HP1 (CCAGAATGCCAAGAAATTTATTGTCCAGCACC), HP2 (ACAATAAATTTCTGGCATTCTGGCAATGG), HP3 (TGGAGAAATTTCTCTGCATTCAAGGTGGTGG) and HP4 (CCTGAATGCAGAGAAATTTCTCCAAGTCT). Primer PDAF S1 adds an *XbaI* restriction site to the 5' end of the product and primer PDAF END adds a *BamHI* restriction site to the 3' end. PCR was performed with pig DAF cDNA as a template with primers PDAF S1 and HP2 to generate the 5' end of pig DAF and HP4 and PDAF END to generate the 3' end of pig DAF. Human DAF SCR4 was amplified with primers HP1 and HP3. The 5'-end PCR product was annealed to the human DAF SCR PCR products and extended–amplified with primers S1 and HP3. This product was subsequently annealed with the 3'-end pig DAF PCR product and amplified with the primers S1 and PDAF END. The resultant product was cloned into the expression

plasmid pDR2ΔEF1α. Fidelity and orientation of all PCR products and the final recombinant cDNA were confirmed by sequencing.

Results

Infection of pig and human cell lines by CVB

The pig cell lines IB-RS-2 and ESK-4, both derived from pig kidney, and the ST cell line derived from pig testis were incubated with CVB serotype 1, 3 or 5 (m.o.i. = 50) and the virus infection was allowed to continue for 24 h. All of the viruses used had been propagated in RD cells and 24 h infection of this cell type resulted in complete destruction of the cell layer (Table 1). Infection of the ST and ESK-4 cell lines with CVB1, 3 and 5 also resulted in complete destruction of the cell layer, and 100% CPE, as characterized by 100% staining for CBV capsid proteins and a non-viable cell layer, was seen in the IB-RS-2 pig kidney cell line. When the infection of IB-RS-2 cells was allowed to proceed for an additional 24 h, complete cell lysis of these cells was also observed.

CVB does not bind pig DAF or pig–human DAF hybrids

Both of the pig kidney cell lines (IB-RS-2 and ESK-4) and the testis cell line (ST) expressed pig DAF on their cell surface, as observed by flow cytometry (Fig. 1). The presence of pig DAF on the cell surface confirmed the possibility that CVB might be able to utilize it as a receptor. To test the ability of CVB to bind pig DAF in the absence of other pig cell-surface proteins, ³⁵S-labelled CVB1, 3 and 5 were incubated with CHO cells transfected to express pig DAF and compared with control CHO cells transfected with empty vector (Table 2). Since the transfected CHO cells expressed 50-fold more pig DAF compared with the endogenous levels observed on the pig cell lines (Fig. 1), it should be possible to detect even low-affinity interactions between the viruses and pig DAF. No

Table 1. Enterovirus capsid detection assay for CVB infection of pig cell lines

Results shown are for virus added at an m.o.i. of 50 and allowed to incubate with cells for 24 h. Results are scored as: lysis, virus infection resulted in complete cytolysis of the cell layer; CPE, 100% of the cells were positive for virus capsid antigen, the cells were rounded and cell death was imminent. No obvious CPE was observed in non-permissive CHO cells. Each condition was replicated in triplicate.

Virus	RD cells	Pig testis (ST)	Pig kidney	
			IB-RS-2	ESK-4
CVB1	Lysis	Lysis	CPE	Lysis
CVB3	Lysis	Lysis	CPE	Lysis
CVB5	Lysis	Lysis	CPE	Lysis

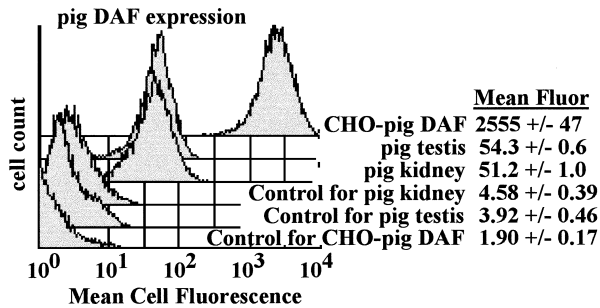


Fig. 1. Representative flow cytometry histograms for the relative levels of pig DAF expression on CHO cells transfected with pig DAF and on pig testis (ST) and kidney (ESK-4) cell lines. All determinations were carried out in triplicate and the mean cell fluorescence and standard deviation for the triplicates are shown on the right for all cell types using the mAb PD3. Background fluorescence (control) from an isotype-matched control antibody raised against human factor H is also shown.

binding of CVB to pig DAF was noted, while large amounts of virus bound to the CHO cells transfected with human DAF (Table 2). Since pig DAF only contains analogues of the first three SCRs, compared to the four SCRs of human DAF, it was possible that the absence of SCR4 was responsible for the absence of CVB binding. To investigate this, we created a recombinant DAF with human DAF SCR4 inserted between pig DAF SCR3 and the Ser-/Thr-rich region (see Methods; Fig. 2A). This recombinant cDNA was also transfected into CHO cells and flow cytometry with monoclonal anti-pig DAF and polyclonal anti-human DAF antibodies (Fig. 2B) confirmed high levels of expression. However, altering the structure of pig DAF to match that of human DAF more closely failed to confer CVB binding to pig DAF (Table 2).

We also measured the binding of the radiolabelled viruses to RD cells, pig kidney cells (IB-RS-2) and pig testis cells (ST) (Table 2). The binding of radiolabelled virus to RD cells was included as a control because they express low levels of DAF and negligible levels of CAR (Shafren *et al.*, 1997), but are still fully permissive to infection by the CVB serotypes we used.

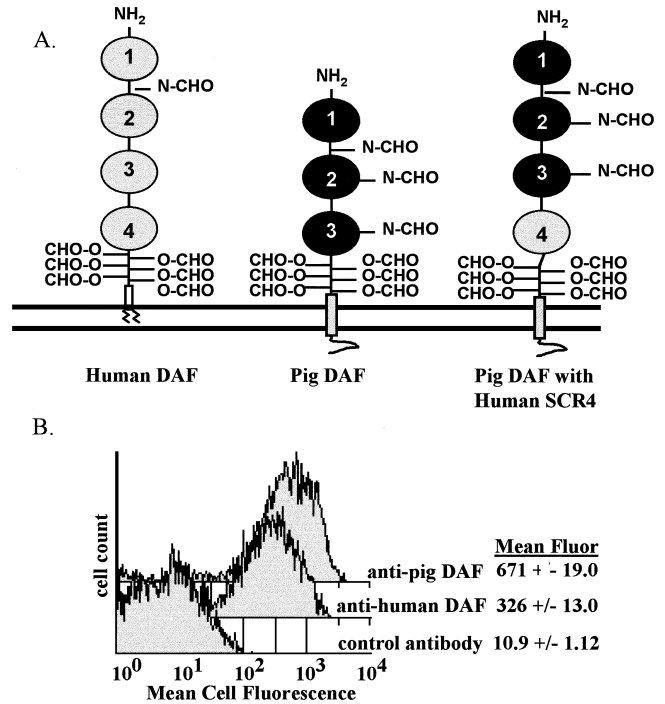


Fig. 2. (A) Simplified schematic representation of human and pig DAF and the hybrid construct. The SCR domains are numbered. Pig DAF lacks a homologue of human DAF SCR4 and has two additional N-glycosylation sites (N-CHO) compared with human DAF. Insertion of human SCR4 was accomplished through PCR with primers designed to have overlapping sequences, as detailed in Methods. (B) Flow cytometry analysis showing high levels of binding of anti-pig DAF mAb and polyclonal anti-human DAF antibody when incubated with CHO cells transfected with recombinant pig-human DAF cDNA.

RD cells and the pig cell lines were all permissive to infection by CVB1, 3 and 5 and all of these cells bound significant amounts of all three CVB serotypes, although the binding of virus to pig cell lines was consistently lower than binding to RD cells, showing comparatively reduced binding capacity for the human viruses (Table 2).

Table 2. Binding of radiolabelled CVB to transfected CHO cells

All values are given as bound c.p.m. ± standard deviation and represent triplicate measurements. Essentially identical results were obtained in two separate experiments. Control cells were CHO cells transfected with empty vector.

Virus	Control	CHO-human DAF	CHO-pig DAF	CHO-pig-human DAF*	Pig testis cells	Pig kidney cells	RD cells
CVB1	537 ± 38	17004 ± 128 [†]	445 ± 22	489 ± 31	1288 ± 41 ^a	1051 ± 22 ^b	2437 ± 215 ^a
CVB3	202 ± 23	18530 ± 538 ^c	264 ± 17	235 ± 28	1362 ± 30 ^b	885 ± 27 ^b	2850 ± 257 ^a
CVB5	127 ± 12	33353 ± 385 ^c	113 ± 11	136 ± 19	586 ± 40 ^b	311 ± 12	437 ± 25 ^b

* CHO cells transfected with recombinant pig DAF with human DAF SCR4 inserted between pig DAF SCR3 and the Ser-/Thr-rich region (see Methods).

† Binding was compared with the level of virus binding to control CHO cells and significant differences are indicated by *a* ($P < 0.05$), *b* ($P < 0.01$) or *c* ($P < 0.001$).

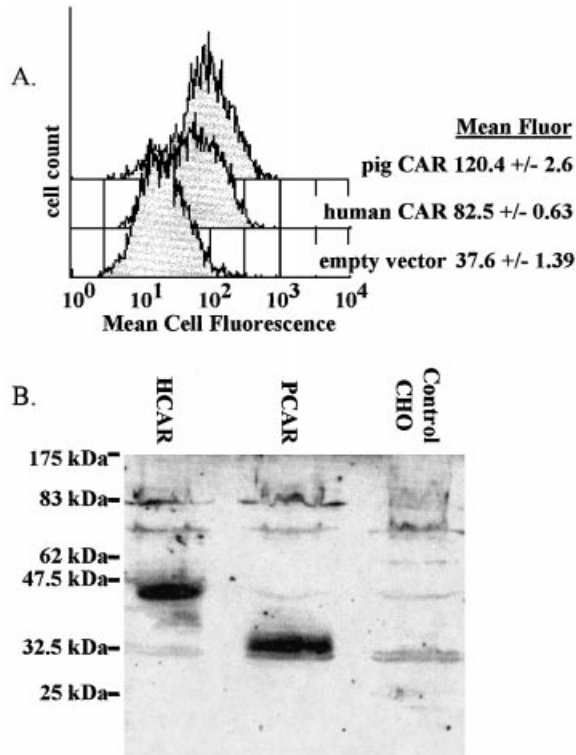


Fig. 3. Flow cytometry (A) and Western blot analysis (B) showing expression of human CAR (HCAR) and pig CAR (PCAR) in transfected CHO cells, as detected by rabbit polyclonal anti-human CAR antibody. The apparent molecular mass of pig CAR is approximately 11 kDa less than that of human CAR, due to truncation of the cytoplasmic tail.

CAR is expressed by pig cell lines and HeLa cells

Total RNA was isolated from the IB-RS-2, ESK-4 and ST cell lines and from human HeLa cells. RT-PCR was performed on all samples with several different primer pairs (see Methods). Full-length human CAR (nt 1–1098) was isolated from HeLa cells and sequenced. The only difference from the published CAR sequence was a G → A change at nt 120, which did not alter the amino acid sequence. This human CAR cDNA was put into two expression vectors, one that expressed the full-length CAR on the surface of transfected CHO cells and a second that produced a truncated soluble human CAR that was used to immunize rabbits (see Methods). The largest PCR product obtained for pig CAR using the human and murine CAR sequence-based primers yielded pig CAR sequence from nt 88 to 802 only for RNA isolated from IB-RS-2, ESK-4 and ST cell lines. The sequence for pig CAR was identical among all cell lines.

Attempts to complete the 5' and 3' cDNA sequence of pig CAR were unsuccessful, so the human cDNA sequence encoding the first 29 aa of CAR, the signal sequence, was engineered onto the pig CAR to allow protein expression. Since this is cleaved co-translationally, it would not affect the mature protein product. Similarly, the missing 3' sequence represented the cytoplasmic tail; a stop codon was therefore

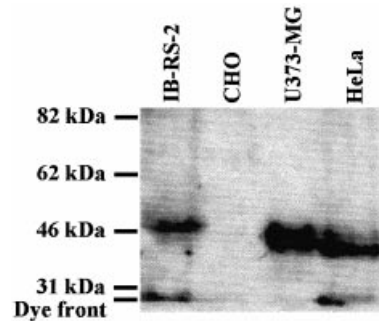


Fig. 4. Western blot analysis of whole-cell lysates from human HeLa and U373-MG cell lines compared with untransfected CHO and pig kidney (IB-RS-2) cell lines. A single band of approximately 46–48 kDa was observed for all but the CHO cells.

inserted at aa 267, which truncates pig CAR but should not affect the ability of CAR to act as a receptor (Wang & Bergelson, 1999). This modified pig CAR cDNA was inserted into the eukaryotic expression vector pDR2ΔEF1α and transfected into CHO cells for further investigation. Expression of human and pig CAR on the transfected CHO cells was tested by flow cytometry with a rabbit polyclonal antibody raised against recombinant soluble human CAR (Fig. 3A). Both pig and human CAR were detected by the anti-human CAR polyclonal antibody and showed uniform expression on the transfected cells. Protein expression was confirmed by Western blot analysis (Fig. 3B). The pig CAR was found to be 11 kDa smaller than full-length human CAR, as expected due to the truncation of 98 residues of the cytoplasmic tail by the insertion of a stop codon at residue 267. Western blot analysis comparing total cell lysates from human HeLa and U373-MG cell lines, both known to express high levels of CAR (Miller *et al.*, 1998; Bergelson *et al.*, 1997a), and the pig kidney IB-RS-2 cell line revealed a single band of approximately 46–48 kDa for all three cell lines (Fig. 4).

CVB infection of pig cells is blocked by anti-human CAR antibodies

Since the rabbit polyclonal anti-human CAR antibody was found to cross-react with native pig CAR, we pre-incubated IB-RS-2 (Fig. 5) and HeLa (data not shown) cells with this antibody prior to addition of a 5-fold dilution series of 10⁸ TCID₅₀ of CVB1, 3 or 5. IB-RS-2 cells were 5- to 25-fold less sensitive to CVB1 than to CVB3 and 5 when equal amounts of virus (based on infectivity for RD cells) were added to cells in the absence of antibody. Infection of IB-RS-2 cells with all three CVB serotypes was blocked completely by the anti-human CAR (Fig. 5). Negligible blocking of CVB infection was observed when IB-RS-2 cells were pre-incubated with rabbit polyclonal anti-pig DAF antibody (Fig. 5). The anti-human CAR antibody was also found to reduce the infection of HeLa cells by all three serotypes by over 3000-fold (data not shown),

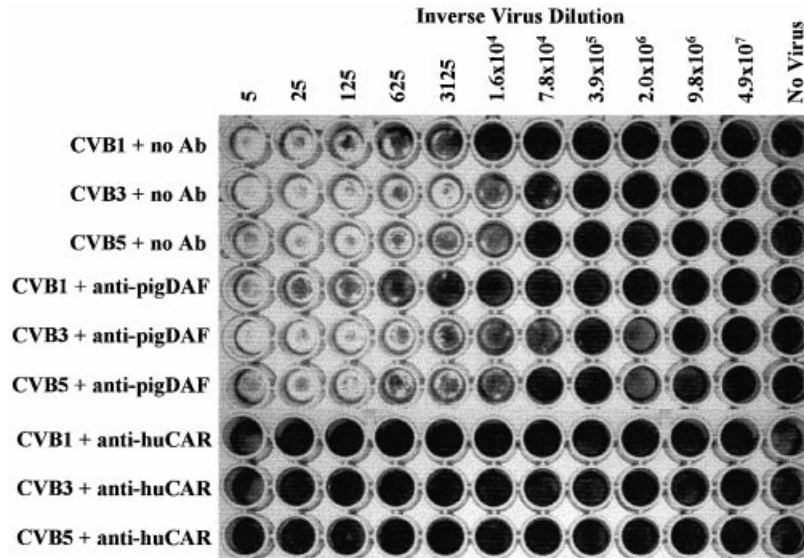


Fig. 5. Blocking of lytic infection of IB-RS-2 pig cells by CVB serotypes. Cells were pre-incubated with rabbit polyclonal Ig purified from antiserum raised against soluble human CAR (anti-huCAR) or pig DAF (anti-pigDAF) and the results were compared with the lysis observed with no addition of antibody (no Ab). Fivefold dilutions of 10^8 TCID₅₀ (as determined on RD cells) of CVB1, 3 and 5 were incubated with IB-RS-2 cells for 3 days and viable cells were visualized by staining with 0.1% crystal violet. No blocking of CVB infection was observed when cells were pre-incubated with anti-pig DAF antibody, but complete blocking of infection was observed with anti-human CAR antibody.

Table 3. Percentages of CVB capsid-positive cells following infection of transfected CHO cells

CHO cells transfected with pig or human CAR cDNA were incubated with CVB serotype 1, 3 or 5 at the m.o.i. shown. Permissive infection of CHO cells was judged by the presence of CVB capsid proteins as detected with β -galactosidase-conjugated antibodies (see Methods). All studies were performed in triplicate and repeated at least once. Percentages were calculated by enumeration of at least 10 fields of microscope examination using a low-power objective lens (approximately 1000 cells per field).

Condition	Infectious virus per cell (m.o.i.)			
	50	10	1	0.1
Control (CHO)	No positive cells observed for any CVB serotype			
CHO-human CAR				
CVB1	100	25	2	0.1
CVB3	100	38	5	0.1
CVB5	100	23	1	0.1
CHO-pig CAR				
CVB1	100	9	0.1	0
CVB3	100	7	0.1	0
CVB5	100	10	1	0.1

but did not block CVB infection completely, likely due to the presence of the second receptor, human DAF, on these cells.

Infection of CHO cells transfected with pig CAR

In order to rule out any cross-reaction of the anti-human CAR antiserum with any possible receptors on pig cells other than pig CAR, CVB infection of CHO cells transfected with pig and human CAR was performed. CHO cells stably

transfected with empty vector, human CAR or pig CAR were subcultured into 24-well plates and then incubated with different amounts of infectious virus (as titrated on RD cells) per cell (m.o.i.). The infection was allowed to proceed for 24 h and cell layers were fixed and stained for the presence of virus capsid proteins, in order to show successful CVB entry and infection. No capsid protein-positive cells were seen for CHO cells transfected with the empty expression vector; however, both human CAR- and pig CAR-transfected cells were susceptible to infection, as measured by the presence of CVB capsid-positive cells (Table 3). Similar susceptibility to CVB1, 3 and 5 infection of pig CAR-transfected CHO cells was observed, indicating that pig CAR on its own can act as a receptor for these CVB serotypes.

Discussion

The ability of all serotypes of CVB to infect and cause CPE in the pig kidney cell line IB-RS-2 was first reported by Knowles *et al.* (1979). We here confirm that CVB serotypes 1, 3 and 5 lytically infect multiple pig cell lines. All six CVB serotypes have been found to share a common receptor, referred to as CAR (Bergelson *et al.*, 1997b; Martino *et al.*, 2000; Lonberg-Holm *et al.*, 1976; Hsu *et al.*, 1988). CVB serotypes 1, 3 and 5 have also been found to use human DAF as a receptor (Shafren *et al.*, 1995; Bergelson *et al.*, 1997a). It has been reported that the murine CAR homologue can act as a receptor for human CVB (Bergelson *et al.*, 1998), while the murine DAF homologue could not (Spiller *et al.*, 2000). Furthermore, Zhang *et al.* (1999) reported that, while the swine pathogen SVDV contains sequences that distinguish it from all other coxsackieviruses, genetic analysis suggests that SVDV evolved from a cross-species jump of CVB5 between 1945 and 1965, and SVDV has recently been found to retain its ability to bind human CAR and DAF (Martino *et al.*, 2000). All of this

evidence suggested that CVB1, 3 and 5 might be able to use the pig CAR and/or DAF homologues as receptors. Here, we investigate the binding of CVB1, 3 and 5 to these homologues.

Rabbit polyclonal anti-pig DAF antibodies were not able to block CVB infection and radiolabelled CVB did not bind CHO cells expressing pig DAF. This is similar to our findings for CVB binding to mouse DAF (Spiller *et al.*, 2000). While mouse DAF has the same extracellular structure as human DAF (four SCRs separated from the membrane attachment by a Ser-/Thr-rich region; Spicer *et al.*, 1995), pig DAF contains homologues for only the first three SCRs followed by a Ser-/Thr-rich region and is approximately 64% identical to human DAF at the amino acid level (Perez de la Lastra *et al.*, 2000). Sequence comparison indicates that pig DAF is missing the homologue for human DAF SCR4. Despite this major structural difference, our previous characterization of pig DAF demonstrates that it is the homologue of human DAF and not a distinct member of the complement regulatory protein super-family. For example, several key structural features unique to DAF are conserved, including the critical tandem lysine residues (K125–127) and adjacent hydrophobic residues at the junction between SCRs 2 and 3, known to be important for complement-regulating function (summarized in Perez de la Lastra *et al.*, 2000). Pig DAF also expressed decay-accelerating activity to regulate human complement activation, albeit to a lesser extent than human DAF.

Bergelson *et al.* (1995) have shown, by binding of radiolabelled CVB3 to human DAF SCR-deletion constructs, that SCR2 played the dominant role in CVB3 binding; however, Shafren *et al.* (1995) found that only monoclonal anti-human DAF antibodies recognizing SCR3 could block CVB3 infection, and it has been shown that removal of SCR4 eliminated the binding of other related picornaviruses to human DAF (Powell *et al.*, 1998). Other evidence has been reported that shows that deletion of human DAF SCR4 does not effect binding of CVB3 (Bergelson *et al.*, 1995). In order to eliminate the possibility that failure of CVB to bind pig DAF was due to altered spacing resulting from the absence of SCR4, we constructed a recombinant form of pig DAF that had human DAF SCR4 inserted between pig DAF SCR3 and the Ser-/Thr-rich region. Incorporation of human SCR4 did not confer binding of any CVB serotype to pig DAF, confirming that pig DAF was intrinsically incapable of binding CVB and also indicating that CVB do not bind human SCR4 alone.

Since CVB serotypes did not bind pig DAF, we investigated whether CVB could bind pig CAR. By using primers based on the existing human and murine CAR sequences, we isolated cDNA from three separate pig cell lines encoding the pig CAR homologue. This sequence was approximately 90% identical to human CAR at the amino acid level. Based on the structure of human CAR and the other members of the CTX homologue family predicted by Chretien *et al.* (1998), it was evident that this pig CAR cDNA was missing the 5' signal sequence and a large portion of the 3' cytoplasmic tail of the mature predicted

protein, so a construct including the human CAR signal sequence and a truncated cytoplasmic tail was generated for expression in CHO cells. The loss of the cytoplasmic tail from pig CAR is unlikely to affect its ability to act as a receptor, as it has been demonstrated previously that exchanging the transmembrane region of human CAR for a glycolipid anchor does not affect receptor function (Wang & Bergelson, 1999). While this work was in progress, Fechner *et al.* (1999) published a partial sequence for pig CAR that matched our sequence exactly.

Transfection of the isolated pig CAR cDNA into CHO cells conferred permissive infection by CVB, as assessed by virus entry and production of CVB capsid proteins, demonstrating that pig CAR was a receptor for CVB. Rabbit polyclonal anti-human CAR antibodies generated against recombinant CAR cross-reacted strongly with pig CAR expressed on CHO cells and identified a single 46–48 kDa protein in human and pig cell lines. Following pre-incubation with pig cell lines, this antibody blocked CVB binding, confirming that a functional CAR homologue was present on pig cells.

In summary, we show that pig DAF cannot function as a receptor for CVB serotypes that utilize human DAF as a receptor, but a homologue of CAR is present on pig cells and can mediate CVB binding and infection. While we cannot rule out the presence of additional CVB receptors on pig cells, our infection-blocking studies with CAR-specific antibodies suggest that pig CAR is the dominant receptor.

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