Endogenous low-level expression of the coxsackievirus and adenovirus receptor enables coxsackievirus B3 infection of RD cells

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Cells in which the appropriate viral receptor cannot be detected may paradoxically act as a host to the virus. For example, RD cells are often considered to be non-permissive for infection with coxsackievirus and adenovirus receptor (CAR)-dependent group B coxsackieviruses (CVB), insofar as inoculated cell monolayers show little or no cytopathic effect (CPE) and immunohistochemical assays for CAR have been consistently negative. Supernatants recovered from RD cells exposed to CVB, however, contained more virus than was added in the initial inoculum, indicating that productive virus replication occurred in the monolayer. When infected with a recombinant CVB type 3 (CVB3) chimeric strain expressing S-Tag within the viral polyprotein, 4–11 % of RD cells expressed S-Tag over 48 h. CAR mRNA was detected in RD cells by RT-PCR, and CAR protein was detected on Western blots of RD lysates; both were detected at much lower levels than in HeLa cells. Receptor blockade by an anti-CAR antibody confirmed that CVB3 infection of RD cells was mediated by CAR. These results show that some RD cells in the culture population express CAR and can thereby be infected by CVB, which explains the replication of CAR-dependent CVB in cell types that show little or no CPE and in which CAR has not previously been detected. Cells within cultures of cell types that have been considered non-permissive may express receptor transiently, leading to persistent replication of virus within the cultured population.

INTRODUCTION

Studies of coxsackievirus and adenovirus receptor (CAR)-dependent group B coxsackievirus (CVB) tropism and replication commonly utilize HeLa and RD cells as positive (permissive) and negative (non-permissive) controls, respectively (e.g. Reagan et al., 1984). Such experiments can provide convenient end-point readout within 24–48 h, by which time HeLa monolayers are typically destroyed and RD monolayers have reached confluence. Consistent with these results, CAR protein is detected readily by immunofluorescence and on Western blots of HeLa cells, but not RD cells, in agreement with results from Northern blots (Cunningham et al., 2003; Polacek et al., 2005; Tomko et al., 1997). The conclusion is that CAR is necessary and sufficient to support CVB infection. This conclusion has been complicated, however, by lytic, DAF-binding CVB strains selected for replication in RD cells, and blockade of CVB infection of polarized epithelial cells by anti-DAF antibodies (Bergelson et al., 1995; Shieh & Bergelson, 2002). Various clinical isolates replicate in RD cells (e.g. She et al., 2006), and some of us have presumed that these viruses must use alternative receptors, such as DAF. Whilst some of these CVB may use receptors other than CAR, in this report, we show again that CVB3 does indeed replicate in RD cells, and show further that replication of the strains used here is CAR-dependent.

METHODS

Cells and viruses. HeLa, rhabdomyosarcoma (RD) (ATCC), RD-HCAR (expressing complete CAR), RD-HCARt3 (also called RDt3, expressing CAR with truncated and non-native cytoplasmic sequence) and RDtHis (expressing CAR with truncated cytoplasmic domain and added His6) (Cunningham et al., 2003) cells were propagated in monolayer culture in Dulbecco’s minimal essential medium containing 10% fetal bovine serum, 90 units penicillin ml\(^{-1}\), 90 μg streptomycin ml\(^{-1}\), 67 μg gentamicin ml\(^{-1}\) and supplementary glutamine (0.9 mM) (all from Gibco). All cell cultures were propagated at 37 °C in a humidified 5% CO\(_2)/air mixture. Viruses used included CVB3/0, CVB3/28, CVB-S-Tag and CVB-GFP (described below). To harvest virus for determination of titre, cultures were frozen and thawed three times, cell debris was removed by low-speed centrifugation (4500 g for 30 min) and virus titre was determined (TCID\(_{50}\) ml\(^{-1}\)) on HeLa cell monolayers (Hofling et al., 2000). For one-step growth curves, cells were inoculated for 1 h and then washed to remove unbound virus. In other experiments, CVB was added to the medium and left throughout the incubation.
Generation of virus genome and virus stocks. A reporter tag, S-Tag, was cloned into the open reading frame of CVB3/28 (Tracy et al., 2002) to allow ready detection of infected cells. S-Tag is a 15 aa sequence with high affinity for the RNase S protein that can be used for purification and detection of proteins to which it is fused (Raines et al., 2000). The infectious CVB3 cDNA genome encoding S-Tag was generated by using a subcloned fragment of an infectious genome of CVB3 containing a polylinker sequence in the region between the VP1- and 2Apro-encoding sequences (Chapman et al., 2000; Hoffling et al., 2000). The polylinker was flanked by sequences encoding the VP1/2Apro cleavage site in which the 3' sequence was altered to have 70% nucleotide sequence identity with the upstream site (Chapman et al., 2000; Hoffling et al., 2000). Overlapping oligonucleotides (STAGPCR1, 5'-CCGGTGATACAAAGGAGGGCTAAGAAAGAGCAGGAGACGGAGTTTTG) and PL3PCR2, 5'-CCGGACATCTCGGCTGCTGCTGCTGTTGAAATTTAGCGGCGGCT) encoding the peptide tag were ligated in and cloned into the BamHI site of the polylinker by using encoded BamHI and BgII sites. The subclone was then ligated into the full-length infectious cDNA genome of CVB3/28, using BgII and XhoI sites unique in that genome.

To generate progeny chimeric CVB3-S-Tag virions, 2 μg pCVB3-PL2-S-Tag DNA was transfected into 10^6 monolayer HeLa cells by using an Effectene transfection reagent kit (Qiagen) according to the manufacturer's protocol. Three days post-transfection, cultures were frozen and thawed three times and cleared of cell debris by centrifugation (4500 g for 30 min). Lysate was used to infect 2x10^9 HeLa cells, which were then incubated until all cells were lysed (virus passage 2). This cell lysate was cleared of cell debris as before. Passages of CVB3-S-Tag were performed similarly, with infected cells incubated at 37 °C for 72 h or until >95% of cells showed cytopathic effect (CPE), followed by freeze-thaw lysis and centrifugation as described above. Titre was determined as TCID50 on HeLa monolayers (Tracy et al., 1992) and aliquots were stored at -74 °C. The same process was used to generate stocks of the parental CVB3/28 and CVB3/0 viruses from infectious cDNA cloned in a plasmid (Tracy et al., 2002; Tu et al., 1995). CVB3/0 and CVB3/28 differ in a single nucleotide position (nt 234), a site affecting VP1-2Apro cleavage site in which the 3' nucleotide sequence was altered to have 10-15% nucleotide sequence identity with the upstream site (Chapman et al., 2000; Hofling et al., 2000). Overlapping oligonucleotides (STAGPCR1, 5'-CGGCACATCTCGGCTGCTGCTGTTGAAATTTAGCGGCGGCT) and PL3PCR2 (5'-GATACTTCGCTGCTGCTGCTGCTGTTGAAATTTAGCGGCGGCT) encoding the peptide tag were ligated in and cloned into the BamHI and BstEII sites of a subclone of CVB3/28 (pBSPL3, 2000). The infectious CVB3 cDNA genome encoding S-Tag was then ligated into the full-length infectious cDNA genome of CVB3/28 (Tracy et al., 2002) by using these sites.

To generate CVB3 virus encoding this protein tag, 2 μg pCVB3-PL3-GFP was transfected into HeLa cells and progeny virus was harvested as described above for CVB3-S-Tag.

S protein detection and stability. HeLa cell monolayers on glass slides were inoculated with CVB3-S-Tag at an m.o.i. of 25, then incubated at 37 °C for 6 h. Slides were rinsed in PBS, fixed in cold 1:1 acetone: methanol for 15 min, rinsed in PBS and incubated in a 1:2000 dilution of S protein-horseradish peroxidase (HRP) conjugate (EMD Biosciences, Inc.) and rinsed with PBS. The reaction product was visualized by using Harker Yates reagent (Polysciences). Slides were counterstained lighty with Mayer's haematoxylin. For Western blots of infected HeLa cells at 4 and 8 h post-inoculation (p.i.), culture medium was removed and cells were lysed in 2x Laemmli buffer (Laemmli, 1970) containing 2-mercaptoethanol, and processed as described previously (Chapman et al., 2000), using S protein–HRP to probe for S-Tag. To assay the maintenance of the S-tag encoding sequence in the CVB3 genome, CVB3-S-Tag was passaged serially 10 times in HeLa monolayers and viral RNA was assayed for the presence of insert by RT-PCR using flanking primers (ID9 and ID10; Chapman et al., 2000). No deletion was seen in 10 passages of the virus, indicating a very high degree of stability for this insert.

Flow cytometry. For detection of CVB3-expressed S-Tag, RD, RD-HCAR or HeLa cells, with or without CVB3, were collected by brief incubation in PBS containing 5 % trypsin and 20 μM EDTA. The cells were washed once by centrifugation (3500 r.p.m. for 2 min in a Dade Immunofose II) with PBS containing 0.1 % NaCl and 5 % BSA (PBS/NaCl/BSA), resuspended in 1 ml PBS/NaCl/BSA, counted and aliquotted at 0.5 x 10^7 into 5 ml plastic test tubes. The cells were then stained with S protein–fluorescein isothiocyanate (FITC) (Novagen/EMD Biosciences) using permeabilization reagents from Caltag Laboratories according to the manufacturer's specifications. S protein–FITC was utilized at a 1:2000 dilution during the permeabilization step. Cells were washed and resuspended in 0.5 ml PBS for analysis. Cells cultured in the absence of CVB were utilized as a negative control for S-Tag binding.

CAR was detected by using the Rmcb monoclonal antibody (mAb) (Hsu et al., 1988). RD and HeLa cells were detached from the plastic by incubation in PBS containing 20 μM EDTA for 20 min at 4 °C, followed by gentle vortexing to release the cells. The cells were washed once by centrifugation (3500 r.p.m. for 2 min in a Dade Immunofose II) with PBS/NaCl/BSA, resuspended in 1 ml PBS/NaCl/BSA, counted and aliquotted at 0.2 x 10^6 into 5 ml plastic test tubes in 200 μl PBS/NaCl/BSA. Cells were incubated with or without 5 μg Rmcb antibody for 30 min at 25 °C. The cells were washed once with 1 ml PBS/NaCl/BSA and then incubated with 200 μl goat anti-mouse FITC (F(ab')2 (Sigma-Aldrich) at a 1:500 dilution in PBS/NaCl/BSA for 30 min at 25 °C. The cells were washed and resuspended in 0.5 ml PBS for analysis. Cells prepared in the absence of primary antibody were utilized as a negative control for Rmcb binding.

Flow cytometry was performed by using a Beckman Coulter FC500 flow cytometer run with Beckman Coulter CXP software. The negative-control cells were utilized to assign photomultiplier and amplifier settings so that negative-control cell fluorescence was situated in the first decade of the four-decade fluorescence scale. Percentage positivity for the S-Tag- or Rmcb-positive cells was determined by subtraction of the percentage of negative-control cells using a linear gate on the FITC fluorescence axis. Approximately 10,000 cell events were counted for each sample.

Western blot detection of CAR. Octylglucoside cell lysates were prepared as described previously (Carson et al., 1999). Proteins were separated by electrophoresis on 10-15 % polyacrylamide gels (Laemmli, 1970) and transferred to Immobilon-P membranes
Low-level CAR mediates CVB infection of RD cells

CVB3 at m.o.i. >1 typically produces a visual cell-lysis end point in HeLa cells (Fig. 1a, HeLa), but not in RD cells (Fig. 1a, RD), within 24 h of infection. However, RD cells engineered to express CAR, or CAR with cytoplasmic domain deleted, are lysed readily by CVB3 within 24 h of infection (Cunningham et al., 2003; Fig. 1a, RDt3), indicating that the apparent resistance of RD monolayers to lytic CVB infection is related to lack of receptor expression. Although the visual end-point assay indicated that RD cells do not appear to be permissive for CVB3, additional experiments in which progeny virus was titrated indicated that CVB3 replicates in RD cultures (Fig. 1b). RD cells produced approximately 50-fold lower CVB3 titres at 24 h than similarly inoculated HeLa cells. However, by 48 h (when CPE in control cultures was complete), CVB3

RT-PCR. Viral genomes were assayed for the presence of the S-Tag sequence by using primers flanking the inserted polylinker (ID9 and ID10) (Chapman et al., 2000). Viral genomic RNA from chimeric virus stocks from each passage was isolated by using Trizol LS reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was transcribed from total cellular RNA with SuperScript II reverse transcriptase (Invitrogen) and primer ID10 essentially as described previously (Chapman et al., 2000), followed by PCR amplification with Tag polymerase (Promega) with both primers. cDNA from insert-containing genomes generates a 574 bp fragment, and revertants to wild-type (passage of CVB3 viruses with inserts at this site causes recombination, deleting both insert and polylinker; Hofling et al., 2000) generate a 344 bp fragment.

Determination of CAR RNA copy number by quantitative RT-PCR. T7 RNA polymerase (positive-sense) transcripts were synthesized from a NotI-digested plasmid containing nt 119-899 (numbering as in GenBank accession no. NM_001338) of human CAR cDNA cloned in the pTracer plasmid (Invitrogen). Transcripts were treated with RNase-free DNase I (Ambion), collected by ethanol precipitation and quantified spectrophotometrically. RNA was prepared from monolayers by using a mini RNA isolation II kit (Zymo Research Corporation). An aliquot of the cells was used for enumeration. cDNA was synthesized from RNA annealed to HMCAR1 (5’-ACCTGAAGGCCTAACAAGAA, reverse complement of nt 528-547) in 20 μl reactions containing 1 U ImProm-II reverse transcriptase (Promega) and 1 U Rnasin (Promega) in ImProm-II buffer supplemented with 3 mM MgCl2 and 0.5 mM dNTPs for 1 h at 42 °C. Real-time quantitative PCR was performed essentially as described previously (Kim et al., 2005) using cDNA reactions diluted fivefold with water so that 10% of a reverse transcription reaction volume was used with HMCAR1 and HMCAR2 (5’-AGTCCCC-GAAGACCCAGGACC, nt 254-273) at 0.125 OD260 units ml⁻¹ in DyNaMo SYBR green qPCR mix (Finnzyme) according to the manufacturer’s instructions. Cycling times were as follows: one cycle at 95 °C for 15 min; 45 cycles at 95 °C for 20 s, 55 °C for 20 s and 72 °C for 20 s; and a final extension at 72 °C for 10 min. To generate a standard curve, quantitative PCRs were carried out with cDNA from defined quantities of T7 transcripts of CAR plasmid by using an Opticon 2 DNA engine (MJ Research). The equation of the standard curve is y = −0.2795x + 9.523 (where y is the log of the number of RNA molecules and x is the critical threshold cycle, Cx). CAR Cx was determined for each cellular RNA sample and numbers of CAR RNA molecules were determined based on the standard curve.

RESULTS

(Fig. 1. (a) RD cultures are macroscopically resistant to CVB3 CPE. HeLa, RD and RDt3 cells were grown to near-confluence in wells of a 24-well plate. CVB3/0 cells (10⁶ per well) were added to cells in one row. After 24 h incubation at 37 °C, wells were rinsed with Tris-buffered saline and adherent cells were stained with Coomassie blue R250. Wells were de-stained with 10% 2-propanol, 10% acetic acid in water, air-dried and photographed. (b) CVB3 replicates in RD cells. Four 24-well plates were seeded with 10,000 cells per well in 2 ml medium. After overnight incubation, medium was removed and 2x10⁵ CVB3/0 cells were added in 100 μl medium. After 1 h at 37 °C, the inoculum was removed and the wells were washed three times with fresh medium. Wells were replenished with 0.5 ml fresh medium and returned to the incubator. At 4, 8, 24 and 48 h after inoculation, one plate was removed from the incubator and stored at −20 °C. The plate contents were frozen and thawed three times, and centrifuged to remove cell debris. Supernatants were assayed for infectious CVB (TCID₅₀). ○, HeLa; □, RD; △, RD-HCAR; ◊, RDtHis.)
titres in RD cultures approached those obtained from the CAR-positive cell lines, including HeLa, although the RD monolayer remained intact. As CAR has been reported to be necessary for CVB3 infection, with few exceptions (Bergelson et al., 1995; Shafren et al., 1997), this result suggested that the RD cells probably express some CAR protein.

A direct assay for expression of CAR protein by RD cells was therefore carried out. Flow cytometry readily detected CAR in HeLa cells, but not in RD cells (Fig. 2). On Western blots of equal amounts of protein from lysates of RD and HeLa cells (Fig. 3), the chemiluminescence associated with CAR was detected readily in the HeLa cell sample after 6 min exposure, at which time the RD lane remained clear. After 60 min exposure, however, the RD sample revealed faint bands corresponding to CAR and a higher-mobility band, which may be a CAR proteolysis product (Carson, 2004) or incompletely glycosylated protein (Excoffon et al., 2007).

These results, showing very different amounts of CAR protein detected in RD cultures compared with HeLa cultures, were substantiated by detection of CAR mRNA in the two cell lines. Quantitative real-time RT-PCR was performed with cellular RNA from samples of $10^4$ HeLa cells and $10^6$ RD cells. The amount of CAR mRNA measured in HeLa cells in two experiments (with eight and seven samples, respectively) averaged $5.2 \times 10^4$ ($\pm 0.5 \times 10^4$) and $6.9 \times 10^4$ ($\pm 0.4 \times 10^4$) copies of CAR mRNA per cell. CAR mRNA was detected consistently in the RD cells, but at less than one copy per cell. In two experiments with replicates of eight in each, means were $2.9 \times 10^{-3}$ ($\pm 0.4 \times 10^{-3}$) and $2.1 \times 10^{-3}$ ($\pm 0.3 \times 10^{-3}$) copies of CAR mRNA per cell (equivalent to one copy in 345 cells and one copy in 475 cells, respectively). The ratio of CAR mRNA copy number in HeLa cells to that in RD cells is thus very large ($1.2 \times 10^7 : 1$), demonstrating that

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**Fig. 2.** Flow cytometry of HeLa cells (a, b) and RD cells (c, d). (a) and (c) represent cells stained with the secondary antibody alone. The anti-CAR mAb Rmcb detected CAR in HeLa cells (b), but not in RD cells (d). Darker lines with grey fill correspond to cells labelled with Rmcb. The lines with no fill correspond to control cells. Overlapping regions are lighter grey.

**Fig. 3.** Western blot detection of CAR in HeLa cells and RD cells. Equal amounts (292 μg) of protein from HeLa and RD cells were applied to SDS/polyacrylamide gels, blotted to PVDF and probed for CAR antigen. HeLa cell CAR was readily detectable after only a few minutes of exposure, at which time the RD lane remained blank. After 1 h exposure, faint bands corresponding to CAR and a putative degradation product were visible in the RD lane. Post-processing of the photograph in this figure included contrast enhancement applied uniformly across the samples.
less-sensitive assays for CAR, which were successful for
detection of CAR in HeLa, would be very likely to fail with
RD cells.

Considering the detection of CAR in RD cells and the
absence of overt CVB CPE, it was important to assess the
cellular distribution of replicating CVB. Although it is
possible to use viral antigens to follow infection by
immunochemical methods (e.g. Schmidtke et al., 2000;
Shieh & Bergelson, 2002), CVB3 engineered to express S-
Tag provided a direct method to detect virus replication
with a single reagent (e.g. FITC-labelled S protein). We
used S-Tag-expressing CVB3 and flow cytometry to
compare the infection of HeLa, RD-HCAR and RD cells
over time. As cells can round up and be released from the
monolayer during mitosis or in the early stages of viral
CPE, floating and adherent cells were combined and
analysed for S-Tag expressed by replicating CVB. At 13 h
p.i., S-Tag was detected in >30% of the HeLa cells. This is
consistent with previous reports of cell-cycle influences on
receptor availability and viral replication (Feuer et al.,
2002; Seidman et al., 2001). S-Tag was also detected in
>30% of RD-HCAR cells at 13 h p.i. (Fig. 4a), suggesting
similar kinetics for virus-dependent S-Tag expression, even
though CAR in these cells was expressed under the control
of the cytomegalovirus promoter. Remarkably, S-Tag was
detected in 4% of the RD cells at 13 h p.i. (Fig. 4c). As
anticipated from results in Fig. 1, at 24 h p.i., few HeLa
cells remained adherent and too few intact floating cells
were available for analysis. S-Tag was detected in nearly
50% of the RD-HCAR cells that remained intact (Fig. 4b).
S-Tag was detected in 6% of the RD cells, most of which
remained adherent at 24 h p.i. (Fig. 4d). At 48 h p.i., S-Tag
was detected in 11% of the RD cells (not shown). There is
no argument that 4% is different from 11% in this
experiment (i.e. the range is 4–11% of RD cells infected
over 48 h and the mean is 7%). In one experiment (K.-S.
Kim & S. D. Carson, unpublished data), levels of CAR
mRNA did not differ in RD cells compared with RD cells
that had been incubated with CVB for 24 h, indicating that
exposure to virus did not induce CAR transcription.

log(TCID50) titres of CVB-S-Tag in the medium reached
7.5 at 13 h post-infection for HeLa cells, 7.2 at 24 h for
RD-HCAR cells and 7.2 for RD cells at 48 h. These
combined results show that CVB can infect and replicate in
all three cell lines, but the infection of RD progressed
slowly in a small percentage of the cell population. This
further supports the conclusion that the CAR detected
in the RD cells is present only on a subpopulation within
the culture, which would be those cells susceptible to
infection.

Fig. 4. Expression of S-Tag following CVB-S-Tag infection of RD-HCAR cells (a, b) and RD cells (c, d). Cells were seeded on
day 1 at 0.5×10⁶ cells per T25 flask. Flasks were inoculated with 5×10⁶ CVB-S-Tag at staggered times to allow all samples to
be harvested and analysed at 48 h after the initial inoculation. The first inoculation was done on day 2 after seeding. S-Tag was
readily detectable in both cell lines within 13 h of infection (a, c), when 34% of RD-HCAR cells and 4% of RD cells were S-
Tag-positive. At 24 h post-infection (b, d), 48% of RD-HCAR and 6% of RD cells were S-Tag-positive. Darker lines with grey
fill correspond to the inoculated cultures. The lines with no fill correspond to the uninfected-control cultures. Overlapping
regions are lighter grey.
In one experiment, medium from RD cells cultured in 25 cm² flasks, with and without CVB, was sampled and assayed for lactate dehydrogenase. At 24 and 48 h after inoculation \((2 \times 10^6 \text{ CVB3/28})\), medium contained 484 and 740 IU ml\(^{-1}\), respectively. Samples from uninfected RD cultures contained LDH at 299 and 294 IU ml\(^{-1}\). This result indicates that, as with RD cells engineered to express CAR (Cunningham et al., 2003), CVB-infected wild-type RD cells are also lysed, although the RD monolayer was confluent when sampled at 48 h.

To confirm that CVB was infecting the RD cells via CAR, HeLa and RD cells were inoculated with CVB after addition of the anti-CAR mAb Rmcb, which inhibits CAR-mediated infection (Hsu et al., 1988), or mAb E1.2D3, which binds CAR on Western blots but does not block infection (Carson et al., 1999), and compared with cells inoculated with virus without antibody (Fig. 5). At 20 h p.i., the 70% of untreated HeLa cells that were infected by CVB3-S-Tag (Fig. 5a) was reduced to nearly 7% by Rmcb (Fig. 5b). S-Tag was not detected in any RD

![Fig. 5. Rmcb, an inhibitory anti-CAR antibody, blocks CVB-S-Tag infection of HeLa and RD cells. Cells were seeded at 0.5×10^6 per T25 flask in 6 ml medium. Two days later, the medium was replaced with 3 ml fresh medium and an additional 3 ml spent medium containing the Rmcb antibody or mAb E1.2D3. Control flasks received 6 ml fresh medium without antibody. Following 30 min incubation, flasks were inoculated with 1.7×10^7 CVB-S-Tag. Cells were harvested for flow cytometry 13 h after infection. Dark lines with grey fill represent data from cells inoculated with CVB. The lines without fill represent data from untreated- and uninfected-control cultures. Overlapping regions are lighter grey. (a–c) Data for HeLa cells that were untreated, treated with Rmcb or treated with mAb E1.2D3, respectively. (d–f) Data for RD cells that were untreated, treated with Rmcb or treated with mAb E1.2D3, respectively. Insets show results of a similar experiment in which CVB-GFP was used in place of CVB-S-Tag and cells were examined at 7.5 h after infection. Treatment of cells with Rmcb prior to inoculation blocked infection and expression of S-Tag or GFP [insets in (b) and (e)].]
cells when Rmcb was present (Fig. 5e), compared with in 4% of cells infected in the absence of Rmcb (Fig. 5d). As expected, the control mAb had no notable effect on infection of either cell line (Fig. 5c, f). These results were reproduced qualitatively by using CVB3-S-GFP (Fig. 5, insets). RD infection by CVB3-S-Tag or CVB-GFP was clearly mediated by binding of virus to CAR, to which Rmcb provided an effective blockade.

**DISCUSSION**

The limiting impediment to widespread infection of RD cells by many CVB is the lack of an appropriate receptor expressed by most or all cells. RD cells that express CAR following multiple high-density passages or as a result of expressed by many CVB is the lack of an appropriate receptor. RD infection by CVB3-S-Tag or CVB-GFP was clearly mediated by binding of virus to CAR, to which Rmcb provided an effective blockade.

Various studies have failed to detect CAR in RD cells. Tomko et al. (1997) detected no CAR on Northern blots; Cunningham et al. (2003) failed to detect CAR in RD cells by Western blot, yet reported CVB3 replication similar to that shown in Fig. 1(b), Polacek et al. (2005) selected a strain of CVB2 (which does not bind DAF), were reported many years ago (Argo et al., 1992; Reagan et al., 1984) and studies of clinical samples have reported CVB replication in RD cells (e.g. 4–11% of CVB1–6 isolates caused CPE in RD cells; She et al., 2006). How these infections are initiated in the apparent absence of CAR has remained inadequately explained, although the alternative-receptor rationale is convenient.

Serial passage of CVB in RD cells can result in selection for a DAF-binding CVB strain that replicates in RD cultures with less CPE (Bergelson et al., 1995, 1997; Reagan et al., 1984). Bergelson et al. (1997) suggested that receptor binding can vary among CVB isolates, and may evolve within infected tissues or cultured cells. This raises the possibility that each CVB inoculum may contain viruses with a spectrum of capacities for binding alternative receptors, including CAR, and which experience selection for receptor usage most successful with the current host. This is quite feasible, as quasispecies mutant swarms are diverse by definition (Domingo et al., 2006). If chance brings together a viral strain that can bind a receptor different from that of the dominant quasispecies population, this could lead both to successful infection and propagation of a potentially newly adapted viral strain. Even though the derivation of DAF-binding CVB strains validates the argument, the event selecting for use of alternative receptors must be rare, as the RD monolayers survived, often reaching confluence in competition with multiple CVB replication cycles and high viral yields (e.g. Fig. 1).

Our results show that the perceived absence of CAR in RD cells is due to prior use of insufficiently sensitive methods. Compared with HeLa and other highly permissive cells, RD cell cultures can be considered to be relatively, but not absolutely, CAR-deficient. As Rmcb blocked S-Tag and GFP expression in the RD cultures inoculated with tagged CVB, it must be the CAR-positive RD cells that are permissive to at least the initial CVB infection. Expression of CAR in RD cells passed at 3–5 day intervals before reaching confluence, rather than at high density (Shafren et al., 1997), is low and difficult to detect (e.g. Fig. 3; Cunningham et al., 2003; Polacek et al., 2005). The RT-PCR presents a quantitative measure of the level of CAR mRNA per cell at a fixed time point. By assuming transcription of only a single CAR mRNA molecule per cell, at most 1 in 400 RD cells could have been translating new CAR protein. Flow cytometry detected CVB-expressed S-Tag in 7% of RD cells on average at 13, 24 and 48 h after inoculation.

CAR expression must be transient in most of the RD cells. Assuming that CVB infects cells as they present CAR, and that infected cells detected at previous time points had been lysed and were not resampled, CAR-dependent infection of 7% of RD cells, in which CAR mRNA is expressed by no more than 0.25%, requires that cells expressing CAR protein exceed those expressing CAR mRNA by at least 28-fold. This could be accomplished if the CAR protein persists 28 times longer than the mRNA is expressed. Trypsin-treated HeLa cells remove cleaved CAR within 15 h of trypsin treatment (Carson, 2000) and glycosylated CAR has been observed after 24 h tunicamycin treatment of CAR-expressing COS cells (Excoffon et al., 2007), showing that CAR protein may be turned over very slowly. Assuming a comparable rate of CAR removal for RD cells (e.g. 15–30 h), CAR mRNA expression for 30–60 min in 0.25% of the cells could provide a population in which about 7% of cells expressed CAR protein in a steady state. The number of CAR-expressing RD cells may vary among experiments, influenced by cell density (Shafren et al., 1997) or by as-yet-undiscovered environmental factors. It appears that RD cells throughout the culture may up- and downregulate CAR expression continuously.

From these results, it is clear that the reported failure to detect CAR does not preclude low-level expression, especially for cell lines and tissues that have been shown to replicate CVB or adenoviruses that utilize CAR. In culture systems, despite extremely low levels of CAR, high titres of virus may accumulate and remain available to infect the cells as they transiently express CAR. Mutant viruses should also accumulate, while the majority of the cells in the culture remain uninfected and viable. This presents an opportune environment for in vitro selection of viruses able to exploit alternative receptors and to outcompete the CVB waiting for the next CAR to be offered.

**ACKNOWLEDGEMENTS**

This work was supported in part by grant R01 AI54551 from the NIH and by American Heart Association Grant-in-Aid 0555697Z.
REFERENCES


