The Coxsackie-Adenovirus Receptor (CAR) Is Used by Reference Strains and Clinical Isolates Representing All Six Serotypes of Coxsackievirus Group B and by Swine Vesicular Disease Virus

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Group B coxsackieviruses are etiologically linked to many human diseases, and cell surface receptors are postulated to play an important role in mediating their pathogenesis. The coxsackievirus adenovirus receptor (CAR) has been shown to function as a receptor for selected strains of coxsackievirus group B (CVB) serotypes 3, 4, and 5 and is postulated to serve as a receptor for all six serotypes. In this study, we demonstrate that CAR can serve as a receptor for laboratory reference strains and clinical isolates of all six CVB serotypes. Infection of CHO cells expressing human CAR results in a 1000-fold increase in CVB progeny virus titer compared to mock transfected cells. CAR was shown to be a functional receptor for swine vesicular disease virus (SVDV), as CHO-CAR cells but not CHO mock transfected controls were susceptible to SVDV infection, produced progeny SVDV, and developed cytopathic effects. Moreover, SVDV infection could be specifically blocked by monoclonal antibody to CAR (RmcB). SVDV infection of HeLa cells was also inhibited by an anti-CD55 MAb, suggesting that this virus, like some CVB, may interact with CD55 (decay accelerating factor) in addition to CAR. Finally, pretreatment of CVB or SVDV with soluble CAR effectively blocks virus infection of HeLa cell monolayers.

Key Words: enterovirus; coxsackievirus B; swine vesicular disease virus; Coxsackievirus adenovirus receptor (CAR); decay accelerating factor (DAF; CD55).

INTRODUCTION

Group B coxsackieviruses are etiologically implicated in human disease whose clinical manifestations include mild gastrointestinal or upper respiratory tract symptoms, myocardiitis, meningitis, encephalitis, and pulmonary disease (Chonmaitree and Mann, 1995; Grist et al., 1978; Liu et al., 1996; Martino et al., 1995a; Rotbart, 1995; Woodruff, 1980). Infection is mediated by cell surface receptors, which facilitate binding and entry of CVB into susceptible host cells. Receptors may have a major role in determining organ and cell tropism in patients infected with these viruses and may account for some of the clinical manifestations and disease sequelae (Crowell and Landau, 1983; Holland, 1961; Rotbart and Kirkegaard, 1992).

It had been proposed that all CVB1-6 serotypes shared a common receptor molecule. This hypothesis was based on the observation that prototype strains of CVB1-6 competed with each other for binding to the HeLa cell surface but did not interfere with cell binding by other enterovirus types (Lonberg-Holm et al., 1976). A monoclonal antibody termed RmcB, directed against this putative common receptor molecule, was subsequently shown to block binding and infection by CVB reference strains (Hsu et al., 1988). However, it was also demonstrated that RmcB blocked only some clinical isolates of CVB from infecting HeLa cells (Bergelson et al., 1997b), throwing into question the nature of MAb blocking studies and the premise of the common CVB receptor molecule.

A common receptor molecule termed the coxsackievir...
The adenovirus receptor (CAR) was recently cloned and characterized. It functions as a cell surface receptor for CVB, as well as an attachment molecule for adenovirus fiber proteins (Bergelson et al., 1997a; Carson et al., 1997; Roelvink et al., 1998; Tomko et al., 1997). CAR is a 46-kDa transmembrane glycoprotein with two extracellular immunoglobulin-like domains. Selected strains of CVB3 and CVB4 and CVB5 were shown to bind and to productively infect nonsusceptible hamster cells transfected with CAR cDNA but not control cells lacking the CAR receptor molecule (Bergelson et al., 1997a; Tomko et al., 1997).

It still remains to be proven that CAR can function as a common host cell receptor for CVB. In this study, we have tested whether CAR functions as a receptor for a variety of clinical and prototype isolates representing the six CVB serotypes. In addition we have examined whether swine vesicular disease virus (SVDV), which is antigenically and genetically related to CVBs (Graves, 1973; Martino et al., 1999; Zhang et al., 1993, 1999), is capable of utilizing the same CVB receptors, namely CAR and DAF.

RESULTS

Confirmation of CAR mRNA in cells

We first confirmed that the cell lines used in this study that were susceptible to virus infection expressed CAR RNA, while cell lines not susceptible to virus did not express CAR. CAR transcription was monitored by RT–PCR analysis using CVB-susceptible HeLa cells, Vero cells, and transiently transfected CHOP-CAR cells and the nonsusceptible untransfected CHOP cells. We also examined the SVDV-susceptible porcine cell lines ST and PK-15. Figure 1 is a composite picture demonstrating that CAR cDNA was amplified from all the cell lines that were susceptible to CVB (HeLa, Vero, CHOP-CAR) and SVDV (ST, PK-15) but not from nonsusceptible untransfected CHOP cells. Although not shown, positive controls using cloned CAR cDNA were run along with the original PCR reactions and were consistently positive as expected. Also, negative water controls containing all reagents except for CAR cDNA were consistently negative. PCR amplification of the entire coding region of CAR produced a DNA product that ran just above 1 kb on gel analysis (Fig. 1, see arrow), consistent with the ~1.1 kb size of the mRNA coding region of CAR (Bergelson et al., 1997a; Tomko et al., 1997).

CVB induces cytopathic effect on CAR-positive cells

All CVB strains tested induced cytopathic effect (CPE) on monolayers of HeLa cells and stably transfected CHO-CAR cells (Table 1 and Fig. 2). Some CVB strains also produced CPE on Vero cell monolayers, although only CVB5 (Faulkner), CVB3cs-N, CVB3cs-NR, and CVB3cs-20 were observed to produce a comparable CPE in Vero cells as they did in HeLa cells (not shown).

Genetically manipulated chimeric viruses generated from the cardiovirulent CVB3cs-CG strain and the avirulent CVB3cs-Ø strain (Lee et al., 1997) also produced CPE on monolayers of HeLa cells and stable CHO-CAR cells (not shown). In contrast, none of the CVBs caused CPE in CHO-control cells lacking the CAR receptor molecule. The control enteroviruses, coxsackievirus A9 and echovirus 9, did not cause CPE on any cell line tested (Table 1 and Fig. 2).

CVB1-6 reference strains, wild-type clinical isolates, and laboratory variants produce progeny virus in transiently transfected CHOP-CAR cells

The yield of reference CVB1-6 strains was 3–6 logs greater in transiently transfected CHOP-CAR cells as compared to control CHOP cells transfected with partial CAR sequence cDNA or to mock transfected cells (Fig. 3a). Similarly, the yield of all nine clinical CVB isolates was 2–3 logs greater in cells expressing the entire CAR protein as compared to controls (Fig. 3b). Finally, the yield of the six CVB3 strains known to be cardiovirulent in mice was 2–6 logs greater in cells expressing full-length CAR as compared to control cells (Fig. 3c). These results indicate that all CVB1-6 strains tested use CAR as a functional cellular receptor.

Small amounts of virus could be detected at 24 h in control cell cultures infected with CVB clinical isolates and cardiovirulent variants CVB3-N, CVB3-NR, and CVB3cs-20 (Figs. 3b and 3c). To confirm that this was not due to virus replication in control cells, we compared virus titers at 24 h and 1 h p.i. Virus titers were generally unchanged or lower at 24 h as compared to those detected immediately after virus absorption with the exception of a few clinical isolates (Figs. 3b and 3c). However, even for these isolates, virus titer changes were never as substantive as when the cells were expressing the CAR.
receptor. Moreover, CPE was never detected in control CHO cell monolayers for any of the virus strains tested (Table 1). Based on these observations, we believe that the low virus titers in control cultures at 24 h p.i. most likely reflects residual input virus from the inoculum. Since the cell monolayers were washed three times, it is unclear as to whether further washing would help to decrease the residual virus levels. It is tempting to speculate that they may even remain constant because of interactions between some virus strains and the CHO cell surface. A previous report of a putative molecule on CHO cells that interacts with the cardiovirulent CVB3-N strain but does not allow for productive lytic infection provides some support for this hypothesis (Kramer et al., 1997).

SVDV utilization of CAR and DAF receptors

Strong antigenic and phylogenetic relationships between CVB and swine vesicular disease virus (SVDV) led us to examine if CAR can serve as a functional receptor for SVDV. As shown in Table 1 and Fig. 2, SVDV infection receptor. Moreover, CPE was never detected in control CHO cell monolayers for any of the virus strains tested (Table 1). Based on these observations, we believe that the low virus titers in control cultures at 24 h p.i. most likely reflects residual input virus from the inoculum. Since the cell monolayers were washed three times, it is unclear as to whether further washing would help to decrease the residual virus levels. It is tempting to speculate that they may even remain constant because of interactions between some virus strains and the CHO cell surface. A previous report of a putative molecule on CHO cells that interacts with the cardiovirulent CVB3-N strain but does not allow for productive lytic infection provides some support for this hypothesis (Kramer et al., 1997).

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**TABLE 1**

Cytopathic Effect in Designated Cell Lines

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell line</th>
<th>HeLa</th>
<th>CHO-CAR</th>
<th>CHO</th>
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<tr>
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<td></td>
<td></td>
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<tr>
<td>CVB1 (Conn-S)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
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<tr>
<td>CVB2 (Ohio)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
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<tr>
<td>CVB3 (VR30-Nancy)</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>CVB3-N (RLC)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>CVB4 (Benschoten)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CVB5 (Faulkner)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CVB6 (Schmitt)</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>Cardiovirulent CVB3&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CG</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>SH</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CH</td>
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<tr>
<td>N</td>
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<tr>
<td>NR</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>BD</td>
<td>+</td>
<td>+</td>
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<td>Clinical isolates</td>
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<td>CVB1 (5010107)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</tr>
<tr>
<td>CVB1 (5010607)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CVB1 (86-2299)*</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>CVB1 (84-0503)*</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>CVB1 (95-0004)*</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>CVB2 (5010159)*</td>
<td>+</td>
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<td>-</td>
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<td>+</td>
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<td>+</td>
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<tr>
<td>CVB3 (4012530)*</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CVB3 (19141255)&lt;sup&gt;v&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CVB3 (19281109)&lt;sup&gt;v&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>CVB3-R (20270361)&lt;sup&gt;v&lt;/sup&gt;</td>
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<td>+</td>
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<tr>
<td>CVB3 (20310308)&lt;sup&gt;v&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CVB3 (86-1799)*</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>CVB3 (86-1990)*</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>CVB3 (86-2327)*</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>CVB3 (86-2424)*</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CVB3 (86-2751)*</td>
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<td>+</td>
<td>-</td>
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<tr>
<td>CVB4 (5010936)&lt;sup&gt;v&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td>CVB4 (87-1026)*</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CVB4 (88-0842)*</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CVB6 (88-0578)*</td>
<td>+</td>
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<td>-</td>
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<td>CVB6 (88-0870)*</td>
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<tr>
<td>CVB6 (89-0895)*</td>
<td>+</td>
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<tr>
<td>CVB6 (89-0516)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
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<tr>
<td>CVB6 (95-0005)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
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<tr>
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<td>+</td>
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<tr>
<td>CA9 (4012720)&lt;sup&gt;v&lt;/sup&gt;</td>
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<tr>
<td>EV9 (22030957)&lt;sup&gt;v&lt;/sup&gt;</td>
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<tr>
<td>Swine vesicular disease virus</td>
<td>SVDV UK 27/72</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<sup>a</sup> Experiments were performed as described in Fig. 2.

<sup>b</sup> Enterovirus specimens maintained in the laboratory of P.P.L. and SVDV were tested on CHO-control cells transfected with empty pcDNA3.1 vector. CVBs maintained in the laboratory of J.F.M. were tested on CHO-control cells transfected with human integrin α2 subunit.

<sup>c</sup> Reference strains obtained directly from the ATCC by T.A.M. unless otherwise noted.

<sup>f</sup> Specimens isolated at the Hospital for Sick Children, Toronto, Canada.

<sup>v</sup> Specimens described in Bergelson et al. (1997b), maintained in the laboratory of J.F.M.

<sup>†</sup> Additional specimens from the laboratory of J.F.M.
produced cytopathic effects on monolayers of stably transfected CHO-CAR cells and on monolayers of HeLa cells. In addition, SVDV produced CPE on monolayers of porcine ST and PK-15 cells from which a CAR homolog was amplified by PCR (Fig. 1). In contrast, no SVDV CPE was observed in the receptor-negative CHO-control cells (Table 1 and Fig. 2). Anti-CAR MAb RmcB decreased SVDV plaque formation by ~75% in stably transfected CHO-CAR cells and in HeLa cells (Table 2 and Fig. 4). In contrast, control serum did not decrease SVDV plaque formation.

DAF is a coreceptor molecule used by some CVB strains (Bergelson et al., 1995; Martino et al., 1998; Shafren et al., 1995). Previous studies have shown that HeLa cells express DAF at the cell surface and that anti-DAF antibodies specifically block CVB interactions with DAF on HeLa cells (Bergelson et al., 1995, 1997b; Martino et al., 1998; Crowell et al., 1986; Mohanty and Crowell, 1993; Shafren et al., 1995). Because SVDV, like the CVBs, was able to use the CAR receptor, its ability to also use DAF as a receptor was explored. As shown in Table 2, pretreatment of HeLa cell monolayers with anti-DAF MAb 914 resulted in a reduction of SVDV yield by 75%. The antibody to DAF was therefore as efficient at suppressing the replication of SVDV as antibody to CAR. These findings are consistent with the hypothesis that, as is the case for CVB1, -3, -5, both DAF and CAR have receptor functions.

Soluble CAR blocks CVB3 and SVDV infection of HeLa cells

To examine whether CAR alone can block virus infection of cells, we produced a soluble form of the CAR molecule (sCAR) and demonstrated that it is a specific inhibitor of CVB and SVDV infection. sCAR lacking the transmembrane and cytoplasmic domains was produced by fusing DNA encoding the extracellular portion of CAR to DNA encoding the Fc region of rabbit immunoglobulin. The DNA was inserted into a eukaryotic expression vector and transiently transfected into 293 cells. sCAR secreting into the supernatant was purified by chromatography. The ability of sCAR to inhibit CVB and SVDV infection of cells was examined using a quantitative in vitro assay for virus cytopathic effect (CPE). sCAR was preincubated with virus, and then the mixture was added to HeLa cell monolayers. As shown in Fig. 5, sCAR blocked CVB3- and SVDV-induced CPE on the HeLa cell monolayers in a dose-dependent manner. In contrast, a control immunoadhesin consisting of avian leukosis virus envelope glycoprotein fused to rabbit Fc (ALV–env) did not block virus infection of the cells.

DISCUSSION

In these studies, evidence is provided that reference strains and clinical isolates representing all six serotypes of group B coxsackieviruses (CVB) interact with the coxsackievirus adenovirus receptor (CAR) during infection. All CVB strains were capable of replicating and causing CPE in hamster cells expressing CAR but not CAR-negative control cells. In contrast, other enteroviruses such as coxsackievirus A9 and echovirus 9 did not infect CAR-expressing cells. These findings are consistent with the hypothesis that all CVB use a common receptor molecule (Bergelson et al., 1997a; Hsu et al., 1988; Lonberg-Holm et al., 1976). Moreover, use of CAR by CVB clinical isolates strongly suggests that CAR may be a CVB receptor in vivo. Virus strains isolated by only one passage through primary GMK cells were capable of utilizing CAR as well as virus strains that had been maintained for many years in cell culture.

In these experiments, CAR was shown to be a functional receptor for all CVB strains tested. In a previous study using a panel of CVB clinical and prototype isolates (Bergelson et al., 1997b), it was found that infection by many—but not by all—isolates was inhibited by the anti-CAR MAb RmcB. Eighteen of these isolates were subsequently shown to infect stably transfected CHO-CAR cells and CHO-control cells, and all were found to use CAR as a receptor. Why some of these viruses are not inhibited by RmcB is not certain. It is possible that they interact with CAR in a way that cannot be inhibited stERICALLY by RmcB; it is also possible that they make use of additional receptor molecules. Coreceptors or accessory molecules for CVBs that have been identified to date include decay accelerating factor (Bergelson et al., 1995; Shafren et al., 1995) and the integrin αvβ6 (Agrez et al., 1997). Two additional putative receptors are a 100-kDa nucleolin-like membrane protein (Raab de Verdugo et al., 1995) and a hamster cell binding molecule (Kramer...
CAR IS RECEPTOR FOR CVB SEROTYPES AND REFERENCE STRAINS

A

VIRUS TITER (TCID₅₀/ml)

10^6

10^5

10^4

10^3

10^2

10^1

CVB REFERENCE STRAIN

CVB1 Conni-5
CVB2 Ohno
CVB3 Nancy
CVB4 Benschoien
CVB6 Faulkner
CVB6 Schmitt

B

VIRUS TITER (TCID₅₀/ml)

10^6

10^5

10^4

10^3

10^2

10^1

CVB CLINICAL ISOLATE

CVB1 5010107
CVB2 5950607
CVB3 5010259
CVB4 4023510
CVB5 19141235
CVB6 19281109
CVB7 20270361
CVB8 20303008
CVB9 5010936

C

VIRUS TITER (TCID₅₀/ml)

10^6

10^5

10^4

10^3

10^2

10^1

CARDIOVIRULENT CVB3 STRAIN

03
SH
Ø
VR30
N
NR
20
et al., 1997), although a role for these molecules in CVB infections in humans is not known.

Previous studies have indicated that SVDV is antigenically and phylogenetically related to CVB and may have arisen within the past century from a single transfer of CVB5 into pigs (Graves, 1973; Zhang et al., 1993, 1999). These observations prompted us to examine whether SVDV could use the CVB receptor CAR. We found that SVDV could replicate and cause CPE in hamster cells expressing CAR but not mock transfected cells lacking CAR. Moreover, pretreatment of CAR-positive cells with anti-CAR monoclonal antibody RmcB blocked virus infection in plaque assays. These observations indicate that CAR can serve as a functional receptor for SVDV, a characteristic shared only with CVBs to date. It is tempting to speculate that the ability of SVDV to use CAR is an inherent characteristic from the evolutionary past that may have facilitated virus entry into porcine hosts. Indeed, when two porcine cell lines commonly used in SVDV propagation were examined by PCR, they both were found to express CAR RNA. Porcine CAR transcripts have also been detected recently in pig liver tissue (Fechner et al., 1999). Porcine CAR seems a likely candidate receptor for SVDV in pigs, and future transfection studies with this molecule are indicated to resolve this concept. Moreover, interactions of CVBs with porcine CAR homolog could provide further insight into receptor interactions with these viruses. This may also be of some concern in xenotransplantation of porcine organs.

Our studies provide evidence that SVDV also uses the CVB receptor molecule DAF. We found that pretreatment of DAF-positive HeLa cells with anti-DAF monoclonal antibody decreased SVDV infection by 75% in plaque assays, suggesting that DAF may be a coreceptor molecule. Binding studies such as those done with CVB (Bergelson et al., 1995; Martino et al., 1998; Shafren et al., 1995) would help define the nature of this receptor interaction and confirm that there are specific interactions and that the blocking is not just due to steric hindrance. DAF receptor usage may also be a characteristic retained from the evolution of CVB5 into SVDV, although it is not known whether a homolog of human DAF is expressed in pigs. The SVDV UK/27/72 strain used for this study was collected in 1972 and was one of the first isolates reported. It would be interesting to determine

### Table 2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Anti-CAR RmcB</th>
<th>Anti-DAF MAb 914</th>
<th>Anti-albumin (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-CAR</td>
<td>72 ± 7</td>
<td>n/a</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>HeLa</td>
<td>72 ± 15</td>
<td>75 ± 5</td>
<td>9 ± 7</td>
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</tbody>
</table>

*CHO-CAR or HeLa cells plated in six-well Costar dishes at a density of 1 × 10⁶ cells/well were preincubated with 1 ml of medium containing a 1:1000 dilution RmcB antibody as described previously (Bergelson et al., 1997) or 50 μg/ml anti-DAF MAb 914 (Martino et al., 1998) or 50 μg/ml of control rabbit anti-bovine albumin antiserum, at room temperature for 1 h. After washing, 1 ml containing ~50 PFU/ml of SVDV was added and cells were incubated at room temperature for 1 h. Monolayers were washed, plaque overlays were added for 2-3 days, and the plaques were counted. All experiments were performed in triplicate.
whether DAF receptor usage is also retained in more recent SVDV strains.

These experiments confirm that all CVBs share a common host cell receptor. The fact that clinical isolates use CAR as a receptor suggests that CAR may function as a receptor in the host and that it may play an important role in the pathogenesis of coxsackievirus disease. The role in pathogenesis of possible coreceptors such as DAF remains poorly understood, and the relation between virus tropism and tissue-specific receptor expression remains to be defined. Since it appears that CAR is the major receptor for CVB, structural studies of virus–CAR interaction could be important in the development of new antiviral agents. Also, soluble CAR decreases virus infection of host cells, possibly opening new avenues for treating CVB- and SVDV-induced disease.

MATERIALS AND METHODS

Cell lines

HeLa cells (CCL-2) and Vero cells (CCL-81) were obtained from the American Type Culture Collection (ATCC). They were grown in RPMI 1640 medium supplemented with 0.5% penicillin and streptomycin and 10% fetal calf serum (FCS). CHOP (Chinese hamster ovary) cells were from the laboratory of Dr. Jim Dennis (Heffernan and Dennis, 1992) and used for transient transfection of CAR cDNA (CHOP-CAR) or control cDNAs. Transiently transfected CHOP-CAR cells were grown in DMEMα medium supplemented with 0.5% penicillin and streptomycin and 10% FCS. CHO-CAR cells were stably transfected with CAR cDNA (Bergelson et al., 1997a), and CHO-control cells were stably transfected with empty pcDNA3.1 vector (Wang and Bergelson, 1999) or with human integrin α2 subunit (Bergelson et al., 1993). Stable CHO-CAR transfectants and CHO-control cells were cultured in nucleoside-free alpha minus MEM supplemented with NaHCO3 to pH 7.5, 0.5% penicillin and streptomycin, and 10% FCS. GMK cells (primary tube cultures of African green monkey kidney) were obtained from Viromed, Minneapolis, MN. They were cultured in ELY medium (Earle’s balanced salt solution, supplemented with lactalbumin, yeast hydrolysate, and 0.5% penicillin and streptomycin). Swine testis (ST) cells were obtained from the American Type Culture Collection. Porcine kidney (PK-15) cells were from NVLS, Ames, IA. ST and PK-15 cells were maintained in alpha MEM supplemented with 0.5% gentamicin and 10% FCS. Media and supplements were purchased from Gibco/BRL Life Technologies Canada, Burlington, Ontario, Canada.

Viruses

Reference virus strains CVB1 (Conn-5), CVB2 (Ohio), CVB3-VR30 (Nancy), CVB4 (Benschoten), CVB5 (Faulkner), and CVB6 (Schmitt) were obtained from ATCC.

Virus stocks were prepared by one passage in HeLa cells (with Vero cells used for CVB6). Stocks were frozen and thawed three times, were clarified by centrifugation, were titrated by plaque assay on HeLa cells, and were stored as aliquots at −70°C. Enterovirus isolates collected over a 5-year period at the Hospital for Sick Children, Toronto, Canada are shown in Table 3. These viruses were isolated by culture on GMK cells and were typed using diagnostic antisera at the Canadian National Centre for Enteroviruses, Halifax, Canada. Additional CVB clinical specimens were maintained in the laboratory of J.F.M., and some of these samples were described previously by Bergelson et al., 1997b (see Table 1). CVB3 strains known to be cardiovirulent in mice (CVB3cs) -SH, -NR, -N, -CG, -20, and -Ø were maintained in our laboratory, as previously described (Martino et al., 1998). SVDV (UK 27/72) was obtained from the Institute for Animal Health, Pirbright, UK, and maintained at the National Center for Animal Diseases, Winnipeg, Manitoba, Canada.

Antibodies

Anti-CAR RmcB monoclonal antibody was prepared as mouse ascites fluid (Hsu et al., 1988) and was provided by J.M.B. Anti-CAR rabbit polyclonal antiserum was prepared as described below. Anti-DAF MAb 914 (clone BRIC 216) was obtained from Serotec Canada, Toronto, Ontario, Canada. Rabbit affinity purified anti-bovine albumin antiserum was obtained from Cappel/ICN, Toronto, Ontario, Canada.

Primers

Primer TM39 was used for first-strand cDNA synthesis of CAR. TM39 (5’-TTGAGGCTAGTAACACAAAT-3’) was derived from the CAR 3’ nontranslated region (Bergelson et al., 1997a). Primers TM31 and TM32 were used for PCR of CAR. Primer TM31 (5’-ACTTTATCTAGGATCCATG-3’) consists
of a BamHI restriction site (underlined), followed by the first 33 nucleotides encoding hCAR protein (Bergelson et al., 1997a). Primer TM32 (5′-CTATACTATAGACCCATTGCCTGTCGTTGCG-3′) consists of an XhoI restriction site (underlined), a single cytosine nucleotide, and 33 nucleotides complementary to the CAR coding sequence upstream of the termination codon (Bergelson et al., 1997a).

PCR amplification of CAR cDNA

RNA extracted from HeLa, Vero, transiently transfected CHOP-CAR cells, untransformed CHOP cells, and the porcine cells lines ST and PK-15 was subjected to reverse transcription followed by PCR to amplify the CAR cDNA. Total cell RNA was purified using TRIzol reagent according to the manufacturer’s directions (Gibco BRL). For first-strand cDNA synthesis, a 15-μl aliquot of RNA was heated for 5 min at 65°C, was chilled on ice, then was added to a 25-μl volume containing 1 [multi] AMV reverse transcription buffer (Pharmacia), 1 μl RNasin (Pharmacia), 4 μl of 10 mM dNTP mixture (Pharmacia), 2 μl of 10 mM spermidine HCl, 2 μl of 80 mM sodium pyrophosphate, 4 μl of downstream primer TM39, and 1 μl of AMV reverse transcriptase (Pharmacia). Samples were incubated at 37°C for 1 h. For PCR, a 4-μl aliquot of this reaction mixture was added to a 46-μl volume containing 1 [multi] reaction buffer (Pharmacia Biotech Inc., Baie d’Urfe, Quebec, Canada), 0.2 mM of dNTP (Pharmacia), and 2 μM each of primers TM31 and TM32. The primer TM31 and TM32 were annealed at 50°C, 2 min; extension 72°C, 2 min; and denaturation 94°C, 1 min.

Expression of CAR and production of rabbit polyclonal antisera

HeLa cell CAR cDNA was amplified by PCR and purified by agarose gel electrophoresis. A band corresponding to the ~1.1-kb coding region of CAR was excised from the gel and was purified with Sephaglas Band Prep (Pharmacia), was digested with BamHI and XhoI (Pharmacia), was ligated into a pET28a (+) cloning vector (Novagen, Madison, WI), and was transformed into INVαF’ One Shot cells (Invitrogen). hCAR-pCDNA-1 was purified by Qiagen chromatography (Qiagen Inc) and was sequenced by Thermosequenase (Amersham Canada Ltd.). Two clones were selected of which one, hCAR7, contained full-length coding sequence and the other, hCAR2, encoded the extracellular region only (amino acids 1–189). CHOP cells were transfected with hCAR-hCDNA-1 or hCAR-pCDNA-1 or mock transfected, using Lipofectamine reagent (Gibco/BRL Life Technologies Canada, Burlington, Ontario, Canada) according to the manufacturer’s instructions. CAR expression was monitored by immunohistochemical staining and Western blot analysis of transfected cells, using rabbit anti-CAR polyclonal antibody. Transfection efficiency was also monitored using a reporter vector containing GFP (jellyfish green fluorescent protein) (kindly supplied by Dr. Peter Backx, University of Toronto, Canada). CVB replication in transiently transfected CHOP-CAR cells (or controls) was monitored by inoculating cell monolayers with each test virus at room temperature for 1 h, washing three times, and further incubating in growth medium for 0 h (to monitor for residual virus in the inoculum) or 24 h at 37°C. Dishes containing cells and virus were frozen and thawed, and progeny virus titer was determined by TCID₅₀ assay on HeLa cells.
CVB-induced cytopathic effect on CAR-positive cells

Confluent monolayers of HeLa cells, stably transfected CHO-CAR cells, or CHO-control cells transfected with empty pcDNA3.1 vector were plated in 96-well Costar dishes. In experiments with enteroviruses maintained in the laboratory of F.P.P., cell monolayers were exposed to a 50-μl aliquot of serial twofold dilutions of virus, and incubated at room temperature for 1 h. Cell monolayers were washed and were further incubated at 37°C. Cytopathic effect was monitored after 3 days by removing the supernatant and staining the cells with a 2% formaldehyde/0.1% crystal violet dye solution. For SVDV, the experiments were performed as described above except that serial 10-fold dilutions of virus were tested. For CVBs maintained in the laboratory of J.F.M., confluent cell monolayers in 24-well plates were exposed to a 100-μl aliquot of virus for 1 h at 37°C. Monolayers were washed and were further incubated at 37°C and examined for cytopathic effect over 6 days.

Soluble CAR blocking of CVB-induced cytopathic effect

For production of soluble CAR immunoadhesin (sCAR), DNA encoding the CAR extracellular domain (ending with PPSNK) was fused to DNA encoding the Fc region of rabbit immunoglobulin, derived from plasmid pKZ374 (provided by Dr. John Young, Harvard Medical School) and was inserted in the mammalian expression vector pcDNA 3.1 (Invitrogen). Fusion protein was purified from the supernatant of transiently transfected 293 cells by chromatography on protein A–Sepharose (Zingler and Young, 1996). The control immunoadhesin, avian leukosis virus envelope glycoprotein fused to rabbit Fc (ALV–env) was produced using plasmid pKB201 provided by Dr. Young. For sCAR blocking of CVB3-induced CPE, sCAR (or control) was added to a fixed concentration of virus and was warmed to 37°C for 1 h. The mixture was then added to confluent monolayers of HeLa cells plated in 96-well plates. Monolayers were examined after 24 h for cytopathic effect by staining cells using the methods described above.

Studies with SVDV

SVDV-induced cytopathic effect was monitored on CAR-positive monolayers of HeLa cells, porcine ST and PK-15 cells, stably transfected CHO-CAR cells, and CAR-negative CHO-control cells transfected with empty pcDNA3.1, using the methods described above for CVB. For antibody blocking experiments, stably CHO-CAR transfectants or HeLa cells were preincubated with anti-CAR MAb RmcB or anti-DAF MAb 914 or antibody to bovine albumin (control) and then were infected with ~50 PFU/ml of SVDV. Infection was monitored by plaque assay. The ability of soluble CAR to block SVDV-induced CPE of HeLa cells was performed as described above for CVB.

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