A Single Coxsackievirus B2 Capsid Residue Controls Cytolysis and Apoptosis in Rhabdomyosarcoma Cells

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Received 12 November 2009/Accepted 26 March 2010

Coxsackievirus B2 (CVB2), one of six human pathogens of the group B coxsackieviruses within the enterovirus genus of Picornaviridae, causes a wide spectrum of human diseases ranging from mild upper respiratory illnesses to myocarditis and meningitis. The CVB2 prototype strain Ohio-1 (CVB2O) was originally isolated from a patient with summer gripe in the 1950s. Later on, CVB2O was adapted to cytolysis in rhabdomyosarcoma (RD) cells. Here, we present analyses of the correlation between the adaptive mutations of this RD variant and the cytolysis in RD cells. Using reverse genetics, we identified a single amino acid change within the exposed region of the VP1 protein (glutamine to lysine at position 164) as the determinant for the acquired cytolysis trait. Moreover, this cytopathic virus induced apoptosis, including caspase activation and DNA degradation, in RD cells. These findings contribute to our understanding of the host cell adaptation process of CVB2O and provide a valuable tool for further studies of virus-host interactions.

Virus infections depend on complex interactions between viral and cellular proteins. Consequently, the nature of these interactions has important implications for viral cell type specificity, tissue tropism, and pathogenesis. Group B coxsackieviruses (CVB1 to CVB6), members of the genus Enterovirus within the family of Picornaviridae, are human pathogens that cause a broad spectrum of diseases, ranging from mild upper respiratory illnesses to more severe infections of the central nervous system, heart, and pancreas (61). These viruses have also been associated with certain chronic muscle diseases and myocardial infarction (2, 3, 12, 13, 22).

The positive single-stranded RNA genome (approximately 7,500 nucleotides in length) of CVBs is encapsidated within a small T=1, icosahedral shell (30 nm in diameter) comprised of repeating identical subunits made up of four structural proteins (VP1 to VP4). Parts of VP1, VP2, and VP3 are exposed on the outer surface of the capsid, whereas VP4 is positioned on the interior. The virion morphology is characterized by a star-shaped mesa at each 5-fold icosahedral symmetry axis, surrounded by a narrow depression referred to as the "canyon" (69). All six serotypes of CVB can use the coxsackie and adenovirus receptor (CAR) for cell attachment and entry (9, 55, 82). Some strains of CVB1, -3, and -5 also use decay accelerating factor ([DAF] CD55) for initial attachment to the host cells (66). Two amino acid changes were identified in the capsid-coding region, and one was identified in the 2C-coding region, and one was identified in the 2C-coding region of the adapted virus. Further characterization of the virus-host interaction showed that the infection was not affected by anti-DAF antibodies, indicating the use of an alternative receptor.

In this study, the amino acid substitutions associated with the adaptation of CVB2O to cytolysis in RD cells were evaluated. Site-directed mutagenesis studies showed that a single amino acid change in the VP1 capsid protein was responsible for the cytolytic RD phenotype. In addition, as
indicated by caspase activation and DNA degradation, the apoptotic pathway was activated in RD cells infected by the cytolytic virus.

**MATERIALS AND METHODS**

**Cells and viruses.** Cultures of a local variant of green monkey kidney (GMK) cells, human epithelial (HEp-2) cells, and human RD cells, obtained from the American Tissue Culture Collection (ATCC), were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10% newborn calf serum (NCS) at 37°C in 5.0% CO2. The prototype strain CVB2 Ohio-1 (VR-29; ATCC) (56, 67) and the CVB2ORD variant (66) were propagated in GMK and RD cells, respectively, as previously described (66).

**Flow cytometry.** The flow cytometry procedure has been described previously (66). RD cells were stained with an anti-CAR (RmcB) antibody (37) (hybridoma kindly provided by L. Philipson and R. Pettersson, Karolinska Institute, Sweden; also available as ATCC CRL-2379) or an anti-DAF (BRIC110) antibody (Cymbus Biotechnologies). A monoclonal mouse IgG1 antibody (X0931; Dako) was used as a negative control. After 1 h of incubation at 4°C, the cells were stained with a secondary R-phycocerythrin-conjugated polyclonal rabbit anti-mouse antibody (R0439; Dako). Data were acquired using a FACSCalibur (Becton Dickinson) and analyzed with CellQuest, version 3.3, software (Becton Dickinson).

**Infectious viral cDNA clones.** The complete CVB2O genome was amplified and cloned into the pcR-Script Direct SK+ vector (Stratagene) by using the Ascl and Not1 restriction enzyme cleavage sites, as previously described (Fig. 1A) (49, 67). In this pcR2Owt (where wt is wild type) construct, the individual CVB2ORD substitutions in the protein VP1 (I to F and Q to K) and 2C (K to R) were introduced by site-specific mutagenesis (Fig. 1). The PfluI and SalI sites were used to generate the single-amino acid mutant pcR2OVP1I118F clone (vP1I118F) encoding the VP1 I118F substitution (names of viruses derived from the infectious cDNA clones are given in parenthesis). The SalI and EcoRI restriction sites were used to construct the pcR2OVP1Q164K clone (vP1Q164K), while EcoRI and BamHI sites were used to produce the pcR2O2CK185R clone (v2CK185R). The CVB2O constructs were propagated in Escherichia coli, isolated, and verified by sequencing.

**Generation of viruses from infectious viral cDNA clones.** Viruses were generated by transfection (Lipofectamine 2000; Invitrogen) of 2.5 μg of the prototype (pcR2Owt) or mutant (pcR2OVP1I118F, pcR2OVP1Q164K, and pcR2O2CK185R) DNA into RD cells according to manufacturer’s protocol. At 5 days posttransfection, the virus from transfected cells was released by three freeze-thaw cycles and further propagated by one subsequent passage in RD cells as described previously (57). Viral RNA was extracted from infected cell cultures (QiAamp viral RNA mini kit; Qiagen), reverse transcribed (Superscript III; Invitrogen), and PCR amplified (PicoMaxx high fidelity PCR system; Stratagene) using virus-specific primers. PCR amplicons were visualized on an agarose gel, purified (QIAquick gel extraction kit; Qiagen), and sequenced. Virus titers were determined by 50% tissue culture infectious dose (TCID50) assays in GMK cells, according to standard procedures (34).

**Viruses infection.** Cells were infected with the different CVB2O variants according to standard procedures (57). Briefly, subconfluent monolayers of RD cells grown in 25-cm2 flasks were inoculated with clone-derived viruses at a multiplicity of infection (MOI) ranging from 1 to 100 TCID50s/cell. After a 1-h adsorption at room temperature, the inoculum was removed, and cells were washed three times to remove unbound virus before addition of DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Incubation was continued for 7 days or until cytopathic effect (CPE) was observed.

In order to determine the number of mutations that were introduced in the vP1Q164K genome during serial passages in RD cells, infected cells were incubated until complete CPE was observed. These samples were subjected to three freeze-thaw cycles and further passaged five times in RD cells at an apparently high MOI (in two parallel experiments). After the fifth passage, the viral progeny was sequenced.

For studies of virus production during repeated noncytolytic infections, RD cells infected with pcR2Owt were detached by EDTA treatment and passaged every fourth day at a 1/3 dilution. The titer of virus released into the medium of each passage was measured by the TCID50 method in GMK cells. After the 10th passage, the viral progeny was sequenced.

**Release of CVB2Owt from RD cells.** Confluent RD cells cultured in 24-well plates were infected with CVB2Owt at an MOI of 1 TCID50s/cell. In order to assess both the intra- and extracellular virus production, samples (only medium or medium together with cells) were frozen at different time points postinfection.
Monolayers of RD cells in six-well plates. Following adsorption for 1 h at 37°C, the inoculum was aspirated and replaced with an overlay medium, which consisted of 100% DMEM and antibiotics. Infected cell samples were harvested at 0 h and 96 h p.i. and virus titers were determined by the TCID50 assay in GMK cells. The replication of viral plus-strand RNA in infected RD cells was analyzed as described previously (80). Briefly, EDTA-tREATED samples were lysed with 0.2% Triton X-100 and 1 mM EDTA in 10 mM Tris- HCl, pH 7.4. The nucleic acid components were separated by centrifugation and resuspended in lysis buffer (2% SDS, 35 mM β-mercaptoethanol, 50 mM Tris- HCl, pH 6.8) supplemented, immediately before use, with 1 mM phenylmethylsulfonyl fluoride and a Complete Mini protease inhibitor cocktail (Roche). The obtained lysate was incubated in boiling water for 10 min and sonicated. The total protein concentration was determined with a spectrophotometer (Nanodrop ND-1000, Saveen Werner). The protein samples (5 to 40 μg) were fractionated by SDS-PAGE (12 or 14% polyacrylamide) and electroblotted onto a 0.45-μm-pore-size polyvinylidene difluoride membrane (Amersham). Nonspecific sites were blocked with 5% nonfat dry milk and 0.1% Tween-20 in Tris-buffered saline (TBS) for 1 h at room temperature and incubated overnight with primary antibodies diluted in TBS with 5% bovine serum albumin (BSA) and 0.1% Tween-20. The blocking solution was also used for the dilution of secondary antibodies. For Western blot analyses, the following primary antibodies were used: anti-caspase-3 (9665; Cell Signaling Technology), anti-Bid (2002; Cell Signaling Technology), anti-caspase-8 (551242; BD Bioscience), anti-caspase-9 (ab28131; Abcam), and anti-actin (ab8227; Abcam). Immunoreactive proteins were visualized by using secondary antibodies conjugated with horseradish peroxidase (P0448 and P0260; Dako) and a chemiluminescence detection system (ECL; Amersham).

In vivo infection assay. Male A/JolaHsd mice with the H-2d locus (Harlan, United Kingdom) and weighing 15 to 17 g were housed three per cage in sterile, pathogen-free conditions and supplied with sterile water and commercial food pellets (Topdovo, Trnava, Slovak Republic). Groups of mice (4 weeks of age) were infected intraperitoneally with CVB20 (2 × 106 TCID50s/mouse), a corresponding amount of CVB2ORD, or PBS (control) as described previously (11). Mice were sacrificed at day 5 postinoculation, a time point selected based on the previously published studies of coxsackieviruses (87, 88). Pancreas and heart tissue specimens were fixed in 4% formalin, embedded in paraffin, and sectioned (5- to 7-μm thickness) for subsequent staining with hematoxylin and eosin. Pancreatic tissues were subjected to three freeze-thaw cycles and homogenized in PBS to an ~10% organ suspension. After removal of cell debris by centrifugation and addition of antibiotics (200 U/ml penicillin and 0.2 mg/ml streptomycin), the virus titer was deter- mined by the TCID50 assay in Hep-2 cells. The animal study was conducted according to directives of the European Commission and approved by the State Veterinary and Food Administration of the Slovak Republic.

Prediction of amino acid changes in the VP1 protein. The locations of CVB2ORD-specific amino acid substitutions were mapped based on multiple sequence alignment (ClustalW) (79) with the closely related CVB3 (Protein Data Bank [PDB] accession number 1COV) (58) that identified the equivalent CVB3 residues. The X-ray crystal structure of the CVB3 capsid was then modeled and visualized using Chimera (64, 73).

Statistical analysis. Data are presented as means ± standard errors of the mean (SEM) of triplicate observations. One-way analysis of variance (ANOVA) followed by Bonferroni’s test was used for multiple comparisons, and a P value of <0.05 was considered statistically significant.

RESULTS
A surface-exposed VP1 substitution in CVB20 controls cytolysis. CVB2 is an enterovirus that causes CPE in susceptible CAR-expressing cells such as GMK and HeLa (55). A previous study has shown that by serial passages in RD cells, CVB20 can be transformed from a noncytolytic virus to a cytolytic variant, CVB2ORD (66). Sequence analysis revealed that the genome of the RD-adapted virus contained three nonsynonymous mutations, one in the 2C-encoding gene (K to R at amino acid position 185) and two in the gene that codes for the capsid protein VP1 (I to F at position 118 and Q to K at position 164) (Fig. 1A). The structural proteins of CVB2 and CVB3 share a high amino acid sequence identity (77%). Therefore, the published tertiary structure of CVB3 (58) was used to model the capsid locations of amino acid substitutions unique to the CVB2ORD variant. The highly conserved VP1 residue (Q) (67) was mapped to be exposed (εi helix) on the surface of the capsid, whereas the amino acid at position 118 (I) was positioned in proximity to the hydrophobic pocket below the canyon floor surrounding the 5-fold axis of the virion (β strand) (Fig. 1B) (66).
To characterize how individual mutations were associated with the cytolytic RD phenotype, three different CVB2O variants were constructed by employing a reverse genetics approach (Fig. 1A). The substitutions in the VP1 protein (I118F and Q164K) and 2C protein (K185R) were individually introduced into a full-length CVB2O cDNA clone. Viruses were derived from cDNA clones in RD cells that expressed DAF but not CAR (Fig. 2A). Sequencing of virus-derived cDNA clones showed that the mutations were individually tolerated and also that no other substitutions or silent mutations were introduced during viral replication. In RD cells, the different CVB2O variants, regardless of their respective propensities to cause cytolysis (Fig. 3C), were able to replicate with similar efficiency in RD cells infected with each one of the CVB2O variants, three different CVB2O variants, regardless of their respective propensities to cause cytolysis (Fig. 3C). At a later stage of the infection (72 h p.i.), a majority of RD cells subjected to virus infection showed various degrees of staining (data not shown).

The viral positive-sense RNAs of viruses adsorbed to RD cells (0 h) as well as those of synthesized progeny viruses (96 h p.i.) were quantified by real-time PCR (Fig. 3D). This quantification showed that the amount of viral RNA increased approximately 800-fold for the cytolytic vVP1Q164K virus. A significant increase of viral plus-strand RNA (100- to 400-fold) was also detected at 96 h p.i. for the noncytolytic viruses (CVB2Owt, vVP1I118F, and v2CK185R). Further, the production of infectious particles was determined by titration in GMK cells. All CVB2O variants showed similar binding characteristics to RD cells (approximately 10^5 TCID_{50}/ml) and produced similar amounts of viral progeny (approximately 10^8 to 10^7 TCID_{50}/ml) during the following 4 days (Fig. 3E).

Previously, it has been shown that several types of CVB can establish a persistent infection in RD cells (4, 29). To further characterize the noncytolytic CVB2O infection, RD cells infected with CVB2Owt were repeatedly passaged. After 10 passages, no signs of CPE could be observed (Fig. 4A) although infection was verified by detection of CVB2 antigens and by a continuous production of infectious progeny (10^6 to 10^7 TCID_{50}/ml at 96 h p.i. of each passage) (Fig. 4B and C). Sequence analysis of this progeny virus showed that none of the RD-adaptive mutations were introduced during RD cell passages. However, due to the canonical propensity of the viral RNA-dependent RNA polymerase, other mutations were observed in the 5' UTR (C to T at nucleotide position 655) or in the VP3 protein (Y to F at amino acid position 107). It is worth noting that the Q164K substitution in the VP1 protein was retained, indicating that this mutation is conserved during replication in RD cells.

An immunofluorescence assay was employed to further characterize the CVB2O infection. Uninfected and CVB2O-infected RD cells were stained with a CVB-specific antibody (24 h p.i.). Surprisingly, the production of viral antigens was equally efficient in cells infected with each one of the CVB2O variants, regardless of their respective propensities to cause cytolysis (Fig. 3C). At a later stage of the infection (72 h p.i.), a majority of RD cells subjected to virus infection showed various degrees of staining (data not shown).

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CVB2 infection was maintained during serial passages of RD cells and that the virus was released from the RD cells without signs of CPE.

Cytolytic and noncytolytic CVB2O viruses replicate with equal efficiencies. In order to compare the replication kinetics of CVB2Owt and vVP1Q164K in RD cells, one-step growth curves were established. In this analysis, the wild-type virus and the vVP1Q164K variant showed comparable replication kinetics (Fig. 5). Only minor differences at the initial phase of the infection (2 h p.i.) and at the plateau of the growth curve (96 h p.i.) were detected. Conclusively, the growth kinetics analysis did not reveal any major differences between the cytolytic and noncytolytic virus.

The VP1 Q164K substitution induces an apoptotic response. Picornaviruses have been shown to manipulate the apoptotic response of their host cells, a characteristic that is most likely associated with the pathology of these viruses (14, 30, 71). To investigate whether apoptosis plays a role during cytolytic and noncytolytic CVB2O infection in RD cells, a number of apoptotic hallmarks were analyzed. As a first step, we evaluated the level of DNA fragmentation in cells infected with virus. Nuclear DNA extracted from virus-infected cells was compared...
with DNA isolated from uninfected cells and cells treated with a chemical apoptosis inducer, STS (18). At 72 h p.i., degradation of DNA was observed in RD cells infected with the cytolytic vVP1<sup>Q164K</sup>, a result comparable to that of STS-treated RD cells (Fig. 6A). In contrast, no evident fragmentation was detected in cells infected with the noncytolytic parental CVB2O virus.

Cellular caspases, a family of cysteine proteases, are key regulators of apoptosis. They are expressed as inactive proenzymes that become activated by specific cleavage in response to apoptotic stimuli (21, 68). To further examine the CVB2O-induced apoptotic response in more detail, cell lysates from RD cells infected with CVB2Owt (MOI of 1) were cultured, and successive 1/3 passages were performed every fourth day. The release of progeny virus during noncytolytic infection was quantified at passages 1 to 10 by endpoint titration in GMK cells (mean ± SEM; n = 3). (D) Release of virus progeny from RD cells infected with CVB2Owt (MOI of 1). Extracellular (viruses present in the medium) and total (extra- and intracellular) virus yields were determined at different time points after infection by endpoint titration in GMK cells. Error bars represent the SEM (n = 3).

FIG. 5. Growth kinetics of CVB2Owt and vVP1<sup>Q164K</sup> in RD cells. RD cells were infected at an MOI of 1 TCID<sub>50</sub>/cell. Total virus yields were determined at different time points after infection by endpoint titration in GMK cells. The results shown are representative of three independent experiments.
two upstream caspases (caspase-8 and caspase-9) were analyzed. The active forms of caspase-8 (36 and 40 kDa) and caspase-9 (35 and 37 kDa) were detected in RD cells infected with vVP1Q164K. However, only the precursors of caspase-8 and caspase-9 were detected in cells infected with the noncytolytic CVB2O virus. Surprisingly, at 48 h p.i., a downregulation of procaspase-8 was observed in these cells.

The Bid protein, a proapoptotic protein of the Bcl-2 family, is an important regulator of apoptosis. Upon activation by the initiator caspase-8, the active form of Bid translocates to the mitochondria where it triggers Bax activation, which, in turn, is followed by cytochrome c efflux into the cytosol (46). To further assess the CVB2O-triggered apoptosis in RD cells, the processing of Bid was determined. The proform of Bid (22 kDa) was clearly processed into its active form (15 kDa) in RD cells infected with the cytolytic vVP1Q164K while no activation was observed for CVB2Owt-infected cells (Fig. 6B).

The association between apoptosis and the cytolytic infection (vVP1Q164K) in RD cells was also quantified by immunofluorescence based on caspase-3 activation. Only a small fraction of uninfected (~1%) and CVB2Owt-infected (~3.6%) RD cells showed staining of active caspase-3 (24 h p.i.). However, a significantly higher percentage (~12%) of cells infected with the vVP1Q164K mutant stained positively for cleaved caspase-3 (24 h p.i.).

Taken together, these results suggested that the VP1 substitution Q164K in CVB2O was associated with apoptosis in RD cells via a number of key players of the apoptotic cascade.

**CVB2ORD causes pancreatic inflammation in mice.** CVBs are known to have pancreatic and myocardial tropism in
murine models (31, 36, 38, 83, 86). In order to investigate the relevance of the cytolytic RD phenotype (CVB2ORD) in a murine model, A/J mice susceptible to CVB infection (83, 87, 88) were inoculated intraperitoneally with CVB2O or CVB2ORD. At day 5 p.i., CVB2ORD-infected mice showed mild to intense perivascular lymphocyte infiltration with focal vasculopathy (i.e., inflammation observed as bleb formation of endothelial cells in blood vessels) in the exocrine pancreas (Fig. 7A). In contrast, no visible signs of infection were observed in mice infected with CVB2O. Moreover, neither CVB2O nor CVB2ORD induced histopathological changes in the endocrine pancreas or in the heart of infected mice. Titrations of the pancreatic tissue showed that the viral titer was higher in the pancreas from mice subjected to the CVB2ORD virus variant (Fig. 7B).

DISCUSSION

In the present study, we examined specific amino acid changes associated with adaptation of CVB2O to cytolytic infection in RD cells. Our results showed that a single amino acid change on the capsid surface of CVB2O transforms the virus from a noncytolytic variant to a virus causing cytolysis. The characterization of the viral infection suggested that the CVB2O-induced cytolytic was associated with an apoptotic response (Fig. 8).

Previously published results have shown that CVB2O has the capacity to adapt to cytolytic replication in RD cells and that this novel property was associated with three nonsynonymous mutations (66). The results from reverse genetics studies with the cloned single mutants presented here demonstrated that a single surface-exposed amino acid change (Q164K) in the VP1 capsid protein of this virus is sufficient for the transformation to a cytolytic phenotype. This observation supports the view that specific capsid residues influence picornaviral cell type specificity and tissue tropism (1, 6, 15, 39, 44, 45, 62).

In detailed studies of the virus replication in RD cells by real-time PCR and titration on permissive GMK cells, the different cDNA clone-derived CVB2O variants showed similar properties regarding genome replication and virus progeny production. The productive infection of the cytolytic virus and noncytolytic CVB2O variants was further confirmed by immunofluorescence studies. This study also showed, together with the single-step growth curve and the observed onset of CPE, that there is a lag phase between the initial phase of viral replication and the CPE. The same phenomenon has previously been observed for another picornavirus, the Ljungan virus of the Parechovirus genus (28, 80). Possibly, this delay
lier onset of cytolysis than infection with vVP1 expressing all three adaptive substitutions, resulted in an ear-
CVB2ORD. However, infection of RD cells with CVB2ORD, an essential determinant for the cytolytic phenotype of
presented here suggested that this genetic change was not
CVB2O mutant expressing a single 2C substitution (K185R)
virion assembly (48, 65, 78, 84). Results from analyses of the
zymatic nucleotide triphosphatase activity, and involvement in
functional protein with reported activities, including guidance
cytolysis.

to replicate in RD cells, a property that was not linked to
cytolytic CVB2 variant remains to be elucidated. Taken
contrast, CVB2Owt causes a persistent infection in RD cells without
detectable signs of apoptosis.

of CPE is a consequence of a virus that is not yet completely
adapted to its host cell. However, the mechanism(s) in-
volved in this delay of the CPE in RD cells infected with the
cytolytic CVB2 variant remains to be elucidated. Taken
together, the CVB2Owt and all CVB2O mutants were able to
replicate in RD cells, a property that was not linked to
cytolysis.

The nonstructural 2C protein of picornaviruses is a multi-
functional protein with reported activities, including guidance
of viral replication complexes to cytoplasmic membranes, en-
zymatic nucleotide triphosphatase activity, and involvement in
virion assembly (48, 65, 78, 84). Results from analyses of the
CVB2O mutant expressing a single 2C substitution (K185R)
presented here suggested that this genetic change was not
an essential determinant for the cytolytic phenotype of
CVB2ORD. However, infection of RD cells with CVB2ORD,
expressing all three adaptive substitutions, resulted in an ear-
lier onset of cytolysis than infection with vVP1Q164K. Thus,
the additional substitution in VP1 (I118F) together with the
amino acid change of 2C contributed to the cytolytic phenot-
type in RD cells by a mechanism that remains to be elucidated.

Previously, it has been shown that CVB can establish per-
sistent infections in RD cells (4, 29). Monitoring of repeated
passages of the wild-type CVB2O in RD cells revealed a con-
tinuous release of infectious progeny although no signs of CPE
were observed. The ability of the CVB2O wild-type strain to
replicate in RD cells without evident signs of CPE has, to our
knowledge, never been characterized before. Studies of polio-
virus have shown that persistent infections may be established
when HEp-2 cells are subjected to virus at a very low MOI
(62). In contrast, the CVB2O infection of RD cells seems to be
independent of virus dosage since these cells remained persist-
tently infected even when they were exposed to a very high
viral dose (MOI of 100). The release of noncytolytic CVB2Owt
is possibly facilitated by viral proteins such as nonstructural
protein 2B, which has been shown to modify membrane permeability (23, 60, 85). Conclusively, these results suggest that
CVB2O established a persistent infection in cultured RD cells.
This may have implications for CVB2 infections in vivo where
muscle cells possibly serve as virus reservoirs. Indeed, CVB2
RNA has been detected in muscle tissue of patients with
chronic muscle diseases (3, 12, 13, 22).

The virus-host cell system represented by the different
CVB2O variants and the RD cells provided a well-defined
model system for studies of persistent and cytolytic CVB2O
infection. This model system also made it possible to examine
whether apoptosis played a role during CVB2O infection in
these cells. Theiler’s murine encephalomyelitis virus is highly
cytolytic in permissive BHK-21 cells, causing rapid cell destruc-
tion without signs of apoptosis; however, in less permissive
cells, virus growth is markedly reduced, and viral replication is
accompanied by induction of apoptosis (40, 41). Other picorna-
viruses, including CVB3, enterovirus 71, foot-and-mouth dis-
cease virus, poliovirus and avian encephalomyelitis virus, have
also been shown to interact with the cellular apoptotic pathway
(17, 19, 47, 50, 51, 63, 81). Although one-step growth analysis
demonstrated that RD cells were equally susceptible to repli-
cation of the cytolytic and persistent CVB2O variants, major
disparities were revealed when the apoptotic status of infected
cells was examined. Prior to cell cytolysis, distinctive apoptotic
hallmarks, i.e., extensive DNA degradation and activation of
caspase-8, caspase-9, and caspase-3, were observed in RD cells
infected with the cytolytic CVB2O (vVP1Q164K) variant. In
addition, the infection was accompanied by an activation of
Bid, an activation previously described for RD cells infected
with enterovirus 71 (19). Conversely, RD cells persistently
infected with the parental CVB2O virus showed no signs of
DNA degradation and caspase activation. In conclusion, these
data add to the increasing knowledge of the interplay between
picornaviruses and the cellular apoptotic pathway during in-
festation.

Several reports have suggested that the structural proteins of
picornaviruses are involved in the induction of apoptosis (33,
50, 63). For example, the VP1 protein of foot-and-mouth dis-
cease virus has been shown to activate a proapoptotic response by
binding to integrins and by deactivation of the Akt signaling
pathway (63). In other experiments, the VP3 protein of avian
encephalomyelitis virus colocalized with mitochondria (50),
whereas the VP2 protein of CVB3 interacted with a proapop-
totic factor, called Siva (33). Hence, picornaviral structural
proteins are associated with induction of apoptosis by different
mechanisms. Whether any of these mechanisms are involved in
the apoptotic response associated with the VP1 protein of
CVB2O remains to be elucidated.
Although induction of apoptosis in RD cells was linked to caspase activation, the involvement of other apoptotic mechanisms cannot be excluded. For example, the large quantities of viral products that accompany a poliovirus infection result in drastic rearrangement of the endoplasmic reticulum (ER) (72). ER-mediated stress resulting in activation of apoptosis has previously been reported for cells infected with a virus of the *Flaviviridae* family, the Japanese encephalitis virus (77). In addition, a previous study has shown that caspase inhibition does not prevent cell death of CVB3-infected HeLa cells (17). Thus, apoptotic activity is certainly not the only mechanism that can induce cell death and cytolysis during picornavirus infections. For example, the multifunctional 2A and 3C proteins are involved in the breakdown process of the cytoskeleton at the time of cell lysis (5, 42, 75). Consequently, cell lysis induced by a virus infection is probably a result of several different intracellular processes.

CVB infections cause myocarditis and pancreatitis (31, 38). As suggested by serological studies, these viruses also appear to be associated with insulin-dependent diabetes mellitus (38). Several experimental murine models have been developed to study CVB infections. In this report, a comparative study in a mouse model indicated that the cytopalytic CVB2ORG variant induced inflammation in the exocrine pancreas while no signs of inflammation were observed in pancreatic tissue of mice infected with the parental CVB2O strain. This pancreatic inflammation is consistent with other studies of CVB infections in mice, which have shown that virus-induced inflammation was located to the exocrine pancreas and not the endocrine tissue (70). However, these initial observations, including the possible association between the cytopalytic RD phenotype and the increased propensity to induce pancreatic inflammation in mice, need to be explored further.

Knowledge about virus-host cell interactions is important in order to elucidate mechanisms by which the virus causes damage and to enable development of new antiviral treatments. The present study illustrates the adaptive potential of picornaviruses, where a single capsid substitution in CVB2O played a pivotal role for both cytopalytic infection and induction of an apoptotic response in RD cells. In the absence of this amino acid change, CVB2O established a persistent, noncytopalytic infection without signs of apoptosis. Thus, this study shows that the fate of the infected cell depends on a complex balance between the host and the virus and that this balance can be disrupted by a single substitution.

**ACKNOWLEDGMENTS**

We thank Marlene Norrby for outstanding technical assistance and Kjell Edman for valuable discussions. Figure 1B was produced using the UCSF Chimera package from the Computer Graphics Laboratory, University of California, San Francisco (supported by NIH P41 RR-01081). This work was also supported by grants from the Swedish Knowledge Foundation, the University of Kalmar, the Helge Axson Johnsons Foundation, the Sparbanken Kronan Foundation, and the Slovak Ministry of Health (MZR code.2005/23-SZU-01).

**REFERENCES**


ERRATUM

A Single Coxsackievirus B2 Capsid Residue Controls Cytolysis and Apoptosis in Rhabdomyosarcoma Cells

Maria Gullberg, Conny Tolf, Nina Jonsson, Charlotta Polacek, Jana Precechtelova, Miriam Badurova, Martin Šojka, Camilla Mohlin, Stina Israelsson, Kjell Johansson, Shubhada Bopegamage, Susan Hafenstein, and A. Michael Lindberg

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Volume 84, no. 12, p. 5868–5879, 2010. Page 5875, legend to Fig. 7, line 1: “Histological analysis” should read “(A) Histological analysis.”

Page 5877. Acknowledgements, paragraph 2: Add “, the EEA Financial Mechanism, the Norwegian Financial Mechanism, and the State Budget of the Slovak Republic (SK 0082)” to the last sentence.