

## Antiapoptotic Activity of the Cardiovirus Leader Protein, a Viral “Security” Protein<sup>∇</sup>

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**Apoptosis is a common antiviral defensive mechanism that potentially limits viral reproduction and spread. Many viruses possess apoptosis-suppressing tools. Here, we show that the productive infection of HeLa cells with encephalomyocarditis virus (a cardiovirus) was not accompanied by full-fledged apoptosis (although the activation of caspases was detected late in infection) but rather elicited a strong antiapoptotic state, as evidenced by the resistance of infected cells to viral and nonviral apoptosis inducers. The development of the antiapoptotic state appeared to depend on a function(s) of the viral leader (L) protein, since its mutational inactivation resulted in the efflux of cytochrome *c* from mitochondria, the early activation of caspases, and the appearance of morphological and biochemical signs of apoptosis in a significant proportion of infected cells. Infection with both wild-type and L-deficient viruses induced the fragmentation of mitochondria, which in the former case was not accompanied with cytochrome *c* efflux. Although the exact nature of the antiapoptotic function(s) of cardioviruses remains obscure, our results suggested that it includes previously undescribed mechanisms operating upstream and possibly downstream of the mitochondrial level, and that L is involved in the control of these mechanisms. We propose that cardiovirus L belongs to a class of viral proteins, dubbed here security proteins, whose roles consist solely, or largely, in counteracting host antidefenses. Unrelated L proteins of other picornaviruses as well as their highly variable 2A proteins also may be security proteins. These proteins appear to be independent acquisitions in the evolution of picornaviruses, implying multiple cases of functional (though not structural) convergence.**

Cells that are infected with a virus recognize the invader's presence by their innate immunity machinery and switch on a variety of defensive mechanisms. The infecting virus, on the other hand, may possess tools capable of interfering with host antiviral responses. The outcome of the infection, both in terms of the efficiency of virus growth and the extent of host pathology, depends on the trade-off between these defensive and counterdefensive measures.

Cellular innate immunity involves multiple pathways, and one powerful defense is apoptosis, or the programmed self-sacrifice of the infected cell, potentially limiting viral reproduction and spread (10). However, many viruses are able to suppress this defensive mechanism (14, 37). Remarkably, virus-elicited pathology may be specific for a given type of cells and a given virus. Unraveling the interplay between pathways leading to the death or survival of the infected cells is an important task that may provide clues to understanding viral pathogenesis and, possibly, may indicate new directions for searching for antiviral drugs.

Picornaviruses are a family of small nonenveloped animal viruses that includes important human and animal pathogens

such as polioviruses, rhinoviruses, hepatitis A virus, foot-and-mouth disease viruses, and many others (89). Their genome is represented by a single-stranded 7.2- to 8-kb RNA molecule of positive polarity encoding about a dozen mature proteins (generated by the limited proteolysis of a single polyprotein precursor), nearly all of which are directly involved in the replication of the viral RNA and formation of virions (1).

The first picornavirus demonstrated to interact with the host cell apoptotic machinery by both triggering and suppressing the apoptotic response was poliovirus (95). Since then, a wealth of data has been accumulated that shows that the activation of apoptotic pathways is a widespread, though not universal, response to picornavirus infection. Thus, apoptosis-inducing capacity was reported for coxsackieviruses B3, B4, and B5 (22, 54, 82), enteroviruses 70 and 71 (25, 27, 60, 88), human rhinoviruses 1B, 9, 14, and 16 (32, 92, 100), foot-and-mouth disease virus (53, 76), avian encephalomyelitis virus (62, 63), and hepatitis A virus (16, 43) and was the subject of several recent reviews (15, 102). The antiapoptotic activity of picornaviruses was studied predominantly by using poliovirus (3, 8, 13, 72) and coxsackievirus B3 (21, 36, 85).

The present study is focused on the interaction of cardioviruses, which are representatives of a genus in the picornavirus family, with the apoptotic machinery of infected cells. Our interest in this topic stemmed from the fact that these viruses, e.g., encephalomyocarditis virus (EMCV) and its strain menogovirus (MV), as well as the less-related Theiler's murine en-

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cephalomyelitis virus (TMEV), while sharing major features of genome organization and reproductive strategy with other family members, encode a unique protein that is not found in other picornaviruses. Indeed, the leader (L) protein, a derivative of the N-terminal portion of the viral polyprotein (55), appears to be a major player in controlling the virus-host interaction. On the one hand, it is devoid of any known enzymatic activity, and L-lacking mutants are viable, at least in certain cultured cells (19, 57, 106). On the other hand, the L protein appears to inhibit host translation (35, 106), suppresses interferon production (46, 83, 98), and impairs nucleocytoplasmic traffic (11, 30, 61, 80, 81). It has been hypothesized that cardiomyovirus L protein also is involved in the interaction with defensive apoptotic machinery.

Previous studies have demonstrated that TMEV infection may induce apoptosis, especially in partially restrictive cells (50, 51). EMCV also exerted a similar effect in certain cell lines (87, 103). The reason(s) underlying variability in the apoptosis-inducing effects of cardiomyoviruses remains unexplained. Here, we demonstrate that the productive cardiomyovirus infection of susceptible HeLa cells resulted in their cytopathic death, which was not accompanied by clear signs of apoptosis. On the contrary, the infected cells acquired an antiapoptotic state, as evidenced by their failure to develop an apoptotic response to viral and nonviral apoptosis inducers. However, the antiapoptotic state failed to develop in cells infected with a mutant virus with inactivated L, and this mutant instead elicited caspase-dependent apoptosis preceded by cytochrome *c* efflux. These data suggest that the wild-type (wt) L protein is involved, directly or otherwise, in the control of viral antiapoptotic function(s).

#### MATERIALS AND METHODS

**Cells and viruses.** HeLa-B cells (95), human rhabdomyosarcoma RD cells, and baby hamster kidney BHK-21 cells were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum. wt MV was derived from the plasmid pM16.1 (34). An MV mutant encoding an L protein with a destroyed Zn finger, C19A/C22A, was described previously (46). In control experiments, poliovirus type 1 strain Mahoney was used.

**Infection and single-cycle growth experiments.** Cells were detached by EDTA treatment, plated onto 35-mm petri dishes (Corning-Costar), and cultivated overnight at 37°C under 5% CO<sub>2</sub> in Eagle medium with 10% fetal bovine serum. At the end of cultivation, the plates contained  $7 \times 10^5$  to  $9 \times 10^5$  cells. The growth medium was discarded, and the virus was added in a volume of 1 ml to provide the input multiplicity of infection (MOI) indicated. After a 30-min incubation at room temperature, the cells were washed with 2.5 ml Eagle medium, and 1 ml of serum-free Eagle medium was added. After incubation at 37°C for the indicated time intervals, the cells were detached by EDTA treatment, combined with the supernatant medium, subjected to three cycles of freezing-thawing, and frozen at -80°C until viral plaque titration.

**Plaque titration.** For EMCV titration, RD cell monolayers grown on 35-mm petri dishes for 3 days were used. The growth medium was discarded, and sample dilutions were introduced in a volume of 0.1 ml. After virus adsorption for 30 min at room temperature, the cells were overlaid with agarose (Amresco; final concentration of 0.4%), fetal bovine serum (2%), and neutral red (0.002%) in Earle's solution supplemented with 3% sodium bicarbonate and 1 mg/ml kanamycin. Plaque counts were made after a 24-h incubation at 37°C in 5% CO<sub>2</sub>. For the plaque assay of MV and its mutant, 1-day cultures of BHK-21 cells were used. The overlay in this case contained 0.9% agarose and no neutral red. After 2 or 3 days of incubation at 37°C in 5% CO<sub>2</sub>, the cultures were fixed with 10% trichloroacetic acid, the overlay was removed, and the cells were stained with 0.15% crystal violet. BHK-21 cells, rather than RD cells, were used for the titration of MV and its mutant, because the former cells are relatively more permissive for the growth of L-deficient MV mutants (106). It should be noted,

however, that the titration of MVs on BHK-21 cells produced markedly fewer plaques than on RD cells.

**Real-time PCR.** Total RNA was isolated from cell monolayers by TRI reagent (Sigma) according to the manufacturer's protocol, and its concentration was determined spectrophotometrically. Two micrograms of RNA was reverse transcribed with the avian myeloblastosis virus reverse transcriptase (Promega) primed with oligonucleotide MG5 (GAATTCCTCTGCCAATAACC; corresponding to nucleotides [nt] 6997 to 7016). The real-time reverse transcription-PCR assays were performed on a Chromo4 four-color real-time PCR system (Bio-Rad Laboratories) by using a commercially available real-time PCR kit (Syntol, Russia) according to the supplier's protocol with oligonucleotide primers MGVL1 (CGCTAGGAATGCGTAGAACA; nt 6601 to 6620) and MGVR1 (AGCTCGTCCTTGAGGAATGT; nt 6717 to 6736). Oligonucleotide MGVP1 (6-carboxyfluorescein-TGGGAAACCGCCACTTATCCC-black hole quench 1; nt 6633 to 6655) was used as the probe. The standard curve was generated by using serial dilutions (corresponding to 10<sup>2</sup> to 10<sup>7</sup> molecules) of a sucrose gradient-purified (40) full-length RNA transcript of wt MV obtained from plasmid pM16.1. The thermal cycling conditions were as follows: 5 min at 95°C for hot activation, followed by 45 cycles of 15 s at 95°C and 30 s at 61°C.

**Apoptosis induction.** Protein synthesis inhibitor cycloheximide (CHI; 100 µg/ml) or the transcription inhibitor actinomycin D (ActD; 3 µg/ml) was used as a nonviral inducer of apoptosis. To activate the apoptotic program by poliovirus infection, the viral reproduction was interrupted at 1.5 h postinfection (p.i.) by the addition of guanidine-HCl (100 µg/ml) to the infected cells at either the onset of infection or 1.5 h p.i. A broad-spectrum caspase inhibitor, Q-VD-OPH (23), hereinafter referred to as QVD, was used to suppress apoptosis.

**TUNEL assay.** Infected and mock-infected cells were grown on coverslips for the time intervals indicated, stained with Hoechst-33342, and fixed at room temperature with Safe Fix for 30 min. The cells were treated with 96 and 70% ethanol and stored at -20°C. The assay was performed by using the apoptosis detection system fluorescein kit (Promega) according to the manufacturer's instructions. The filter cube I3 was used for the registration of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling)-positive cells.

**DNA fragmentation electrophoretic assay.** DNA fragmentation was assayed essentially as described previously (95). Briefly, the cells were detached from the plastic by EDTA treatment, suspended in a buffer containing 20 mM EDTA and 10 mM Tris-HCl, pH 7.4, and lysed with 0.5% Triton X-100 for 20 min in an ice bath. The suspension was subjected to centrifugation in an Eppendorf minicentrifuge (12,000 rpm, 15 min, 4°C), and the nucleus-free supernatant was treated with phenol-sodium dodecyl sulfate (SDS). The nucleic acids were precipitated with ethanol, dissolved in 10 µl of H<sub>2</sub>O, and treated with RNase A (10 µg/ml, 37°C, 30 min). Samples were subjected to electrophoresis on 1.5% agarose gels.

**Caspase activation assay.** The caspase activity in cellular extracts was determined by measuring the cleavage of peptide Ac-DEVD-pNA, a synthetic analog of the natural substrates of caspase-3 and -7. The determination of this DEVDase activity was performed essentially as described previously (3). The cells were lysed with cell lysis buffer from the Clontech CPP-32 colorimetric detection kit. The enzymatic activity was determined in 100-µl reaction mixtures containing 45 µl of cell lysate, 100 µM Ac-DEVD-pNA (Calbiochem), 50 µl of 2× reaction buffer [100 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 7.0, 0.2 mM EDTA, 20% glycerol, 2 mM dithiothreitol], and where indicated, 10 µM zVAD-(OMe)-fmk. The optical density was measured at 405 nm. For the identification of activated caspases, extracts of virus-infected and mock-infected cells were prepared by sonication and heating for 10 min at 95°C in lysis buffer (2% SDS, 50 mM 2-mercaptoethanol, 50 mM Tris-HCl [pH 6.8]), and the conversion of procaspases into caspases was investigated by Western blotting by using anti-caspase-3 (585), anti-caspase-8 (3-1-3), and anti-caspase-9 (824) antibodies (all from Santa Cruz). Anti-actin antibodies were from Sigma.

**Mitochondria fragmentation and cytochrome *c* efflux assays.** HeLa-B cells or HeLa-B cells expressing the enhanced yellow fluorescent protein fused with the mitochondria-targeting sequence from subunit VIII of human cytochrome *c* oxidase (EYFP-mito) were used. The latter was obtained by transformation with pEYFP-mito vector (Clontech). One-day-old subconfluent monolayers on coverslips were infected and/or exposed to inhibitors for the indicated times. The immunostaining was performed as described previously (11). Briefly, infected and mock-infected cells were fixed for 20 min with 3.7% paraformaldehyde in phosphate-buffered saline (PBS), washed, and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Cells were washed three times with PBS for 5 min each and blocked with 1% bovine serum albumin (BSA) for 1 h at 37°C. The incubations with primary (anti-cytochrome *c*; Pharmingen) and secondary (anti-mouse IgG-Alexa 546 conjugate; Invitrogen) antibodies were performed at 37°C in buffer for immunofluorescence (0.1% BSA, 0.05% Tween-20 in PBS). The

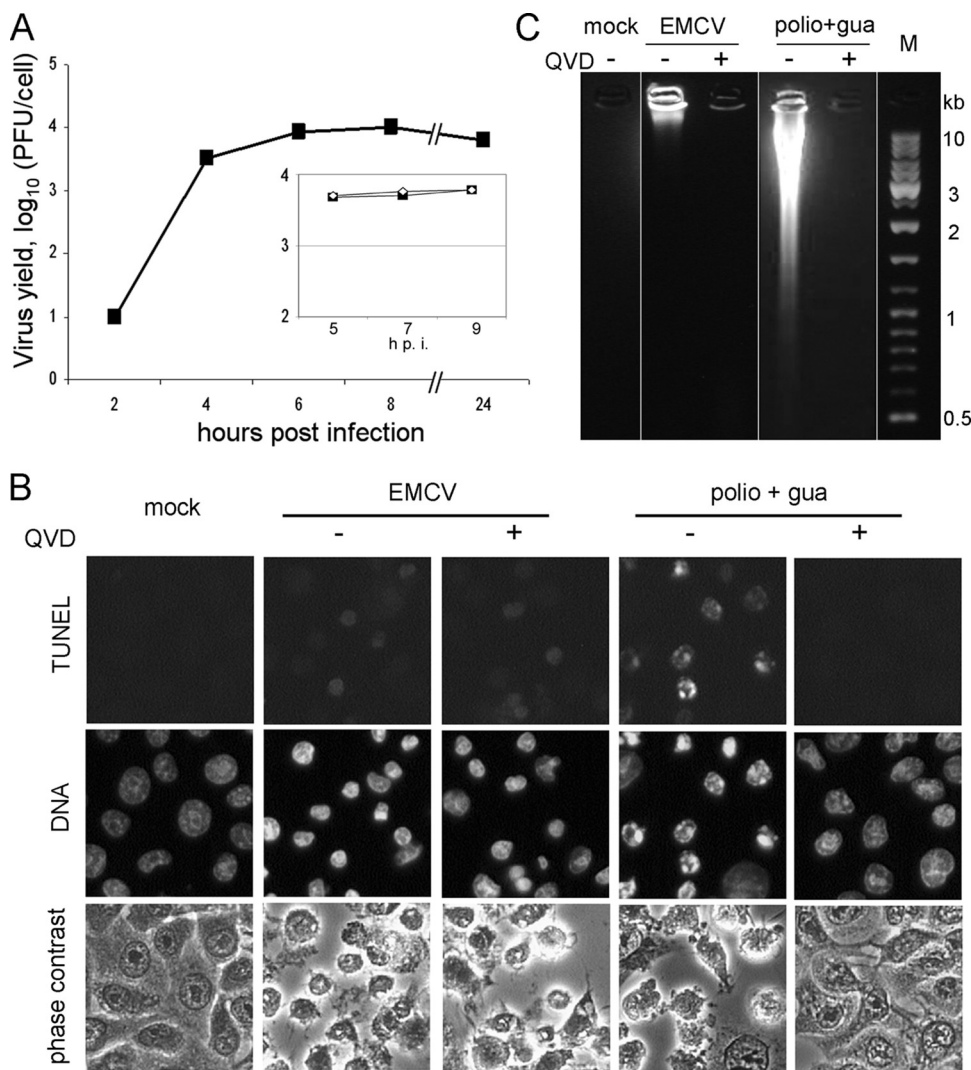


FIG. 1. EMCV reproduction in HeLa cells. (A) One-step growth curve. Cells were infected at an input MOI of ~500 PFU/cell, and the harvest was plaque assayed in RD cells. Results from a separate experiment performed in the absence (solid symbols) or presence (open symbols) of 20 μM QVD are presented in the inset. (B) Mock-infected cells, EMCV-infected cells, and cells abortively infected with poliovirus (i.e., with 100 μg/ml of guanidine-HCl added at 1.5 h p.i. [polio+gua]), all in the absence or presence of 20 μM QVD, were fixed at 7 h p.i. and stained for the TUNEL assay and with Hoechst-33342. (C) Electrophoretic investigation of DNA from samples similar to those shown in panel B.

cells then were washed three times for 5 min with the same buffer. The second wash contained Hoechst-33342 dye. The coverslips were mounted in Mowiol and inspected with Leica DMLS or Zeiss Axiovert 200M microscopes.

**Image processing.** The color images obtained by microscopy were transformed into the grayscale mode by using Adobe Photoshop 7.0. Developed films (in the Western blot assays) were scanned and processed with the same program. When appropriate, the brightness and contrast of the whole row of grayscale pictures in a given panel were equally optimized (to make visible details presented in the color images) by using the Brightness/Contrast tool of Adobe Photoshop 7.0. In the case of Fig. 2, three-dimensional image stacks underwent equal deconvolution for each fluorochrome channel using Axiovision release 3.1 software (Carl Zeiss).

**RESULTS**

**Antiapoptotic state and cytopathic (necrotic) death of HeLa cells productively infected with EMCV.** HeLa cells are permissive for EMCV, and the viral yield may approach 10<sup>4</sup> PFU/cell by 6 to 8 h p.i. (Fig. 1A). By this time, the overwhelming majority of infected cells died, exhibiting typical signs of cyto-

pathic effect, with the rounding up and complete disorganization of the cytoplasmic structure as seen by phase-contrast microscopy, as well as moderate chromatin condensation and nuclear deformation and shrinking as seen upon Hoechst staining (Fig. 1B). No ostensible signs of apoptosis could be detected in the infected cells. Indeed, nuclear fragmentation was not observed and chromatin condensation was not as intense as that seen in typical apoptosis (e.g., induced by restrictive poliovirus infection [Fig. 1B] or the nonviral apoptotic inducer ActD [see Fig. 3A]). When assayed for DNA degradation by TUNEL assay, many infected cells produced only a very faint signal (Fig. 1B). Also, only a slight degradation of cellular DNA to high-molecular-mass species could be observed at late steps of EMCV infection by electrophoretic analysis (Fig. 1C). Neither cytopathic effect nor viral yield was appreciably affected by a pan-caspase inhibitor, QVD (Fig. 1). EMCV infection did not cause any marked cytochrome *c* re-

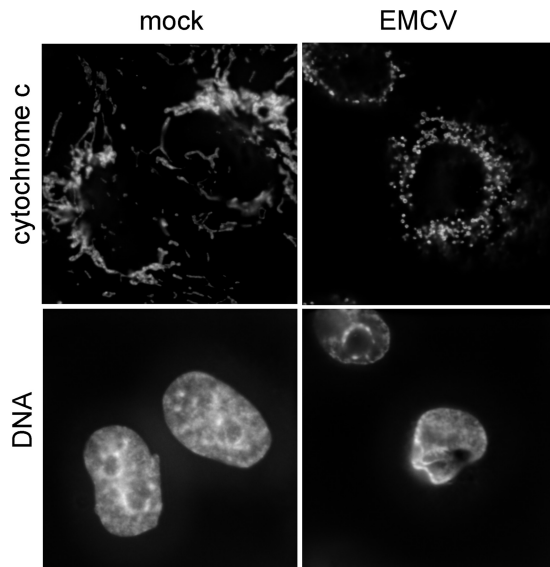


FIG. 2. Fragmentation of mitochondria without cytochrome *c* efflux upon productive EMCV infection. Mock-infected and EMCV-infected HeLa cells were incubated for 5 h, immunostained for cytochrome *c*, and stained for DNA with Hoechst-33342. Alterations in the nuclear shape and structure, typical of cytopathic effect, revealed by Hoechst staining indicated that the cells in the right panel were undergoing viral infection.

lease from mitochondria, another hallmark of apoptosis, but unexpectedly, the extensive fragmentation of mitochondria could be observed (Fig. 2). Indeed, a network of thread-like mitochondria was transformed into a set of punctated structures in the infected cell. Of note, mitochondrial fission is known to be a factor involved in the control of apoptotic machinery (24, 77, 104).

Remarkably, EMCV induced an antiapoptotic state, as evidenced by the resistance of the infected cells to a nonviral apoptotic inducer. The treatment of uninfected cells with 3  $\mu\text{g/ml}$  of ActD for 5 h resulted in the development of apoptosis in a significant proportion of cells, judging by morphological (cytoplasmic blebbing, strong chromatin condensation, and nuclear fragmentation) and biochemical (positive TUNEL signal and DNA fragmentation to oligonucleosome-sized fragments) criteria. All of these alterations could be prevented by QVD, confirming that ActD treatment led to a bona fide caspase-dependent apoptosis. The ActD-induced apoptosis was strongly, if not completely, suppressed in EMCV-infected cells (Fig. 3A to C). EMCV infection also prevented cytochrome *c* efflux from mitochondria caused by ActD treatment (Fig. 3D). Since EMCV prevented mitochondria damage-mediated cytochrome *c* efflux, we conclude that the viral antiapoptotic mechanism operated, at least in part, at the mitochondrial level or upstream of mitochondria. The yield of infectious cardioviruses is known to be essentially unaffected by ActD, and in the experiment shown in Fig. 3,  $\sim 1.2 \times 10^4$  PFU/cell were produced by 5 h p.i. in the presence and absence of the drug. ActD did not prevent cell destruction caused by EMCV (Fig. 3).

As shown previously (2, 95) (Fig. 1), restrictive poliovirus infection induces apoptosis as a result of the activation of a mitochondrion-dependent and caspase-dependent pathway

(13). This apoptosis also was suppressed by coinfection with MV (Fig. 4).

All of these data strongly suggested that EMCV infection switched on an antiapoptotic program in the infected cells.

**Mutant cardiovirus with impaired L protein induced apoptosis.** To ascertain the possible involvement of cardiovirus L protein in the control of apoptotic machinery, an L-deficient variant of MV with two mutations, C19A/C22A, destroying the Zn finger motif of the protein (46) was investigated. Previous studies showed that the destruction of this motif resulted in the functional inactivation of L (46, 61).

At 5 h p.i., the C19A/C22A mutant produced usually, but not always, severalfold fewer infectious virus particles than did its wt counterpart, and this difference was somewhat increased by 7 h p.i. (Fig. 5A). However, in some experiments, the mutant and wt MV exhibited more similar growth curves (not shown). The quantification of viral RNA molecules by real-time PCR (Fig. 5B) demonstrated a good parallelism between RNA replication and the generation of infectious progeny, with very close ratios of the number of RNA molecules to PFU for both the wt and C19A/C22A mutant (not shown). As could be expected, this ratio somewhat decreased with time, likely reflecting the maturation of virions. Infection with the mutant ended up with complete cell destruction (Fig. 6A).

In distinction from the wt MV, the L-deficient mutant elicited a marked apoptosis in HeLa cells, as judged by cytoplasmic blebbing, chromatin condensation, nuclear fragmentation, TUNEL assay (Fig. 6A, B), and DNA laddering (Fig. 6C) criteria. None of these signs of apoptosis could be seen in the mutant-infected cells incubated in the presence of QVD (Fig. 6).

One may argue that the activation of the apoptotic program is due to a somewhat less efficient reproduction of the L-deficient mutant. Indeed, the interruption of ongoing poliovirus reproduction at an early step by the inhibition of translation by a low dose (10  $\mu\text{g/ml}$ ) of CHI (by itself not inducing apoptosis in a significant proportion of cells) resulted in the suppression of the viral antiapoptotic program and the development of apoptosis (3). We investigated whether a similar treatment also would activate the apoptotic program in cardiovirus-infected cells. CHI addition at 1.5 h p.i. diminished the yield of infectious virus by  $\sim 10^3$ -fold. However, such a treatment did not lead to an increase in the proportion of TUNEL-positive cells in the EMCV-infected population, although the fraction of such cells in the uninfected population slightly increased (Fig. 7). As expected (3), CHI addition to poliovirus-infected cells, which were used as a control, resulted in the development of an appreciable apoptotic response. We concluded that a significant suppression of ongoing cardiovirus reproduction did not result, by itself, in the development of apoptosis.

Thus, in distinction from wt cardioviruses inducing an antiapoptotic state, L-deficient MV activated the apoptotic program, suggesting that L protein was involved, directly or otherwise, in the control of the apoptotic machinery of the infected cells.

**The apoptotic pathway activated by L-deficient cardiovirus mutants.** The prevention by QVD of apoptosis triggered by the L<sup>-</sup> mutant (Fig. 6) suggested the involvement of a caspase(s). This conclusion was supported by measuring the DEVDase

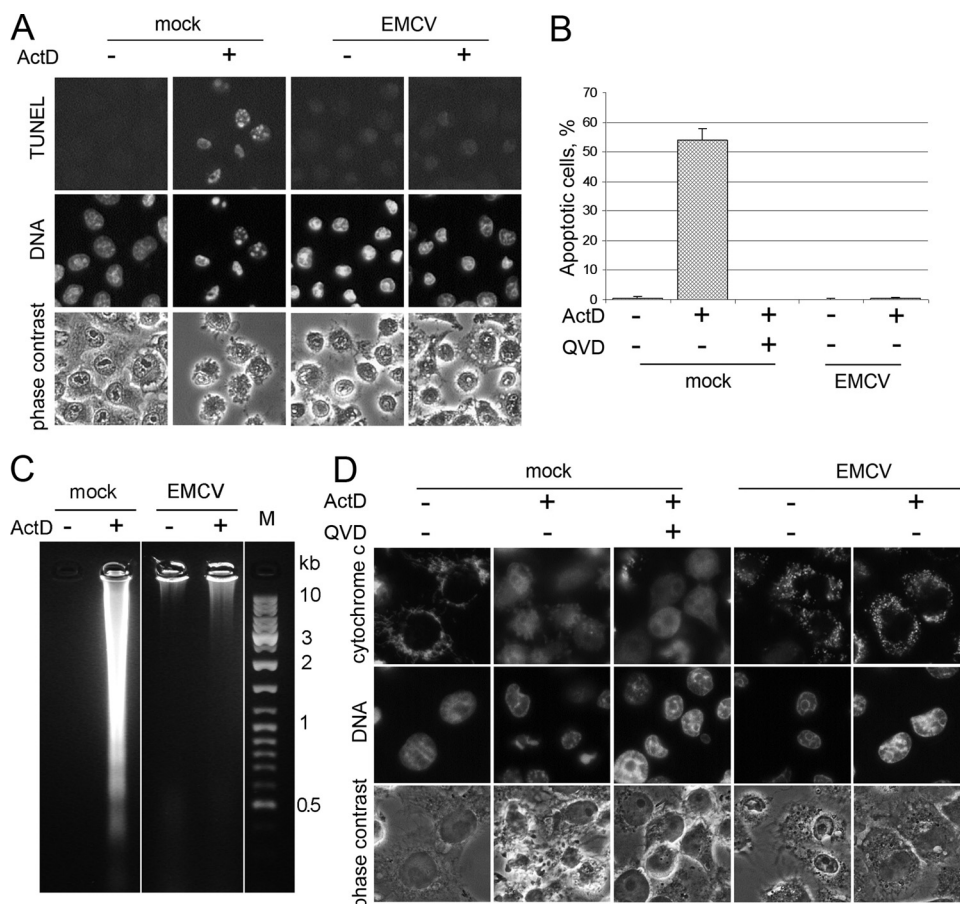


FIG. 3. EMCV inhibition of ActD-triggered apoptosis. (A) HeLa cells were preincubated in serum-free medium for 1.5 h and then mock infected or EMCV infected and incubated at 37°C for 5 h in the absence and presence of 3 μg/ml ActD. The cells were stained for the TUNEL assay and with Hoechst-33342. (B) Quantification of TUNEL-positive cells in the cell populations from the experiment shown in panel A. (C) Electrophoretic investigation of DNA from cell samples shown in panel A. (D) Samples similar to those shown in panel A were immunostained for cytochrome *c*.

activity, i.e., the capacity to cleave DEVD-pNA, a synthetic analog of the natural substrates of caspase-3 and -7. As shown in Fig. 8A, infection with the C19A/C22A mutant resulted in a marked and early (i.e., already at 5 h p.i.) activation of a DEVDase. This activity was only slightly increased by 7 h p.i., and it was practically fully inhibited by QVD. The cells infected with wt MV also demonstrated an increase in DEVDase activity, which, at 5 h p.i., was significantly lower than that of the mutant-infected cells, and approached a similarly high level during the next 2 h. A comparable increase in DEVDase activity also was observed in cells infected with EMCV (not shown). The ability of wt virus to activate DEVDase activity was unexpected, because no obvious signs of apoptosis were observed under such conditions.

To identify the caspase(s) activated upon infection, immunoblot assays were performed. The results (Fig. 8B) demonstrated the proteolytic cleavage (suggestive of activation) of caspase-9 and -3 and, to a lesser extent, of caspase-8 in the L-deficient mutant-infected cells already at 5 h p.i., with more intense bands of proteolytic products at 7 h p.i. Some activation, albeit less prominent and occurring later (i.e., at 7 h p.i.), was observed in wt-infected cells, in line with the results of the

DEVDase assay. Similar proteolytic products also were detected in cells undergoing CHI-elicited apoptosis. QVD prevented the processing of procaspases (in the case of caspase-3, an aberrant proteolytic product was reproducibly observed in samples treated with the inhibitor).

To further characterize the pathway leading to apoptosis in cells infected with the L<sup>-</sup> mutant, we looked at the status of mitochondria and cytochrome *c*. To this end, cells expressing the enhanced yellow fluorescent protein fused with the mitochondria-targeting sequence from human cytochrome *c* oxidase (EYFP-mito) were generated. An advantage of these cells consisted in the possibility to evaluate mitochondrial fragmentation independently of cytochrome efflux. The former could be judged by the altered pattern of fluorescence emitted by EYFP (transition from thread-like to punctated signals), whereas the latter could be seen as diffuse clouds after immunostaining with anti-cytochrome antibodies. In cells infected with the C19A/C22A mutant, mitochondria were fragmented and the extensive exit of cytochrome *c* from mitochondria could be demonstrated by 7 h p.i. (Fig. 9A). The proportion of the mutant-infected cells exhibiting cytochrome *c* efflux was significantly greater than that in cells rendered apoptotic after

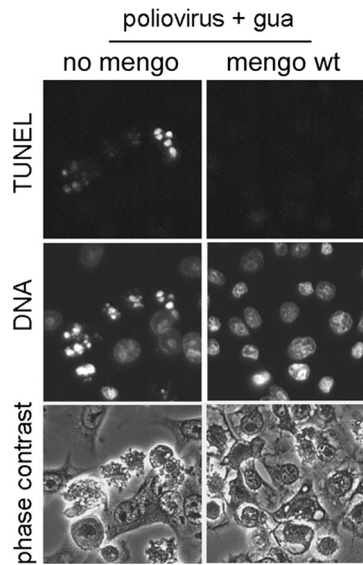


FIG. 4. MV (mengo) inhibition of apoptosis triggered by restrictive poliovirus infection. HeLa cells were infected with poliovirus (MOI,  $\sim 800$  PFU/cell), and simultaneously portions of samples (the right column) were infected with wt MV (MOI,  $\sim 10$  PFU/cell). Guanidine-HCl (100  $\mu$ g/ml) was added at 1.5 h p.i. to all cultures (poliovirus + gua). The cells were fixed at 7 h p.i. and stained for the TUNEL assay and with Hoechst-33342. The yield of MV was not appreciably changed in the presence of guanidine.

treatment with CHI (Fig. 9B). On the other hand, nearly no virus-infected cells demonstrated the exit of cytochrome *c* from fragmented mitochondria (in line with data in Fig. 2). The anti-caspase drug QVD failed to prevent mitochondrion fission in cells infected with wt and L-deficient MV, indicating that this fission took place upstream of caspase activation; the cytoplasmic exit of cytochrome *c* in mutant-infected cells also was not prevented (Fig. 9).

Thus, infection with the L-deficient cardiovirus activated a caspase-dependent, cytochrome *c*-mediated apoptotic pathway. This activation appeared to be due, at least in part, to alterations in the mitochondrial structure, and in particular to

the enhanced permeability of its outer membrane, allowing cytochrome *c* efflux. The mitochondrial injury occurred through a caspase-independent mechanism, since neither fragmentation nor cytochrome *c* efflux triggered by the mutant were prevented by the caspase inhibitor.

## DISCUSSION

**Apoptosis-triggering and apoptosis-preventing functions of cardioviruses.** The results presented here demonstrate that EMCV is capable of switching on two opposing pathways in HeLa cells, the host defensive apoptotic response and a viral antiapoptotic counterdefense. As a result of the dominance of the latter, the cells died without obvious signs of apoptosis. In possessing both apoptosis-triggering and apoptosis-suppressing activities, cardioviruses resemble poliovirus (3, 8, 9, 13, 95) and coxsackievirus B3 (22, 36, 85). It is yet to be determined whether other representatives of the picornavirus family also are equipped with these opposing functions.

The competition between the opposing programs may involve multiple players and is influenced by a variety of genetic (viral and cellular), physiological, and environmental factors. The contribution of host factors is illustrated by the ability of cardioviruses to induce apoptosis in some, but not all, types of cells (5, 50, 51, 87, 103). Furthermore, the L-deficient MV, exhibiting a highly apoptogenic activity in HeLa cells, failed to elicit apoptosis in a subline of BHK-21 cells, which proved to be resistant to certain nonviral apoptotic inducers as well (unpublished data). For poliovirus, variable responses of apoptotic machinery of different cells also were reported: this virus, being unable to induce apoptosis in productively infected HeLa cells (2, 95), triggered apoptosis in others (4, 9, 84). On the other hand, abortive poliovirus infection fails to trigger apoptosis in RD cells (84).

One of the possible reasons for the variation of the outcomes of cardiovirus-cell interactions could be the rate of the accumulation of L in the infected cells as well as the biological relevance of cellular targets of L in specific cells. The time course and efficiency of different steps of viral reproduction in these cells also may affect this outcome. The consequences of

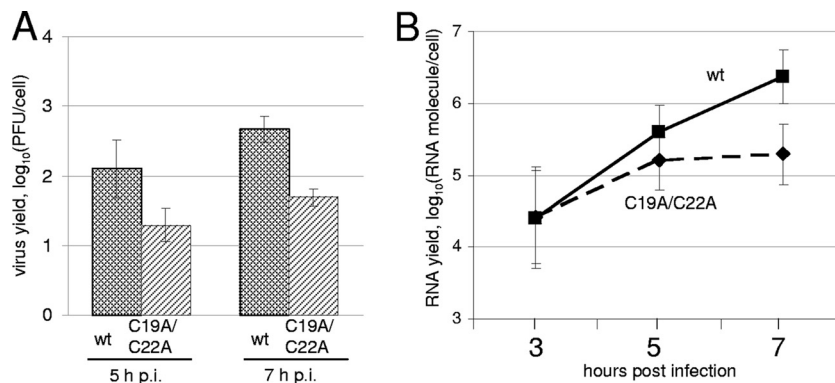


FIG. 5. Effects of mutations in the L protein on MV reproduction in HeLa cells. Accumulation of infectious virus (A) and viral RNA molecules (B) during the one-step growth of wt and C19A/C22A MVs, as determined by plaques assay and real-time PCR, respectively. The input MOI for both viruses was  $\sim 20$  PFU/cell. Average data  $\pm$  standard deviations for seven independent experiments are shown in panel A, and those for two (at 3 h p.i.) and three (at 5 and 7 h p.i.) independent experiments are shown in panel B. At least three replicate cultures were investigated at each time point in each real-time experiment.

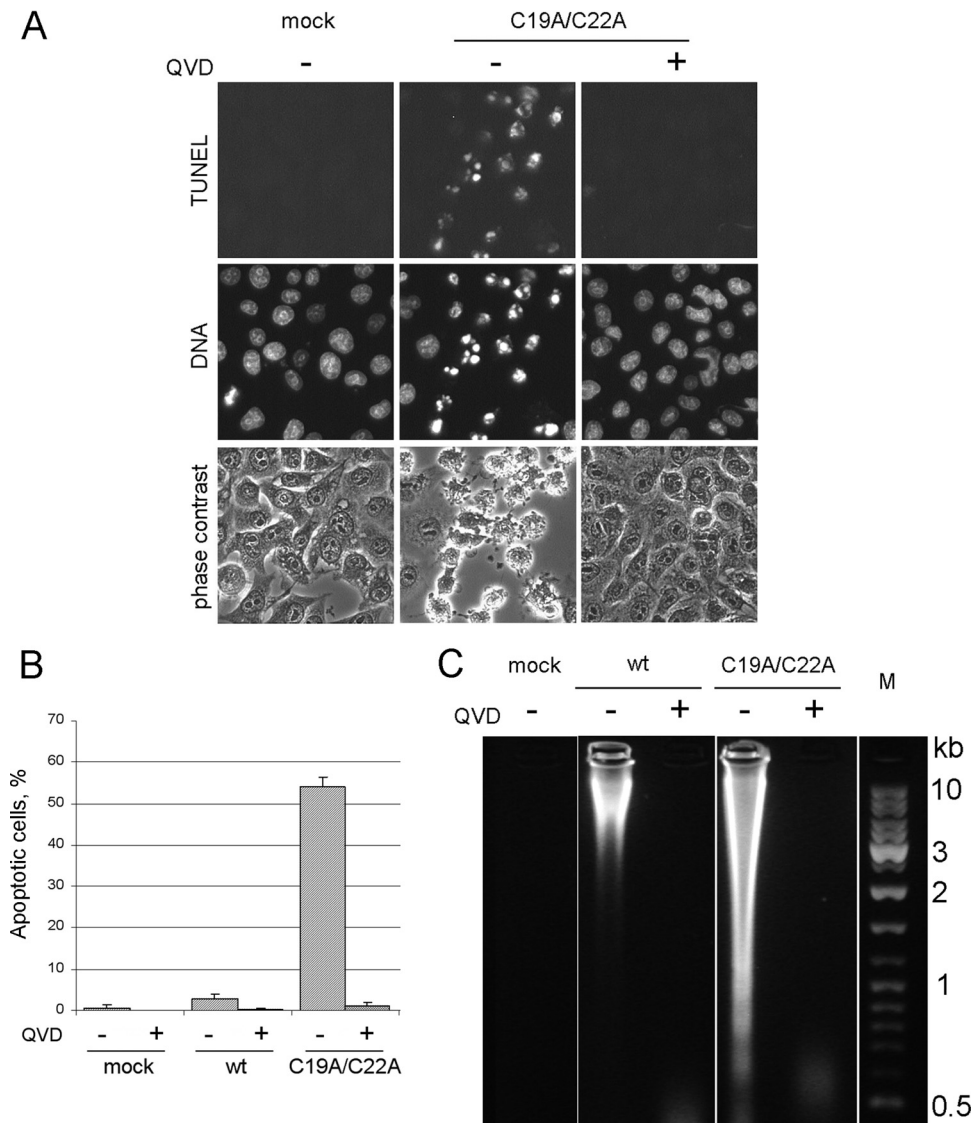


FIG. 6. Apoptosis-triggering activity of L-deficient MV mutant. (A) HeLa cells were mock infected or infected (MOI, ~20 PFU/ml) with wt MV or its L<sup>-</sup> mutant for 7 h at 37°C, and then they were stained with Hoechst-33342 and for the TUNEL assay. Appropriate samples were incubated with 20 μM QVD. The virus yields in the absence and presence of the inhibitor were essentially the same, 257 and 285 PFU/cell, respectively. (B) Quantification of TUNEL-positive cells in samples of cells in the experiment shown in panel A. (C) Electrophoretic investigation of DNA from mock-infected and virus-infected cells.

the trade-off between viral apoptosis-inducing and apoptosis-preventing activities are not expected to be uniform either. In certain picornavirus/cell systems, the inhibition of apoptosis may adversely affect viral reproduction and/or externalization (32, 65). In contrast, the yield of infectious L-deficient MV in HeLa cells was essentially unaffected by QVD, a caspase inhibitor.

Accordingly, one should be cautious in extrapolating observations made in one type of cells to another one, and especially to the events occurring in the infected organism. TMEV (86, 96, 97), porcine EMCV (17), and poliovirus (39) were reported to elicit apoptosis in certain cells of animals. It is not clear if these apoptotic events always developed as a direct consequence of viral reproduction in the target cells or if they were due to some secondary, e.g., immunological, reaction (52). If

the former explanation is true, it means that the viral antiapoptotic function was not efficient enough to overcome the apoptotic responses in these cases. However, this should not necessarily be so in some other in vivo settings.

**The apoptotic program elicited by cardiomyoviruses.** The activation of the apoptotic program upon cardiomyovirus infection becomes evident when the viral antiapoptotic function(s) is suppressed, as is the case in infection with L-deficient mutants (another MV mutant with a nearly complete deletion of L protein exhibited apoptosis-inducing activity similar to that of the Zn finger mutant described above; unpublished data). Under these conditions, numerous hallmarks of apoptosis were observed: cytochrome *c* exit from mitochondria, the activation of caspase-9, caspase-3, and caspase-8, cytoplasmic blebbing, chromatin condensation, nuclear fragmentation, and extensive

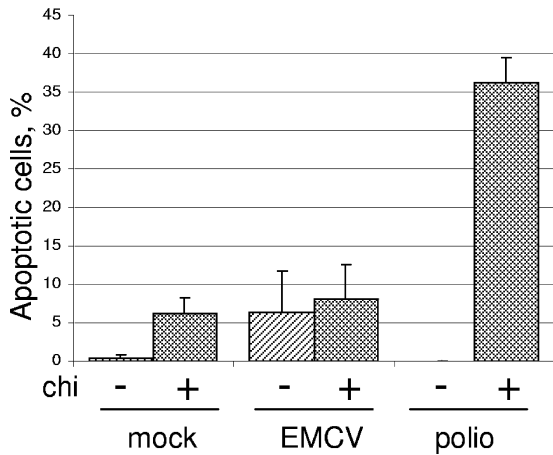


FIG. 7. Effect of the interruption of EMCV and poliovirus (polio) reproduction on the development of apoptotic response. HeLa cells were infected with EMCV or poliovirus (both at an MOI of ~500 PFU/cell), and CHI (final concentration, 10 µg/ml) was added to appropriate samples at 1.5 h p.i. Cells were fixed at 7 h p.i., and the proportion of TUNEL-positive cells was determined.

DNA degradation (as revealed by both TUNEL assay and electrophoretic analysis). Collectively, these alterations pointed to the involvement of a mitochondrion-dependent, cytochrome *c*-dependent, and caspase-dependent apoptotic pathway(s).

Picornaviruses are known to activate apoptotic pathways through interaction with plasma membrane receptors or intracellular sensors (8, 15, 53, 102). Viral capsid proteins were

implicated in triggering apoptosis both from without (9, 53, 76) and from within the cell (47, 62). Other possible players are viral proteases 3C and 2A (18, 26, 42, 60, 105) and viral non-structural proteins 2B, 3A (64), and 2C (63). Double-stranded RNA also may serve as an apoptosis inducer (49, 99). However, the exact nature of cardiовiral activator(s) of the apoptotic pathway(s) and of their immediate cellular targets have yet to be elucidated.

An intriguing observation made in this study was mitochondrial fragmentation in the virus-infected cells. In other systems, mitochondrial fission may take place either upstream or downstream of the mitochondrial membrane permeabilization, a key step in the mitochondrion-dependent apoptotic pathways (24, 77, 104). A possibility that this fission is an upstream event in the cardiовirus-activated apoptotic pathway is in line with the recently reported ability of poliovirus 2B and 3A proteins to induce the fragmentation of the organelle (64). However, further research is needed to ascertain the significance of mitochondrial fission for cardiовirus-triggered apoptosis.

**The antiapoptotic program elicited by cardiовiruses.** The nature of the cardiовirus antiapoptotic program is even less certain. Only limited information is available on the antiapoptotic tools exploited by other picornaviruses. Coxsackievirus B3 (36) and poliovirus (8) were reported to activate the phosphatidylinositol 3-kinase/Akt signaling pathway, thereby promoting cell survival. 2B protein of the former virus may suppress the apoptotic program by changing intracellular distribution of Ca<sup>2+</sup> (21). Other antiapoptotic mechanisms used by enteroviruses may involve the inactivation or degradation of caspases (13, 85) and the depletion of receptors of extracellular pro-

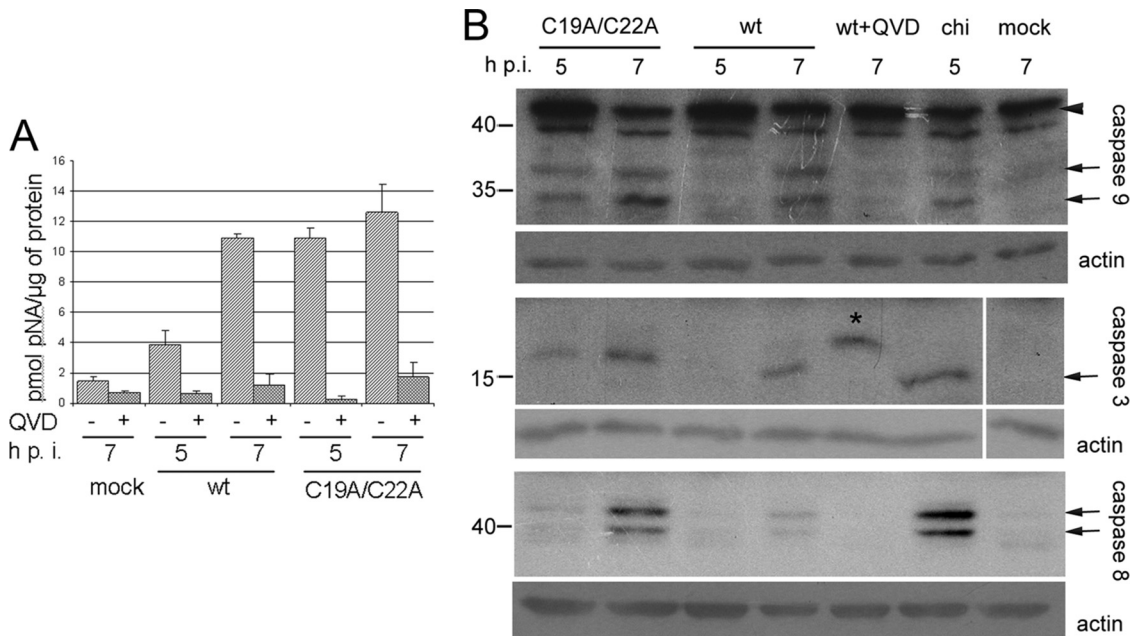


FIG. 8. Effects of infection with wt and C19A/C22A mutant MVs on caspase activation. (A) DEVDase activation. HeLa cells were infected with appropriate viruses for 7 h, and DEVDase activity was assayed in cell extracts prepared at 5 and 7 h p.i. as described in Materials and Methods. (B) Western blot analysis of partial proteolysis of caspase-3, -8, and -9 in virus-infected cells at 5 and 7 h p.i. and in cells treated with 100 µg/ml of CHI for 5 h. The bands corresponding to procaspase-9 and processed caspases are marked by arrowhead and arrows, respectively. A slower-migrating band in the caspase-3 assay in the presence of QVD (asterisk) was reproducible in the inhibitor-containing samples; its nature was not further investigated.



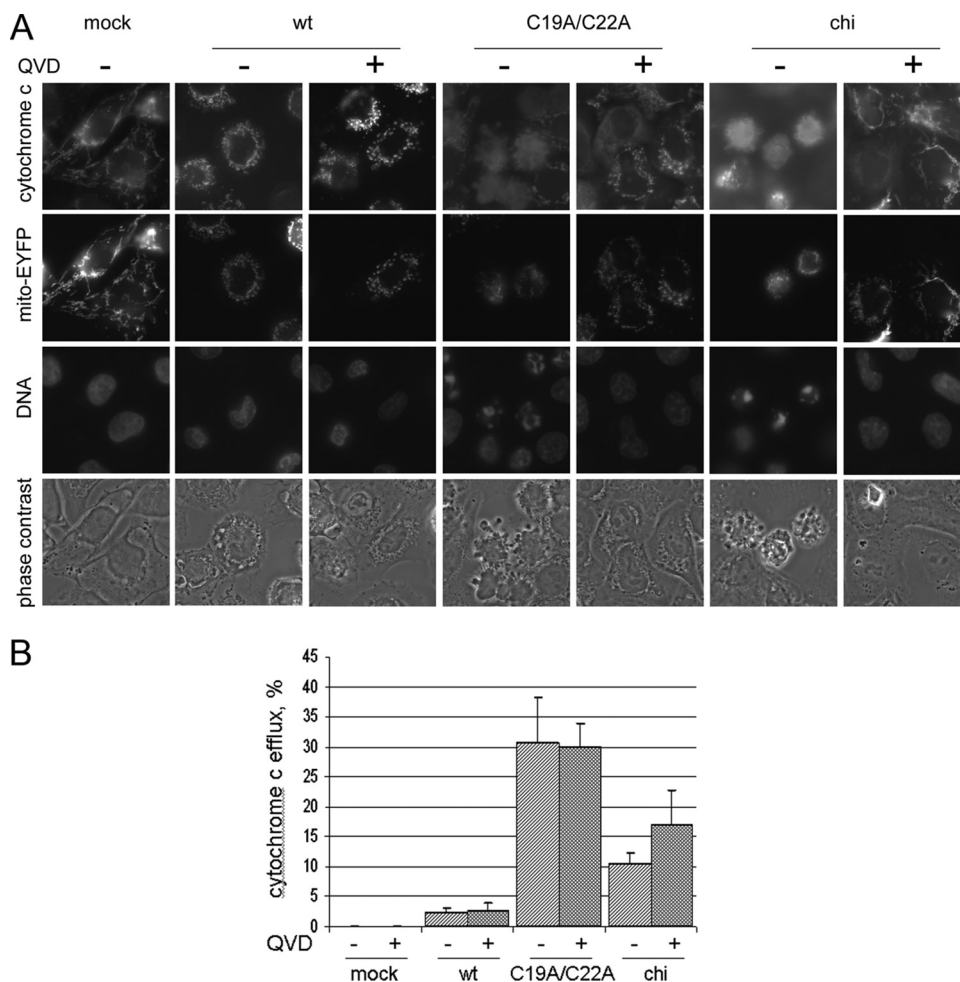


FIG. 9. Fragmentation of mitochondria and cytoplasmic exit of cytochrome *c* in cells infected with the L mutant. (A) HeLa cells expressing mitochondria-targeted EYFP (EYFP-mito) were infected with wt and C19A/C22A MVs for 7 h or were treated with 100 μg/ml of CHI for 4 h and investigated for the localization of cytochrome *c* by indirect immunofluorescence or mitochondrial integrity by the direct observation of EYFP. Appropriate samples were incubated in the presence of 20 μM QVD during infection or CHI treatment. DNA was stained with Hoechst 33342. (B) Percentage of cells demonstrating cytochrome *c* efflux.

apoptotic ligands (72). In other viruses, a much greater variety of viral antiapoptotic tools are known (6, 20, 28, 68, 73, 74, 93, 94, 101). These tools may operate at transcriptional or posttranscriptional levels, with targets in mitochondria as well as upstream or downstream of them. The antiapoptotic state elicited by cardioviruses may exploit some of these mechanisms.

This study implicates cardiovirus L protein in the viral antiapoptotic activity; indeed, its inactivation led to the development of apoptosis. The mechanism of this antiapoptotic function has yet to be determined. It cannot be ruled out that it is associated, at least in part, with the less efficient growth of L<sup>-</sup> mutants. Indeed, the balance of viral apoptosis-triggering and apoptosis-suppressing functions, whatever their nature, could well depend on the time course of the synthesis of relevant proteins. This may explain the apoptotic activity of wt cardioviruses under partially restrictive conditions in certain systems (50, 51). However, the somewhat impaired reproduction of L-deficient mutants is hardly a decisive cause of their apoptotic activity. The difference between the infectious particles (or RNA molecules) of wt and L<sup>-</sup> virus produced by 5 h p.i. (when

there already was a marked apoptosis in the mutant-infected cells; not shown) was relatively small. Moreover, the interruption of cardiovirus reproduction by a low dose of CHI did not lead to apoptosis. We propose that L protein has a more direct target(s) among the components of the cellular apoptotic/antiapoptotic machinery.

As mentioned above, we detected the fragmentation of mitochondria upon infection with wt virus, when the development of apoptosis essentially was suppressed. This fission was not accompanied with the exit of cytochrome *c* into the cytoplasm. Therefore, one may speculate that the wt EMCV-elicited mitochondrial fission is involved in the viral antiapoptotic function rather than representing an upstream apoptotic event. Indeed, it was reported that mitochondrial fission may suppress apoptosis in some cases, e.g., upon ceramide treatment, oxidative stress, or serum deprivation (77, 91). Under these conditions, a major proapoptotic factor is the redistribution of Ca<sup>2+</sup> from endoplasmic reticulum stores to mitochondria. The fragmentation of these organelles would result in impeding the propagation of Ca<sup>2+</sup> waves through the mitochondrial network

(77, 91). If mitochondrial fission plays a role in the cardiovirus antiapoptotic activity, one may further speculate that an alteration in  $Ca^{2+}$  distribution is an important component of the apoptotic response to EMCV infection. It is noteworthy that the expression of EMCV protein 2B leads to a decrease in  $Ca^{2+}$  content within the endoplasmic reticulum (29).

Interestingly, a viral protein is known that exerts its antiapoptotic function via mitochondrial fission. The immediate-early protein vMIA of human cytomegalovirus (41) recruits proapoptotic protein Bax to mitochondria and somehow inactivates it there (7, 79); this process is associated with the disruption of the mitochondrial network (67). The antiapoptotic state elicited by vMIA is effective against nonviral apoptosis inducers (66). However, we were unable to reveal any significant similarity between amino acid sequences of vMIA and cardiovirus L.

Another intriguing observation was the activation of several caspases in nonapoptotic HeLa cells infected with wt cardioviruses. The activation of caspase-8, -9, -3, and -7 late upon EMCV infection (at 7 and 24 h p.i.) also was reported by others (49). A broad group of viral antiapoptotic proteins are known to counteract various caspase activators or caspases themselves (20, 85). However, the interruption of an apoptotic pathway downstream of caspase activation is rather puzzling. It suggests the existence of another cardiovirus antiapoptotic function, operating downstream of caspases and suppressing significant DNA degradation. Further research is needed to clarify this point.

Cardiovirus L exhibits a variety of effects on the innate immunity system (see below). It has yet to be elucidated how and whether these diverse effects are related to each other.

It should be noted that the genome of some, but not all, strains of TMEV contains, within the L-coding gene, an out-of-frame sequence encoding a short polypeptide, called L\* (58), which was reported to exhibit antiapoptotic activity (38, 48). However, EMCV does not express L\*, and EMCV L has no structural resemblance to L\* of TMEV-like viruses.

**Cardiovirus L protein as a security protein.** The leader proteins of cardioviruses are not essential for viral reproduction, as evidenced by the viability of L-deleted mutants in certain cells (19, 57, 106). These proteins appeared to target predominantly host metabolism and infrastructure by inhibiting host translation (35, 106), interfering with the interferon system (46, 83, 98, 107), increasing the bidirectional permeability of the nuclear pores and inhibiting active nucleocytoplasmic transport (11, 30, 61, 80, 81), and suppressing apoptosis (this study). We suggest that such nonessential anti-host proteins could be named security proteins.

Bearing the same name but being structurally unrelated, the L protein of aphthoviruses, a protease, also is not essential (78), but it inhibits host translation by the cleavage of translation initiation factors and disables the host interferon system (31, 33, 45). Remarkably, 2A protein of cardioviruses, being nonessential though important (69, 90, 108), also exhibits anti-host functions by inhibiting cell translation (44). 2A protease proteins of enterovirus and rhinovirus (unrelated to 2A proteins of cardioviruses), though apparently essential (70), also perform a set of anti-defensive functions very similar to those exhibited by L proteins of cardioviruses and aphthoviruses, namely the inhibition of host translation (59), the disruption of

controllable nucleocytoplasmic traffic (12, 75), the suppression of interferon action (71), and others (56). We propose that L proteins of aphthoviruses as well as 2A proteins of cardioviruses, enteroviruses, and rhinoviruses also be included in the group of security proteins. It seems likely that this group will be joined by other picornavirus proteins.

The fact that the picornavirus security proteins, despite their structural unrelatedness, serve similar functions (largely or even solely neutralizing host antidefenses) strongly suggests that they had been acquired independently, and perhaps relatively recently, in the course of evolution, and that the major driving force behind these acquisitions was positive selection for anti-defensive capabilities.

Obviously, the possession of security proteins is not an exclusive privilege of picornaviruses.

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#### ADDENDUM IN PROOF

After the article was revised, we became aware of a paper by Fan et al. (J. Fan, K.-N. Son, S. Y. Arslan, Z. Liang, and H. L. Lipton, *J. Virol.*, **83**:6546–6553, 2009) reporting that ectopic expression of individual Theiler's murine encephalomyelitis virus (BeAn strain) leader protein in BHK-21 cells resulted in the development of apoptosis. It therefore seems that cardiovirus L proteins may, depending on conditions, exhibit either antiapoptotic or proapoptotic activity.

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