The Coxsackievirus 2B Protein Suppresses Apoptotic Host Cell Responses by Manipulating Intracellular Ca\(^{2+}\) Homeostasis*

Michelangelo Campanella\textsuperscript{a}, Arjan S. de Jong\textsuperscript{b}, Kjerstin W. H. Lanke\textsuperscript{b}, Willem J. G. Melchers\textsuperscript{b}, Peter H. G. M. Willems\textsuperscript{b}, Paolo Pinton\textsuperscript{b}, Rosario Rizzuto\textsuperscript{b}, and Frank J. M. van Kuppeveld\textsuperscript{a,\textsuperscript{**}}

From the \textsuperscript{a}Department of Experimental and Diagnostic Medicine, Section of General Pathology and Center for the Study of Inflammatory Diseases, Via Borsari 46, I-44100 Ferrara, Italy and the Departments of \textsuperscript{b}Medical Microbiology and \textsuperscript{c}Biochemistry, Nijmegen Center for Molecular Life Sciences, University Medical Center Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

Enterviruses, small cytoplasmic RNA viruses, confer an antiapoptotic state to infected cells in order to suppress infection-limiting apoptotic host cell responses. This antia apoptotic state also lends protection against cell death induced by metabolic inhibitors like actinomycin D and cycloheximide. The identity of the viral antiapoptotic protein and the underlying mechanism are unknown. Here, we provide evidence that the coxsackievirus 2B protein modulates apoptosis by manipulating intracellular Ca\(^{2+}\) homeostasis. Using fluorescent Ca\(^{2+}\) indicators and organelle-targeted aequorins, we demonstrate that ectopic expression of 2B in HeLa cells decreases the Ca\(^{2+}\) content of both the endoplasmic reticulum and the Golgi, resulting in down-regulation of Ca\(^{2+}\) signaling between these stores and the mitochondria, and increases the influx of extracellular Ca\(^{2+}\). In our studies of the physiological importance of the 2B-induced alterations in Ca\(^{2+}\) signaling, we found that the expression of 2B suppressed caspase activation and apoptotic cell death induced by various stimuli, including actinomycin D and cycloheximide. Mutants of 2B that were defective in reducing the Ca\(^{2+}\) content of the stores failed to suppress apoptosis. These data implicate a functional role of the perturbation of intracellular Ca\(^{2+}\) compartmentalization in the enterviral strategy to suppress intrinsic apoptotic host cell responses. The putative down-regulation of an endoplasmic reticulum-dependent antiapoptotic pathway is discussed.

Many viruses are endowed with the potential to manipulate cell death pathways in order to prevent premature abortion of the infectious cycle (1, 2). The molecular mechanism by which enterviruses manipulate the life span of their host cell is, as yet, poorly understood. Enterviruses (coxsackievirus, poliovirus, and ECHO virus) are nonenveloped, cytoplasmic RNA viruses that replicate their genome at the secretory pathway-derived membrane vesicles that accumulate in the cytoplasm of the infected cell (3). Classically, lytic viral replication is believed to induce canonical cellular necrosis by destruction of the plasma membrane, causing the collapse of ionic gradients. However, evidence is accumulating that the issue of how enterviruses induce cell death is much more complex. Entervirus infection leads to the development of the so-called cytopathic effect (CPE),\textsuperscript{1} a necrosis-like type of cell death that is characterized by rounding up of the infected cells, distortion and displacement of the nuclei, condensation of chromatin, and increased plasma membrane permeability. This type of cell death is the result of a complex interplay between apoptosis-inducing and apoptosis-suppressing functions encoded by the entervirus genome (4–6). Early in infection, i.e. upon translation of the entervirus RNA genome, sufficient quantities of a putative pro-apoptotic function are produced to trigger an apoptotic response. Concomitantly with the onset of viral replication, however, the implementation of the virus-induced apoptotic program is abruptly interrupted, suggesting that enterviruses also encode an antiapoptotic function (6). This antiapoptotic function also renders infected cells resistant against non-viral apoptotic stimuli like cycloheximide and actinomycin D (4). The apoptosis-suppressing function dominates upon productive infection (i.e. conditions that allow efficient virus replication). Under these conditions, only at late stages (i.e. after the development of CPE) can some signs of apoptosis be detected (7). Virus replication and CPE, however, are not sensitive to caspase inhibitors or the overexpression of Bcl-2 (5). The apoptosis-inducing function dominates upon non-permissive infection (i.e. conditions that restrict virus growth). The full-blown apoptosis that is induced under these latter conditions is efficiently suppressed by caspase inhibitors as well as by Bcl-2 overexpression (5).

Recent studies have shed some light on the identity of the putative apoptosis-inducing entervirus proteins. Individual expression of the viral proteinase 2A\(^{pro}\), which inhibits cap-dependent translation of cellular mRNAs, or the expression of 3C\(^{pro}\), which shuts off host cell RNA transcription, results in apoptotic cell death (8, 9). Little is known about the identity of the apoptosis-suppressing factors. The 3A protein suppresses the extrinsic apoptotic pathway by eliminating labile receptors

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\textsuperscript{1} To whom correspondence may be addressed. Tel.: 39-0532-291361; Fax: 39-0532-291361; E-mail: r.rizzuto@unife.it.

\textsuperscript{**} To whom correspondence may be addressed. Tel.: 31-24-3617574; Fax: 31-24-3614666; E-mail: f.vankuppeveld@ncmls.kun.nl.

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\textsuperscript{1} The abbreviations used are: CPE, cytopathic effect; ER, endoplasmic reticulum; aa, amino acid(s); DAPI, 4,6-diamidino-2-phenylindole; KB, Krebs-Ringer buffer; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CHO, Chinese hamster ovary; GFP, green fluorescent protein; EGFP, enhanced GFP; AEQ, aequorin; cyt, cytosolic; er, ER-targeted; Go, Golgi-targeted; mt, mitochondrial targeted; IP3, inositol 1,4,5-trisphosphate; GuHCl, guanidine hydrochloride.
from the cell surface (10) through its ability to inhibit protein secretion (11). The enterovirial protein that is responsible for the suppression of the intrinsic apoptotic host cell responses has not yet been identified.

Ca2+ is one of the most versatile and universal signaling mediators in cells and is required for the activation of many cellular processes. Increasing evidence indicates that alterations in the finely tuned intracellular Ca2+ homeostasis and compartmentalization can lead to cell death, either through apoptosis or necrosis (12). The switch from the control of physiological functions to the death program most likely involves alterations in the tightly regulated spatio-temporal Ca2+ pattern or alterations at the level of organelles (e.g. mitochondria or ER/Golgi) or effector proteins (e.g. calpain or calcineurin) that are activated by Ca2+ (13). Enteroviruses have a profound effect on intracellular Ca2+ homeostasis (14, 15). We have previously shown that infection of HeLa cells with the coxsackievirus results in a reduction of the amount of Ca2+ that can be released from the intracellular stores. In parallel, a gradual increase in the cytosolic Ca2+ concentration ([Ca2+]i) is observed due to the influx of extracellular Ca2+ (15).

The enterovirus 2B protein has been implicated in the virus-induced alterations in intracellular Ca2+ homeostasis (15, 16). The 2B protein is a small (97–99 aa) membrane-integral replication protein (17) that, in infected cells, is localized at the surface of the ER- and Golgi-derived membrane vesicles at which viral replication takes place (3, 18, 19). All enterovirus 2B proteins contain two hydrophobic regions, of which one is predicted to form a cationic amphipathic α-helix (20, 21). This amphipathic α-helix displays characteristics typical for the group of membrane-lytic α-helical peptides that can build membrane-integral pores by forming multimeric transmembrane bundles (22, 23). Homomultimerization reactions of 2B have been demonstrated by yeast and mammalian two-hybrid (24, 25) and biochemical approaches (26) and in living cells by using fluorescence resonance energy transfer (FRET) microscopy (27). These data strongly suggest that 2B is responsible for the reduction of the [Ca2+]i in ER and Golgi ([Ca2+]iER and [Ca2+]iGolgi) by building pores in the membranes of these organelles. Direct evidence that 2B indeed causes a reduction in the [Ca2+]i of the stores is, however, still lacking.

In this study, we investigated Ca2+ homeostasis in 2B-expressing cells and evaluated the physiological importance of the alterations in Ca2+ signaling for the implementation or suppression of the different cell death programs. Using fluorescent Ca2+ indicators and organelle-targeted aequorins (AEQs; genetically encoded Ca2+ sensors), we demonstrate that 2B indeed reduces [Ca2+]iER and [Ca2+]iGolgi in HeLa cells. We show that this leads to a decrease in the amount of Ca2+ that can be released from these stores and, as a consequence, results in the stimulus-induced rise of [Ca2+]i in the mitochondria ([Ca2+]imit). Moreover, the influx of Ca2+ from the extracellular medium is increased, and thus, the [Ca2+]icyt responses are larger. In our studies of the functional importance of the 2B-induced manipulation of intracellular Ca2+ signaling, we found that the expression of 2B suppressed apoptosis induced by certain stimuli, including actinomycin D and cycloheximide.

2B mutants that were unable to reduce the Ca2+ content of the stores failed to protect against apoptosis. These data implicate a functional role of the 2B-induced perturbation of intracellular Ca2+ compartmentalization in the enteroviral strategy to suppress premature abortion of the viral life cycle and provide a physiological example of the regulatory role of Ca2+ signaling in the modulation of apoptotic cell death.
μM digitonin in a hypotonic Ca²⁺-containing solution (10 mM CaCl₂ in H₂O), thus discharging the remaining aequorin pool. The output of the discriminator was captured by a Thorn-EMI photon counting board and stored in an IBM-compatible computer for further analyses. The aequorin luminescence data were calibrated off-line into [Ca²⁺] values, using a computer algorithm based on the Ca²⁺ response curve of wild-

**Fig. 1.** Intracellular Ca²⁺ homeostasis in 2B-expressing cells. A, [Ca²⁺]ₗ of CHO cells expressing GFP or 2B-GFP at 48 h posttransfection. Cells were loaded with Fura-2/AM, GFP-positive cells were identified, the fluorescence at 340 and 380 nm was analyzed, and the ratio 340/380 nm was calculated. B, the amount of thapsigargin-releasable Ca²⁺. The average peak increase in the thapsigargin-induced 340/380 nm ratio is shown (i.e. the increase in the 340/380 nm ratio relative to the basal 340/380 nm ratio in the Ca²⁺-free medium that was recorded just before the addition of thapsigargin; see panel C). C, representative traces showing the peak increases in thapsigargin response of a control cell, a GFP expressing cell, and three 2B-GFP expressing cells. D, average peak increase in the thapsigargin-induced 340/380-nm ratio plotted against the initial 340/380-nm ratio of cells in the presence of extracellular Ca²⁺. Note that the average amount of thapsigargin-releasable Ca²⁺ of 2B-GFP-expressing cells that exhibited an initial 340/380-nm ratio of 0.6–0.9 (which is similar to that of the control cells) was significantly lower than that of control cells. In each experiment, the 340/380-nm ratio of a large number of cells from different coverslips was analyzed. The average ± S.D. of five independent experiments is shown. *, p < 0.05; **, p < 0.01.
type and mutant aequorins (30, 33).

**Cell Death Analysis—** To assay the antiapoptotic effects in coxsackievirus-infected cells, cells grown on coverslips were either mock-infected or infected with coxsackievirus (30 min at room temperature) at a multiplicity of infection of 50. At the indicated times (specified in the relevant figure legends), cells were challenged with guanidine hydrochloride (GuHCl) (2 mM), cycloheximide (100 μg/ml), or actinomycin D (0.5 μg/ml) for the indicated times. For the analysis of the nuclear morphology, cells were fixed and stained with DAPI (10 μg/ml), viewed under an Axiovert epifluorescence-inverted microscope (Carl-Zeiss GmbH), and imaged with a Nikon Coolpix 995 digital camera equipped with an MDC lens. Caspase activation was analyzed using the Apo-ONE™ homogeneous caspase-3/7 assay according to the instructions of the manufacturer (Promega). Analysis of DNA fragmentation was performed as described by Tolskaya et al. (4). Incubations were performed in serum-free DMEM except for the experiments in which caspase-3 activation was assayed (because of the high caspase-3 background in serum-free DMEM in control cells and infected cells).

To investigate the antiapoptotic effects in 2B-expressing cells, cells grown on coverslips were transfected either with 2B-GFP or mt-GFP. At 36 h post-transfection, cells were treated with the apoptosis-inducing drugs for 4 h (for analysis of caspase-3 activation) or 16 h (for analysis of cell survival). Incubation with cycloheximide, actinomycin D, and etoposide was performed in KRB buffer supplemented with 1 mM etoposide was performed in DMEM containing 10% FBS. Incubation of cell survival, cells were washed thoroughly to eliminate apoptotic cells but not its inactive zymogen, and a Texas Red-conjugated goat anti-rabbit antibody. The percentage of fluorescent cells before and after a challenge with the apoptotic drugs was determined de visu by counting at the microscope. To assay cell survival, cells were washed thoroughly to eliminate apoptotic cells after incubation with the apoptotic drugs. The percentage of fluorescent cells before and after a challenge with the apoptotic drugs was determined de visu by counting at the microscope.

**RESULTS**

**Ca²⁺ Homeostasis in 2B-expressing Cells—** To investigate the effects of 2B on subcellular Ca²⁺ homeostasis, we made use of a fusion protein of 2B and GFP. We have shown previously that the fusion of EGFP at the C terminus of the coxsackievirus 2B protein does not interfere with its membrane-active function and localization (17). Because the EGFP fluorescence heavily contaminates that of the fluorescent Ca²⁺ indicator Fura-2 (data not shown) (34), we made use of GFP (S65T), a non-enhanced GFP protein that exhibits 30-fold reduced fluorescence intensity relative to EGFP. Fig. 1A shows the [Ca²⁺]cyt of nontransfected control cells and cells expressing either GFP (S65T) or 2B-GFP (S65T). The [Ca²⁺]cyt in GFP-expressing cells was slightly lower than that of control cells (suggesting that there is some minor contamination of the Fura-2 fluorescence by GFP). Expression of 2B-GFP, however, resulted in a significant increase in [Ca²⁺]cyt (p < 0.01), shown previously to depend on extracellular Ca²⁺ (15).

Next, we addressed the relationship between the [Ca²⁺]cyt and the [Ca²⁺] of the intracellular stores (i.e. ER and Golgi) using thapsigargin, a specific inhibitor of the sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA) pump. The activity of the SERCA is required to compensate for the continuous leakage of Ca²⁺ that takes place from the stores under normal conditions. Upon inhibition of the SERCA, the Ca²⁺ that leaks from the stores is not resequestered and accumulates in the cytosol. The size of the thapsigargin-induced transient increase in [Ca²⁺]cyt reflects the [Ca²⁺] of the stores. Fig. 1B shows that the amount of thapsigargin-releasable Ca²⁺ in cells expressing GFP was similar to that observed in nontransfected control cells. Cells expressing 2B-GFP, however, exhibited a significant decrease (p < 0.01) in the amount of thapsigargin-releasable Ca²⁺. Fig. 1C shows representative traces of nontransfected controls, cells expressing GFP, and cells expressing 2B-GFP. The amount of thapsigargin-releasable Ca²⁺ from cells expressing 2B-GFP exhibited an inverse relationship with the initial [Ca²⁺]cyt (as measured in the presence of extracellular Ca²⁺);
were incubated in Ca\textsuperscript{2+}, a Ca\textsuperscript{2+} ionophore, in the absence of extracellular Ca\textsuperscript{2+}, and then challenged with 100 \textmu M histamine (Hist.). C. HeLa cells transfected with cytAEQ and 2B-GFP or mt-GFP (control) were incubated in Ca\textsuperscript{2+}-free medium (plus 100 \textmu M EGTA) and challenged with histamine, followed by the addition of Ca\textsuperscript{2+}-containing medium. Representative traces in [Ca\textsuperscript{2+}]\textsubscript{cyt} (A) and [Ca\textsuperscript{2+}]\textsubscript{Golgi} (B) in 2B-expressing cells (gray line) and control cells (black line) are shown on the left. On the right is shown the average \pm S.D. of six independent experiments. *, p < 0.005.

Reduced ER and Golgi Luminal Ca\textsuperscript{2+} Concentrations in 2B-expressing Cells—To investigate the effect of 2B on ER and Golgi calcium levels directly and independently, we used chimeras of aequorin, a Ca\textsuperscript{2+}-sensitive photoprotein. To this end, HeLa cells were cotransfected with erAEQ or GoAEQ and 2B-GFP or mt-GFP (which was further used as negative control throughout this study), and the [Ca\textsuperscript{2+}]\textsubscript{ER} and [Ca\textsuperscript{2+}]\textsubscript{Golgi} were compared. To efficiently reconstitute the aequorin chimeras and reliably measure [Ca\textsuperscript{2+}]\textsubscript{ER} and [Ca\textsuperscript{2+}]\textsubscript{Golgi}, the luminal [Ca\textsuperscript{2+}] of these organelles must first be reduced. This was obtained by incubation of the cells in KRB supplemented with 1 mM aequorin (i.e. the prosthetic group of aequorin) and ionomycin, a Ca\textsuperscript{2+} ionophore, in the absence of extracellular Ca\textsuperscript{2+}. Aequorin luminescence signals were collected using a luminometer and calibrated into [Ca\textsuperscript{2+}] values. Under these conditions, the [Ca\textsuperscript{2+}] was <10 \textmu M in both organelles. Upon switching the perfusion medium to KRB buffer supplemented with 1 mM Ca\textsuperscript{2+}, [Ca\textsuperscript{2+}]\textsubscript{ER} and [Ca\textsuperscript{2+}]\textsubscript{Golgi} gradually increased, reaching plateau levels in control cells of ~365 \textmu M in the ER and ~345 \textmu M in the Golgi (Fig. 2). In 2B-GFP-expressing cells, lower steady state levels (~30–40% reduction) were observed in both compartments (~255 and ~215 \textmu M in the ER and Golgi, respectively). The addition of histamine to the cells resulted in a rapid decrease in [Ca\textsuperscript{2+}] in both organelles, confirming that the sensitivity to agonists of the two Ca\textsuperscript{2+} stores was retained, although a smaller amount of Ca\textsuperscript{2+} could be released in 2B-expressing cells. Taken together, these data indicate that 2B is responsible for a reduction of the luminal [Ca\textsuperscript{2+}] in both ER and Golgi.

Mitochondrial and Cytosolic Ca\textsuperscript{2+} Handling in 2B-expressing Cells—Mitochondria play an important role in intracellular Ca\textsuperscript{2+} homeostasis. The mitochondria are localized in close proximity of inositol 1,4,5-trisphosphate (IP3)-gated channels and are capable of taking up the Ca\textsuperscript{2+} that is released by IP3-generating agonists, thereby buffering the [Ca\textsuperscript{2+}]\textsubscript{cyt} (35). We hypothesized that the 2B-induced reduction in the steady state [Ca\textsuperscript{2+}]\textsubscript{ER} and [Ca\textsuperscript{2+}]\textsubscript{Golgi} levels and the ensuing reduction of IP3-induced Ca\textsuperscript{2+} release should decrease the uptake of Ca\textsuperscript{2+} by the mitochondria. To test this hypothesis, HeLa cells were transfected with mtAEQ and either 2B-GFP or mt-GFP and then challenged with histamine (in the presence of extracellular Ca\textsuperscript{2+}). Fig. 3A shows that the peak mitochondrial response is markedly reduced (almost ~35%) in 2B-GFP-expressing cells compared with the control cells. Similar results were obtained when cells were challenged with ATP, an agonist that drives production of IP3 through its action on a G\textsubscript{q} coupled P2Y receptor, or when CHO cells were used as model system (data not shown). Taken together, these data indicate that the 2B-induced reduction of [Ca\textsuperscript{2+}] in the stores leads to a reduction in the stimulus-induced mitochondrial Ca\textsuperscript{2+} uptake.

The 2B-induced reduction in the steady state [Ca\textsuperscript{2+}]\textsubscript{ER} and
surprisingly, we found no difference in the amplitude of 

|Ca\(^{2+}\)|Golgi levels should also be reflected in a decrease in the rise of the |Ca\(^{2+}\)|cyt upon stimulation with IP3-generating ago-
nists. To investigate this supposition, HeLa cells were cotrans-
fected with cytAEQ and 2B-GFP or mt-GFP and then chal-
enged with histamine (in the presence of extracellular Ca\(^{2+}\)).

Fig. 4. C
coxsackieviruses confer an antiapoptotic state to infected cells. A, coxsackievirus-induced cell death upon permissive and
nonpermissive infection. HeLa cells were either mock-infected (left) or infected with coxsackievirus at a multiplicity of infection of 50 in the
ab

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These findings provide further evidence for the existence of an antiapoptotic function in coxsackievirus.

Expression of 2B Protects against Cycloheximide and Actinomycin D-induced Apoptosis—Next, the possible role of the coxsackievirus 2B-induced alterations in Ca\(^{2+}\) homeostasis in the suppression of apoptosis was investigated. For this investigation the following protocol was used. Cells were transfected with 2B-GFP, and the number of fluorescent cells was counted at the microscope before and after a challenge with an apoptotic drug. The rationale, as reported previously for other proteins such as Bcl-2 (36) and VDAC (37), is that if the transfected protein increases the sensitivity to apoptotic agents, the number of fluorescent cells will be lower after the apoptotic challenge (because more transfected cells die), whereas the opposite will happen if the protein has antiapoptotic activity.

To investigate whether 2B is involved in conferring an antiapoptotic state to infected cells, 2B-GFP-expressing cells and mt-GFP-expressing cells were challenged with actinomycin D and cycloheximide at 36 h posttransfection. The results obtained with actinomycin D are shown in Fig. 6A. When mt-GFP was transfected, ~40–45% of the cells showed GFP fluorescence. 16 h after the addition of actinomycin D, the number of viable cells was drastically reduced, but the fraction of fluorescent cells remained the same. Conversely, when 2B-GFP was transfected, the same fraction of fluorescent cells was identified before actinomycin D treatment, but it markedly increased 16 h after the addition of the apoptotic agent (~70%). These data indicate that the 2B-induced alterations in Ca\(^{2+}\) homeostasis confer protection against actinomycin D-induced apoptotic cell death (p < 0.005). Enhanced survival was also observed upon treatment with cycloheximide (p < 0.01) (Fig. 6B).

To test whether the expression of 2B also protects against other apoptotic stimuli, we tested the possible suppressive effect against apoptosis induced by 10 \(\mu\)M ceramide, a lipid signaling mediator that releases Ca\(^{2+}\) from intracellular stores and induces apoptosis via a Bcl-2 sensitive pathway (38), and by 20 \(\mu\)M etoposide, a drug that causes DNA damage by inhibiting topoisomerase II, leading to apoptosis through p53 via a Bcl-2-sensitive, Bax-dependent pathway. Again, the number of fluorescent cells before and after a treatment with the apoptotic drugs was determined. No change in the percentage of living fluorescent cells was observed in HeLa cells transfected with mt-GFP. Cells expressing 2B-GFP were efficiently protected against apoptosis induced by ceramide (p < 0.005) (Fig. 6C) but not by etoposide (Fig. 6D). The failure of 2B to suppress etoposide-induced apoptosis was not due to a lower apoptotic efficacy of this drug (etoposide induced apoptosis in ~60% of the cells, similar to what was observed for actinomycin D and cycloheximide; data not shown). Taken together, these data indicate that 2B protects against some, but not all, Bcl-2-sensitive apoptotic pathways.

To demonstrate that the cytoprotective effect of 2B is indeed due to a reduced activation of caspases, cells expressing either 2B-GFP or mt-GFP were treated either with actinomycin D, cycloheximide, or etoposide and then stained with an antibody that specifically recognizes the active form of caspase-3 but not its inactive zymogen (Fig. 7). In control cells, the vast majority of cells showed extensive caspase-3 activation upon the addition of each of these apoptotic drugs (similarly as in nontransfected cells; data not shown). In 2B-expressing cells, however, caspase-3 activation induced by actinomycin D and cycloheximide, but not by etoposide, was potently suppressed. These findings are in agreement with the results described in Fig. 6 and provide evidence that the enhanced survival observed in 2B-expressing cells is due to the suppression of caspase-3 activation.

Effects on Intracellular Ca\(^{2+}\) Homeostasis and Apoptosis of 2B Mutants—Our data strongly suggest that the 2B protein lends protection against apoptosis through its ability to manipulate intracellular Ca\(^{2+}\) homeostasis. However, it cannot be excluded that the effects of 2B on Ca\(^{2+}\) homeostasis and its anti-apoptotic ability represent two distinct, unrelated functions. To investigate whether the antiapoptotic activity of 2B is functionally related to its ability to manipulate intracellular Ca\(^{2+}\) fluxes, we characterized two 2B-GFP mutants with mutations in the hydrophobic regions that are implicated in pore formation (17). In the first mutant, the amphipathic character of the \(\alpha\)-helix formed by the first hydrophobic region (aa 37–54) is disturbed by the substitution of lysine residues 41, 44, and 48 with hydrophobic leucine residues (mutation K41L/K44L/K48L) (25). In the second mutant, the hydrophobic nature of the second hydrophobic region (aa 63–80) is disturbed by substitution of two hydrophobic residues (isoleucine 64 and valine 66) by polar serine residues (mutation I64S/V66S) (20).

HeLa cells were cotransfected with erAEQ and the indicated 2B-GFP mutants or mt-GFP, and the [Ca\(^{2+}\)]\(_{ER}\) was compared. Fig. 8A shows that both mutants 2B-K41L/K44L/K48L and 2B-I64S/V66S failed to reduce the luminal [Ca\(^{2+}\)] in the ER. Consistent with this, measurement of the [Ca\(^{2+}\)]\(_{ER}\) upon challenge with histamine in cells transfected with mtAEQ showed that the peak mitochondrial Ca\(^{2+}\) response in cells expressing the mutants was similar to that in control cells (Fig. 8B). These data provide evidence that both mutants are defective in manipulating intracellular Ca\(^{2+}\) fluxes. To investigate the anti-apoptotic activity of these mutants, HeLa cells expressing the 2B-GFP mutants were challenged with either actinomycin D or cycloheximide, and caspase-3 activation was analyzed as described above. Fig. 9 shows that the mutants 2B-K41L/K44L/K48L and 2B-I64S/V66S were unable to suppress either acti-
nomicin D-induced or cycloheximide-induced caspase-3 activation. Consistent with this, both mutants also failed to increase cell survival after treatment with these apoptotic drugs (data not shown). Together, these findings demonstrate that the antiapoptotic activity of 2B is functionally linked to its ability to manipulate intracellular Ca\textsuperscript{2+} homeostasis.

DISCUSSION

Viruses induce a number of alterations in the metabolism and structure of their host cell to ensure efficient reproduction. Some of these alterations can be sensed by the host cell and turn on a defensive apoptotic reaction that is aimed at curtailing virus replication. Many viruses have developed countermeasures to prevent premature abortion of the viral life cycle. Enteroviruses are small cytopathic RNA viruses that cause a necrosis-like type of cell death, called CPE, which is the ultimate result of a complex interplay between apoptosis-induced and apoptosis-suppressing functions encoded by the enterovirus genome (4). In this report, we provide evidence that the enterovirus 2B protein plays a major role in suppressing apoptotic host cell responses by manipulating intracellular Ca\textsuperscript{2+} homeostasis.

Using both chemical and genetically encoded Ca\textsuperscript{2+} indicators, we demonstrated that the expression of 2B results in the following effects: (i) a reduction in the luminal [Ca\textsuperscript{2+}] in both ER and Golgi; (ii) a decrease in the amount of Ca\textsuperscript{2+} that can be released from these organelles using either thapsigargin or physiological, IP3-generating stimuli like histamine and ATP; (iii) a reduction in the stimulus-induced amount of Ca\textsuperscript{2+} that is taken up by mitochondria; and (iv) an increase in the influx of extracellular Ca\textsuperscript{2+}, leading to a rise in [Ca\textsuperscript{2+}]\textsubscript{cyt}. The reduction in the Ca\textsuperscript{2+}-filling state of the stores preceded the increase in the influx of extracellular Ca\textsuperscript{2+}, indicating that the stores are the primary target of 2B. Similar results were obtained in HeLa cells and CHO cells, indicating that it is unlikely that the effects of 2B are cell type-specific. These data are consistent with the idea that 2B decreases [Ca\textsuperscript{2+}]	extsubscript{ER} and [Ca\textsuperscript{2+}]	extsubscript{Golgi} by increasing the passive leakage of Ca\textsuperscript{2+} ions from these stores, most likely through the formation of membrane-integral pores, and thereby accounts for a reduction in the amount of releasable Ca\textsuperscript{2+} and the down-regulation of Ca\textsuperscript{2+} fluxes between stores and the mitochondria. The increased influx of Ca\textsuperscript{2+} is most likely due to the increased plasma membrane permeability that is observed in 2B-expressing cells, which allows the passage of ions and normally nonpermeant low molecular weight compounds (11, 39, 40).

The 2B-induced perturbation of intracellular Ca\textsuperscript{2+} distribution and signaling was identified as an important component of the entervoiral strategy to suppress infection-limiting apoptotic host cell responses. These apoptotic responses are most likely triggered by the action of the viral proteinases 3C\textsuperscript{pro} and 2A\textsuperscript{pro}, which inhibit cellular transcription and cap-dependent translation, respectively (8, 9). Enteroviruses interrupt this apoptotic program by encoding functions that implement an antiapoptotic program (4). The antiapoptotic state that is conferred to infected cells can even suppress nonviral apoptotic stimuli such as cycloheximide and actinomycin D (whose effects resemble those of 3C\textsuperscript{pro} and 2A\textsuperscript{pro}, respectively). In our studies of the physiological relevance of the 2B-induced alterations in intracellular Ca\textsuperscript{2+} homeostasis, we observed a role for 2B in suppressing apoptosis. The expression of 2B suppressed caspase-3 activation and apoptotic cell death induced by actinomycin D and cycloheximide. Mutants of 2B that were defective in manipulating intracellular Ca\textsuperscript{2+} fluxes failed to protect against apoptosis. Together, these findings strongly suggest that 2B, by modulating intracellular Ca\textsuperscript{2+} homeostasis, plays a major role in conferring the antiapoptotic state to infected cells and, thereby, in extending the life span of the host cell.

The enterovirus 2B protein represents one of the first antiapoptotic proteins of small genome RNA viruses. Most of our knowledge about viral apoptosis-suppressing functions comes from the group of large genome DNA viruses (herpesviruses, adenoviruses, and poxviruses), which often encode multiple antiapoptotic proteins, including Bel-2 homologs, caspase suppressors, and cell cycle and transcription mediators (1, 2). Relatively little is known about apoptosis-suppressing functions in small genome RNA viruses that exhibit a relatively fast...
replication cycle and have little, if any, genetic capacity to develop individual antiapoptotic functions. It has been suggested that the antiapoptotic activities of RNA viruses may be byproducts of the cellular alterations that are induced by the viral replication proteins to efficiently replicate their RNA genome. Obviously, the ability of 2B to suppress apoptotic host cell responses is not its sole or primary function. Mutations in the hydrophobic domains of 2B, which most likely interfere with pore formation (as shown in this study for the mutants 2B-K41L/K44L/K48L and 2B-I64S/V66S), cause primary defects in viral RNA replication (20, 21). The exact function of 2B is as yet unknown. The ability of 2B to form pore-like structures in secretory pathway membranes may be required for the ability of the 2BC precursor to cause the accumulation of secretory pathway-derived membrane vesicles at which viral RNA replication takes place (3, 41). Thus, the ability of 2B to reduce the Ca\(^{2+}\) filling state of the stores most likely serves two different functions, namely formation of the viral replication complex and suppression of apoptotic host cell responses.

How can the 2B-induced alterations in intracellular Ca\(^{2+}\)
signaling lend protection against certain apoptotic stimuli? The finding that 2B failed to suppress etoposide-induced apoptosis indicates that the alterations in Ca\(^{2+}\) homeostasis suppress a specific apoptotic pathway rather than confer a general blockage of apoptosis. Recent studies on the Bcl-2 oncogene have provided evidence for the coexistence of spatially different apoptotic pathways in the same cell, one ER-dependent and one mitochondrial-dependent, which eventually converge at the mitochondria (42, 43). Bcl-2 is localized at the outer mitochondrial membrane, the nuclear envelope, and the ER membrane. Most of the results published so far have emphasized the importance of Bcl-2 at the mitochondria, where it antagonizes the ability of pro-apoptotic Bcl-2 family members to induce cytochrome C release (44). The function of Bcl-2 at the ER membrane is less clear. An ER-restricted Bcl-2 mutant suppressed apoptosis induced by various stimuli (including ceramide) but failed to protect against etoposide, a drug that acts directly at the mitochondria by causing membrane translocation of Bax (42, 43). The antiapoptotic activity of ER-restricted Bcl-2 argues for the existence of a signaling mechanism between the ER and the mitochondria. Growing evidence indicates that alterations in Ca\(^{2+}\) fluxes between these organelles have a modulatory effect on apoptosis (13). Key events occurring in the mitochondrial matrix such as ATP production, an important source of reactive oxygen species production, and possibly also the opening of the permeability transition pore leading to swelling of the outer mitochondrial membrane and the release of proapoptotic proteins like cytochrome C, are sensitive to increases in [Ca\(^{2+}\)]\(_{\text{mt}}\) (13, 45). Accordingly, down-regulation of Ca\(^{2+}\) fluxes between the ER and the mitochondria can protect the mitochondria from cytotoxic rises in [Ca\(^{2+}\)]\(_{\text{mt}}\). Increasing evidence indicates that Bcl-2 exerts some of its antiapoptotic effects from the ER by reducing [Ca\(^{2+}\)]\(_{\text{ER}}\) and down-regulating Ca\(^{2+}\) fluxes between the ER and mitochondria (36, 46, 47), possibly through the ability of Bcl-2 to form ion channels in the ER membrane (48). Moreover, conditions that lowered [Ca\(^{2+}\)]\(_{\text{ER}}\) protected HeLa cells from ceramide-induced apoptosis, whereas conditions that increased [Ca\(^{2+}\)]\(_{\text{ER}}\) had the opposite effect (38). The findings that 2B down-regulated Ca\(^{2+}\) fluxes between the stores and the mitochondria and suppressed apoptosis induced by ceramide, but not etoposide, strongly suggest that 2B specifically targets a Ca\(^{2+}\)-sensitive, ER-dependent apoptotic pathway.

Is the ability of 2B to decrease Ca\(^{2+}\) signaling between the ER and mitochondria sufficient for conferring an antiapoptotic state to enterovirus-infected cells? In both poliovirus-infected cells (4, 6) and coxsackievirus-infected cells (this study), the antiapoptotic function is expressed early in the infection (i.e. at ~2 h postinfection). Previously, we demonstrated that coxsackievirus infection results in a ~50% reduction of the Ca\(^{2+}\) content of the thapsigargin-sensitive stores within the first 2 h of infection (15). Thus, the virus-induced alterations in intracellular Ca\(^{2+}\) signaling coincide with the implementation of the anti-apoptotic state, lending support to the idea that these events are causally linked. It cannot be excluded that the rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\) also contributes somehow to the suppression of apoptosis in infected cells. A rise in [Ca\(^{2+}\)]\(_{\text{cyt}}\) may lead to the activation of calpains, Ca\(^{2+}\)-sensitive proteases that can exert antiapoptotic effects by cleaving caspases, including key caspases 8 and 9 (49, 50). Interestingly, aberrant processing of caspase-9 (albeit at a low level) was observed in HeLa cells upon productive poliovirus infection but not upon abortive infection (51). However, aberrant processing of caspase-9 was not reported upon productive coxsackievirus infection (52). Our observation that the 2B-induced alterations in Ca\(^{2+}\) homeostasis failed to protect against etoposide-induced caspase-9-de-
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