

Bcl-2 and Bcl-xL overexpression inhibits cytochrome *c* release, activation of multiple caspases, and virus release following coxsackievirus B3 infection

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Abstract

Coxsackievirus B3, a cytopathic virus in the family *Picornaviridae*, induces degenerative changes in host cell morphology. Here we demonstrate cytochrome *c* release and caspases-2, -3, -6, -7, -8, and -9 processing. Enforced Bcl-2 and Bcl-xL expression markedly reduced release of cytochrome *c*, presentation of the mitochondrial epitope 7A6, and depressed caspase activation following infection. In comparison, cell death using TRAIL ligand caused caspase-8 processing prior to cytochrome *c* release and executioner caspases and cell death was only partially rescued by Bcl-2 and Bcl-xL overexpression. Disruption of the mitochondrial inner membrane potential following CVB3 infection was not inhibited by zVAD.fmk treatment. Bcl-2 or Bcl-xL overexpression or zVAD.fmk treatment delayed the loss of host cell viability and decreased progeny virus release following infection. Our data suggest that mitochondrial release of cytochrome *c* may be an important early event in caspase activation in CVB3 infection, and, as such, may contribute to the loss of host-cell viability and progeny virus release.

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Introduction

Coxsackievirus B3 (CVB3) is an enterovirus of the family *Picornaviridae*, which can infect both murine and human hosts. In permissive tissue culture cells, or in vivo tissues such as the myocardium and liver, infection results in cellular degenerative changes observable by light and electron microscopy (Enders, 1954; McManus et al., 1993;

Ukimura et al., 1997; Klingel et al., 1998) with eventual loss of cell viability.

At least two stages of proteolytic events occur within target cells following viral receptor binding, genome internalization, and protein translation. The picornaviral proteases have been shown to directly cleave multiple host proteins early in infection including regulatory proteins involved in host transcription and translation (Etchison et al., 1982; Clark et al., 1993; Yalamanchili et al., 1996, 1997a, 1997b, 1997c and the cytoskeleton (Badorff et al., 1999). Later during the virus life cycle, caspases are activated, resulting in degradation of substrates including poly(ADP-ribose) polymerase (PARP) and DNA fragmentation factor (Carthy et al., 1998). Caspases do not appear to be primarily

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responsible for the characteristic architectural degeneration, or cytopathic effect (CPE) (Carthy et al., 1998). Events such as caspase processing and cleavage of substrates (Carthy et al., 1998), substrate phosphorylation (Huber et al., 1997), and early and late calcium influx (van Kuppeveld et al., 1997; Li et al., 1999) suggest that cells retain a capacity for multiple functions following induction of CPE. Thus, condensed, smooth-surfaced, rounded cells consistent with CPE appear to still be viable, although damaged.

Apoptosis can occur through either extrinsic receptor- or intrinsic mitochondria-mediated pathways or both. Caspase-8 is commonly associated with death receptor signaling (Muzio et al., 1996), caspase-2 and -9 with mitochondrial signaling (Li et al., 1997), and caspase-10 with granzyme B signaling (Talanian et al., 1997).

Ligation of death ligands such as Fas and tumor necrosis factor related apoptosis-inducing ligand (TRAIL) with their appropriate receptors results in the formation of a death-inducing signaling complex (DISC) at the plasma membrane. Caspase-8 associates with surface receptor death inducing signaling complexes, and to a lesser extent caspase-3, and has also been shown to amplify apoptosis through the mitochondria by cleavage of Bid to truncated-Bid (Bossy-Wetzell and Green, 1999; Li et al., 1998; Crompton, 2000). Bid cleavage results in cytochrome *c* (cyt *c*) release, caspase-9 activation, and further amplification of the death proteolytic cascade. It has been shown that cyt *c* release may occur through Bid-mediated intramembranous oligomerization of proapoptosis Bcl-2 family members, Bak (Wei et al., 2000) or Bax (Eskes et al., 2000; von Ahnen et al., 2000; Desagher et al., 1999). Caspase-3, -6, and -7 are considered executioner caspases and their activation results in processing of numerous cellular proteins at internal aspartic acid residues which, in turn, results in an apoptotic phenotype (Nicholson and Thornberry, 1997).

Mitochondria have increasingly become recognized as important organelles in regulating life or death of cells (Ravagnan et al., 2002). Cellular stress can trigger apoptosis through degenerative mitochondrial alterations which may occur in a caspase-2-dependent manner (Lassus et al., 2002). Several prominent alterations occur to mitochondria during apoptosis that include the release of intermembrane space molecules, changes in the membrane potential, ionic changes, and more. Compromise of mitochondrial permeability can release sequestered proapoptotic molecules such as cyt *c* (Liu et al., 1996), Smac/DIABLO (Verhagen et al., 2000; Du et al., 2000), HtrA2/Omi (Hegde et al., 2002; Martins et al., 2002; Suzuki et al., 2001; Verhagen et al., 2002), Endo G (Li et al., 2001), apoptosis-inducing factor (AIF; Susin et al., 1999b), procaspase-9 (Susin et al., 1999a), and others. The best characterized of these, cyt *c*, in the cytosol, functions as a cofactor and in the presence of Apaf-1, caspase-9, and dATP can form an apoptosome to autocatalyze caspase-9, thereby leading to activation of downstream caspases (Li et al., 1997).

The Bcl-2 proteins are central regulators of mitochon-

drial permeability and release of proapoptotic molecules. Bcl-2 and Bcl-xL are antiapoptotic members localized in the mitochondrial and endoplasmic reticular membranes, as well as in the nuclear envelope. In the mitochondria, Bcl-2 and Bcl-xL preserve mitochondrial integrity and prevent the subsequent release of apoptogenic molecules (Liu et al., 1996; Kluck et al., 1997; Yang et al., 1997).

In this study, we demonstrate, for the first time, that cyt *c* is released from the intermitochondrial membrane space followed by activation of caspase-9 and downstream effector caspases. Overexpression of Bcl-2 and Bcl-xL, which blocked cyt *c* release, completely inhibited caspase cleavage, activity, and substrate cleavage following CVB3 infection, which suggests that such activity occurs downstream of mitochondrial permeability alteration. CVB3 infection in HeLa cells overexpressing Bcl-2 or Bcl-xL, or treated with zVAD.fmk, is associated with decreased cell death and progeny virus release, although there is no protection from early cytopathic changes, including cell rounding and detachment.

Results

CVB3 infection leads to cyt c release and activation of multiple caspases

To explore the mechanisms by which CVB3 infection-induced apoptosis occurs, HeLa cells were CVB3- or sham-infected at a multiplicity of infection (m.o.i.) of 10 and examined for processing and activation of caspases and downstream targets at 0 to 14 h following infection. Following CVB3 infection, cyt *c* is released into the cytosol (s-100 cell lysate) and caspase-9 is cleaved beginning at 8 h (Fig. 1A). Subsequently, caspases-2, -3, -6, -7, and -8 are processed 10 to 14 h following CVB3 infection of HeLa cells (Fig. 1B). Caspase activation is confirmed by cleavage of lamin A/C and PARP, substrates of caspase-6 and caspase-3, respectively (Fig. 1B). Cyt *c* release and subsequent caspase activation raises the possibility that the observed apoptosis is triggered at the mitochondria following CVB3 infection.

As a comparative model of receptor-mediated apoptosis, HeLa cells were exposed to TRAIL (150 ng/ml) in the presence and absence of zVAD.fmk. Cells were collected from 0 to 5 h and cytosolic extracts and examined for cyt *c* release and total cell lysates were separated by SDS-PAGE and immunoblotted for antibodies for caspase-2, -3, -6, -7, -8, -9, and PARP and lamin A/C. We found that TRAIL induced caspase-8 processing followed by processing of subsequent executioner caspases and their substrates (Fig. 1C). Treatment with zVAD.fmk prevented cyt *c* release and caspase processing; thus, such events are indeed caspase-dependent.

To determine whether cardiac myocytes also activate caspases following CVB3 infection, HL-1 (murine atrial

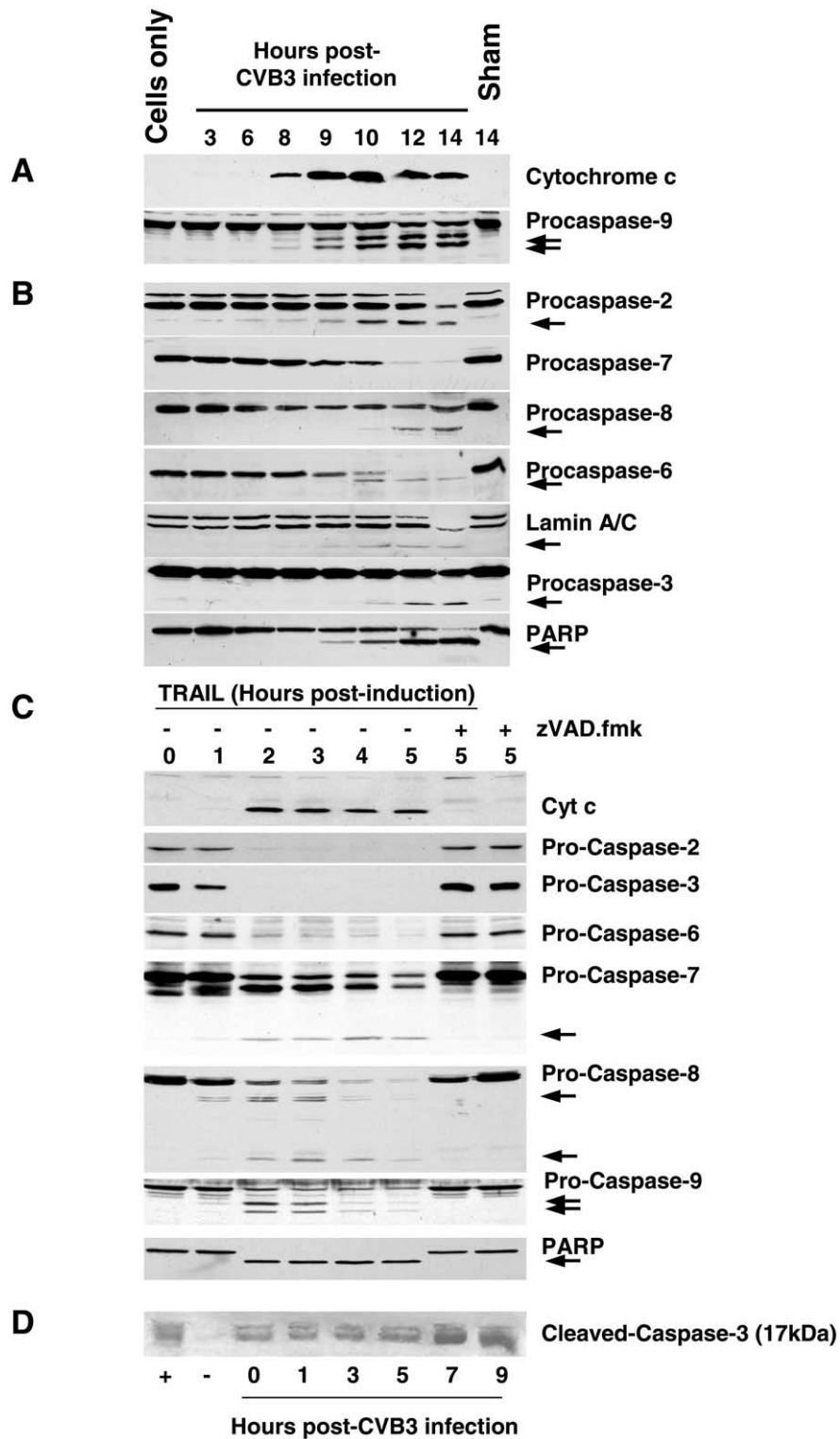


Fig. 1. Cytochrome *c* release and processing of multiple caspases following CVB3 infection. HeLa cells were infected with CVB3 (m.o.i. 10) and harvested 0–14 h postinfection. Cytosolic extracts were separated by SDS–PAGE, and immunoblot analysis was performed using antibodies directed against the caspases and their substrates. (A) Cytochrome *c* is released and caspase-9 is cleaved following CVB3 infection. (B) Downstream cleavage of multiple caspases and substrates PARP and Lamin occurs beginning 10–14 h following CVB3 infection. (C) Cytochrome *c* release, cleavage of multiple caspases, and PARP occur following exposure to TRAIL (150 ng/ml) and zVAD.fmk. Caspase processing can be detected by either disappearance of the proform or appearance of a cleavage product, or both. (D) HL-1 (murine atrial cardiomyocyte) cells were infected with CVB3 (m.o.i. 100) and harvested 0–9 h postinfection. Caspase-3 cleavage was observed to increase at 7–9 h following infection (positive and negative controls are activated and nonactivated Jurkat cell lysates, respectively).

cardiomyocyte) cell lysates were probed for the 17-kDa caspase-3 cleavage product. An increase in caspase-3 cleavage product was observed 7–9 h postinfection (Fig. 1D).

Overexpression of Bcl-2 and Bcl-xL blocks CVB3-induced cyt c release and activation of caspases

Bcl-2 and Bcl-xL are antiapoptotic molecules which regulate mitochondrial permeability and release of cyt *c* into the cytosol. Neo (vector only) and antiapoptotic Bcl-2- and Bcl-xL-overexpressing HeLa cells were either sham or CVB3 (m.o.i. 10) infected and cell lysates were collected 10 h postinfection. Overexpression of Bcl-2 and Bcl-xL was confirmed by immunoblot analysis (Fig. 2A). Bcl-2 and Bcl-xL prevented the release of cyt *c*, processing of caspase-2, -3, -7, -8, -9, and PARP, measured at 10 h following CVB3 infection (Fig. 2B). DEVD(Asp-Glu-Val-Asp)ase-like cleavage activity in total cell lysates peaked in Neo cells 12 h postinfection and decreased in activity thereafter, but was completely inhibited in both Bcl-2 and Bcl-xL cells (Fig. 2C). These results confirm that CVB3-induced apoptosis is initiated by mitochondrial release of apoptogenic molecules such as cyt *c*.

Overexpression of Bcl-2 and Bcl-xL inhibits CVB3-induced mitochondrial membrane protein expression

To further determine mitochondrial function following CVB3 infection, we examined the expression of the mitochondrial epitope 7A6 in cells infected with CVB3 and exposed to TRAIL. Neo, Bcl-2, and Bcl-xL overexpressing cells were exposed to a gradient of TRAIL treatments for 24 h and 150 ng/ml was determined to be an ideal concentration to induce Neo cell apoptosis (data not shown). We used Apo 2.7 antibodies, which reacts with a 38-kDa mitochondrial membrane protein (7A6 antigen) expressed as an early event in apoptosis (Zhang et al., 1996). Expression of the mitochondrial epitope 7A6 was induced by TRAIL (150 ng/ml) in Neo cells and blocked by zVAD.fmk treatment, and Bcl-2 and Bcl-xL overexpression (Fig. 3). Infection by CVB3 induced 7A6 expression in Neo cells which was unaffected by zVAD.fmk treatment but exhibited nearly a complete ablation, with Bcl-2 and Bcl-xL (Fig. 3). These observations suggest that CVB3-induced apoptosis is dependent on mitochondrial alterations upstream of caspase activity. Uninfected Neo, Neo, and zVAD.fmk, Bcl-2, and Bcl-xL cells did not exhibit any basal level of 7A6 expression.

Overexpression of Bcl-2 and Bcl-xL enhances cell viability

To examine the effect of Bcl-2 and Bcl-xL expression on CVB3-induced target cell death, we examined cell viability over 18 h, as measured by the 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. We

demonstrate that zVAD.fmk, and Bcl-2 and Bcl-xL overexpression, significantly reduced cell death following virus infection (Fig. 4A). As compared to a complete loss in cell viability following infection with CVB3 alone, treatment with zVAD.fmk reduced loss in cell viability to approximately 45%. Expression of Bcl-2 and Bcl-xL also preserved overall viability at approximately 70 and 60%, respectively. Mitochondrial release of caspase-independent proapoptotic molecules such as AIF and Endo G may account for the increased cell viability for Bcl-2 and Bcl-xL overexpression as compared to zVAD.fmk following CVB3 infection. Cell viability may not be directly related to CPE as characterized by the apoptotic phenotype of cell shrinking, rounding, and eventual detachment from the culture surface despite treatment with zVAD.fmk or expression of Bcl-2 and Bcl-xL as compared to Neo cells alone (Fig. 4B). Thus, cell viability is partially dependent on mitochondria-mediated apoptosis which can be rescued by Bcl-2 and Bcl-xL expression and by zVAD.fmk.

Enforced expression of Bcl-2 and Bcl-xL only partially restored cell viability following addition of TRAIL as compared to Neo cells (Fig. 4C). However, Bcl-2 and Bcl-xL expression did preserve cellular morphology, thus cell viability and morphology are separate phenomena. The inability of Bcl-2 and Bcl-xL to completely prevent TRAIL-induced cell death confirms that receptor-mediated cell death is only partially mitochondria-dependent.

Overexpression of Bcl-2 and Bcl-xL prevents viral release

We used immunoblot to probe for virus VP1 expression and found that there was no change in VP1 expression between Neo and Bcl-2 and Bcl-xL overexpressing cells 10 h p.i. (Fig. 5A). Treatment with the general caspase inhibitor zVAD.fmk, or Bcl-2 and Bcl-xL expression, showed a decrease in the amount of progeny virus released from the supernatant at 10 h p.i. as determined by plaque assay. Plaque assay for infectious virus on intracellular extracts confirms that the decrease in released virus is accounted for by an increase in cytoplasmic virus (Fig. 5B). Bcl-2 and Bcl-xL overexpression and zVAD.fmk reduction of extracellular virus suggest that host cell apoptosis facilitates the release of progeny virus from infected cells.

Discussion

Viruses may either prevent host cell death by expression of antiapoptotic viral proteins (Teodoro and Branton, 1997; Granville et al., 1998) or benefit by the initiation of rapid cell death following infection. Picornaviruses have a short replication life cycle and are thus capable of quickly parasitizing the host and controlling normal cellular functions. Host transcription and translation are inhibited to allow for maximal use of host accessory factors for viral RNA transcription and polyprotein translation. Within hours of infec-

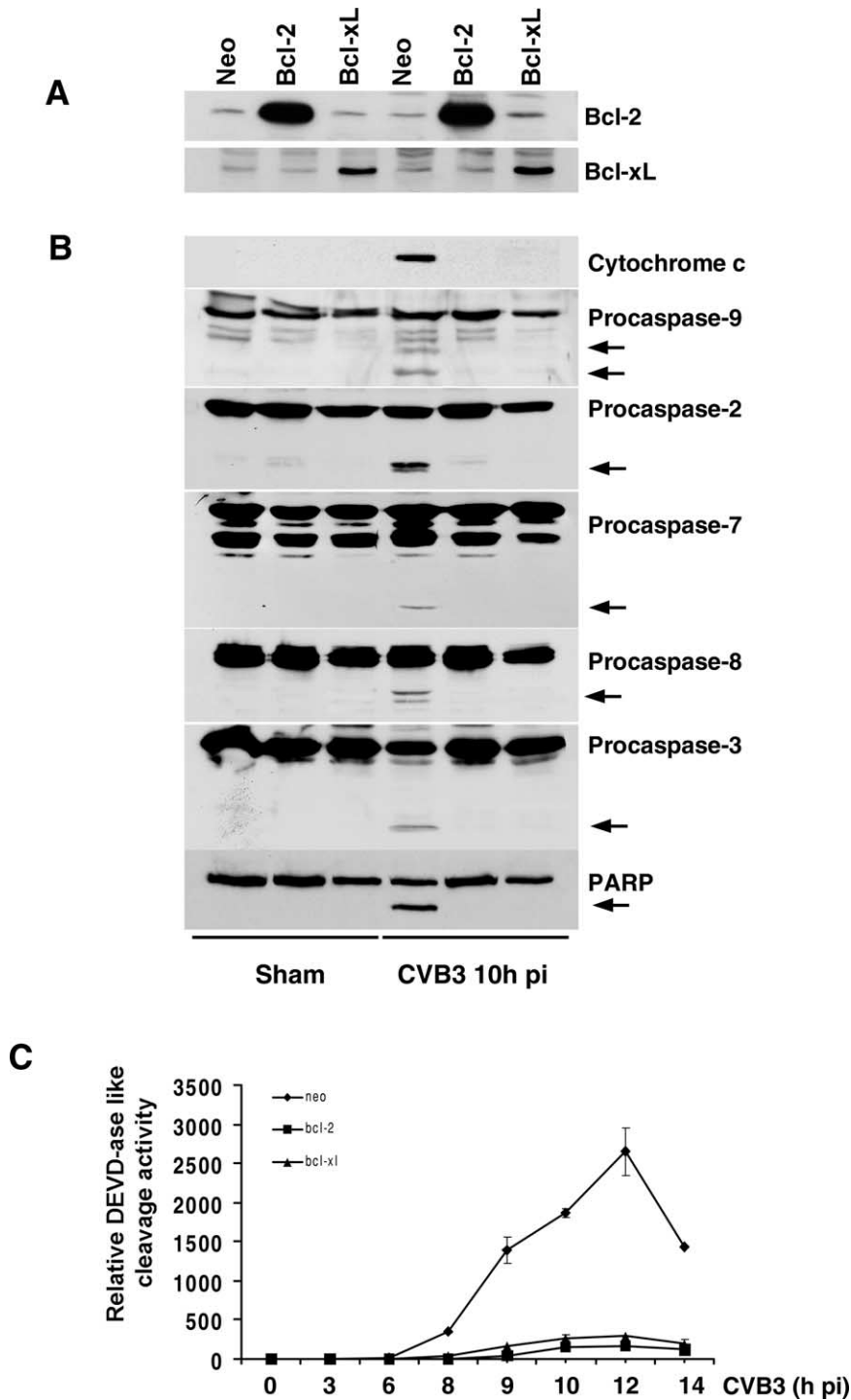


Fig. 2. Bcl-2 and Bcl-xL overexpression prevents *cyt c* release and processing and activation of multiple caspases. Neo, Bcl-2, and Bcl-xL overexpressing HeLa cells were either CVB3 (m.o.i. 10) or sham infected and assessed for caspase cleavage and activation. Cytosolic extracts were assessed for (A) presence of Bcl-2 and Bcl-xL and (B) cytochrome *c* release from the intermitochondrial membrane space to the cytosol, multiple caspase activation, and cleavage of PARP at 10 h following CVB3 infection. (C) Neo cells, Bcl-2 overexpressing HeLa cells, and Bcl-xL overexpressing HeLa cells were infected with CVB3 (m.o.i. 10), harvested 0–14 h postinfection, and submitted for DEVDase-like cleavage activity assay.

tion, infectious progeny virus can be detected in the cytosol of the host cell. Thus, it is becoming evident that apoptosis in picornavirus-infected cells may be a process triggered or accelerated by the virus to facilitate viral progeny release.

Activation of the apoptotic machinery can be demonstrated in tissue culture following picornavirus infection (Carthy et al., 1998). We further demonstrate that in addition to caspase-3 activation, PARP, and lamin A/C cleav-

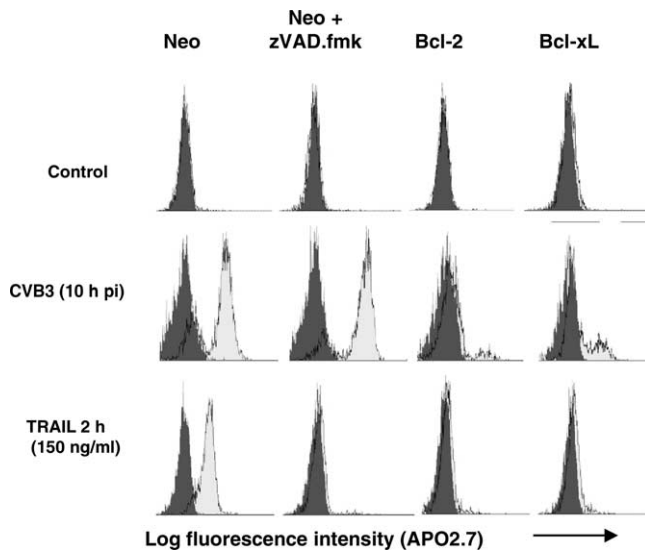


Fig. 3. Bcl-2 and Bcl-xL overexpression but not zVAD.fmk decreases CVB3-induced 7A6 expression. Neo cells, neo cells treated with zVAD.fmk, Bcl-2 overexpressing HeLa cells, and Bcl-xL overexpressing HeLa cells were either infected with CVB3 (m.o.i. 10) for 10 h, exposed to TRAIL (150 ng/ml) for 2 h, or control treated, and flow cytometry with APO2.7 was utilized to detect the mitochondrial epitope 7A6.

age, multiple members of the caspase family are activated. Apoptosis-related caspases -2, -3, -6, -7, -8, and -9 are all activated or degraded into their characteristic large and small subunit components during CVB3 infection. Effector caspases -3, -6, and -7 are cleaved and activated as shown by substrate cleavage including PARP, a nuclear protein involved in DNA repair, and Lamin A/C, a structural protein. Caspase-8 and -3 can process Bid (Bossy-Wetzel and Green, 1999; Li et al., 1998) and caspase-9 can activate caspase -3, -6, or -7 (Pan et al., 1998). To show that apoptosis signaling is not confined to HeLa cells, we show that CVB3-infected murine atrial cardiomyocytes also exhibit caspase-3 cleavage late during the infectious process. Once caspases are activated, a rapid proteolytic cascade occurs and the cell is quickly dismantled and prepared for phagocytosis.

Caspases can cleave multiple substrates to regulate translation, transcription, DNA synthesis and repair, stress signaling, and to disrupt the cellular cytoskeleton by degradation of important structural proteins. Data presented here suggest that caspase activation in picornavirus-infected cells enhances viral release from cells. Such a consequence would support the virus life cycle by allowing the virus to find a new host cell and possibly to decrease the ability of the host to circumvent a virus-induced mobilization of a nonspecific immune response. Further caspase activation would decrease inflammation, since molecules such as phosphatidylserine expressed on apoptotic cells are known to induce rapid phagocytosis with limitation of inflammation.

Mitochondria are central regulators of caspase activation during picornavirus infection. Cyt *c* is released and initiator

caspase-9 activation may occur through cytosolic procaspase-9, which suggests that this is an important event in CVB3-induced apoptosis. Cytoplasmic cyt *c* can associate with Apaf-1 in the presence of dATP and trigger caspase-9 autocatalysis. Activated caspase-9 may either be cytosolic pro-caspase-9 which is recruited to or activated by the apoptosome or be mitochondrially sequestered procaspase-9 that is released with cyt *c*, or a combination of both. Anti-7A6 defines an epitope on the mitochondrial membrane protein that appears to be exposed on cells undergoing apoptosis (Zhang et al., 1996). We demonstrate here that expression of the mitochondrial epitope 7A6 following infection occurs despite zVAD.fmk treatment; thus, it is unlikely that mitochondrial permeability transition occurs following Bid processing by caspase-8 or -3. The synthetic general caspase inhibitor zVAD.fmk does not inhibit caspase-2 nor does it have any effect on downstream caspase-independent events. In our infection model, processing of caspase-8, -2, and -3 is a relatively late event. In addition, since zVAD.fmk did not affect mitochondrial expression of 7A6, CVB3-mediated mitochondrial alterations are likely upstream of caspase-dependent pathways.

TRAIL is a death ligand that binds to death domain containing TRAIL-R1 and TRAIL-R2, induces oligomerization, recruitment of adaptor proteins, and subsequent caspase activation (Schulze-Osthoff et al., 1998). Contrary to infection by CVB3, TRAIL induces processing of caspase-8 and subsequent cyt *c* release and processing of executioner caspases, as well as mitochondrial membrane alterations and expression of the 7A6 epitope.

As alluded to earlier, there are several proapoptogenic materials that have been identified in addition to cyt *c* such as AIF, procaspase-2, -3, and -9, Smac/DIABLO, Endo G, HtrA2/Omi, and others, which are normally sequestered within the mitochondria. As previously shown, cell viability was only partially restored with general caspase inhibition (Carthy et al., 1998). Taken together, proapoptogenic molecules such as AIF and Endo G which translocate to the nucleus and induce DNA fragmentation in a caspase-independent manner may be involved (Li et al., 2001; Susin et al., 1996). Other mitochondrial alterations induced during picornavirus infection may relate to apoptotic signaling; previous studies have demonstrated the generation of reactive oxygen species (Hiraoka et al., 1993), downregulation of various mitochondrial transcripts (Taylor et al., 2000), and a decrease in ATP levels (Waldenstrom et al., 1993).

The Bcl-2 and Bcl-xL are antiapoptotic molecules which are localized to mitochondrial, endoplasmic reticular, and nuclear membranes (Tsujimoto and Shimizu, 2000). We found that overexpression of Bcl-2 and Bcl-xL prevented cyt *c* release from the mitochondria to the cytosol for at least the first 10 h following infection in HeLa cells. This inhibition of cyt *c* release delays and decreases DEVDase-like cleavage activity. However, incomplete preservation of morphology suggests cell bursting during viral release, inhibition of host transcription and translation, and loss of

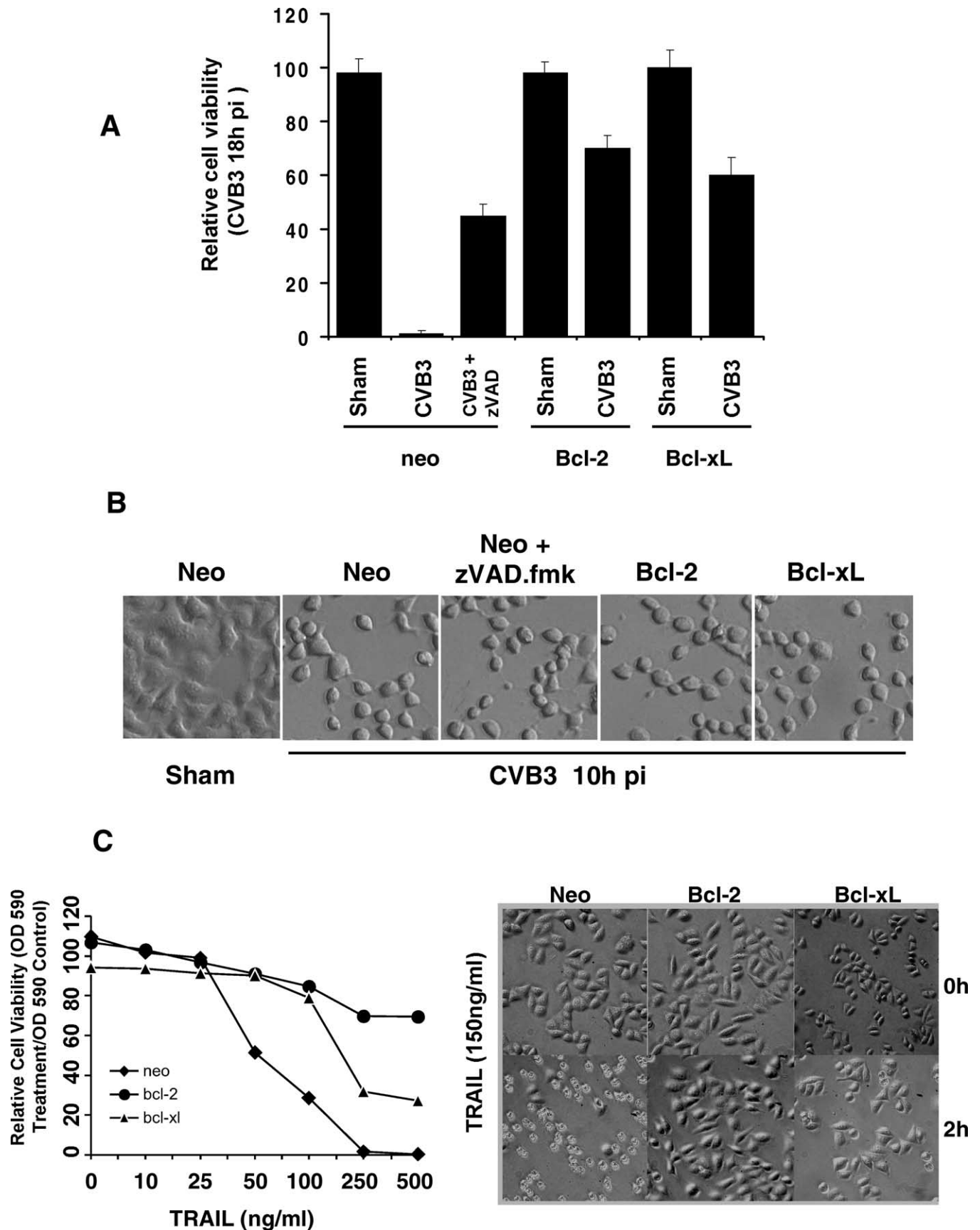


Fig. 4. Bcl-2 and Bcl-xL overexpression and zVAD.fmk restores cell viability but not cytopathic effect. Neo cells, neo cells treated with zVAD.fmk, Bcl-2 overexpressing HeLa cells, and Bcl-xL overexpressing HeLa cells were either infected with CVB3 (m.o.i. 10) or sham infected. (A) Cell viability was measured by MTT dye reduction assay and (B) morphological analysis by differential interference contrast microscopy. (C) Neo cells, Bcl-2, and Bcl-xL overexpressing HeLa cells were exposed to TRAIL (0–500 ng/ml) and MTT analysis was performed at 24 h. Morphological analysis by differential interference contrast microscopy of Neo cells, Bcl-2 overexpressing HeLa cells, and Bcl-xL overexpressing HeLa cells was also performed at 0 and 2 h postinduction.

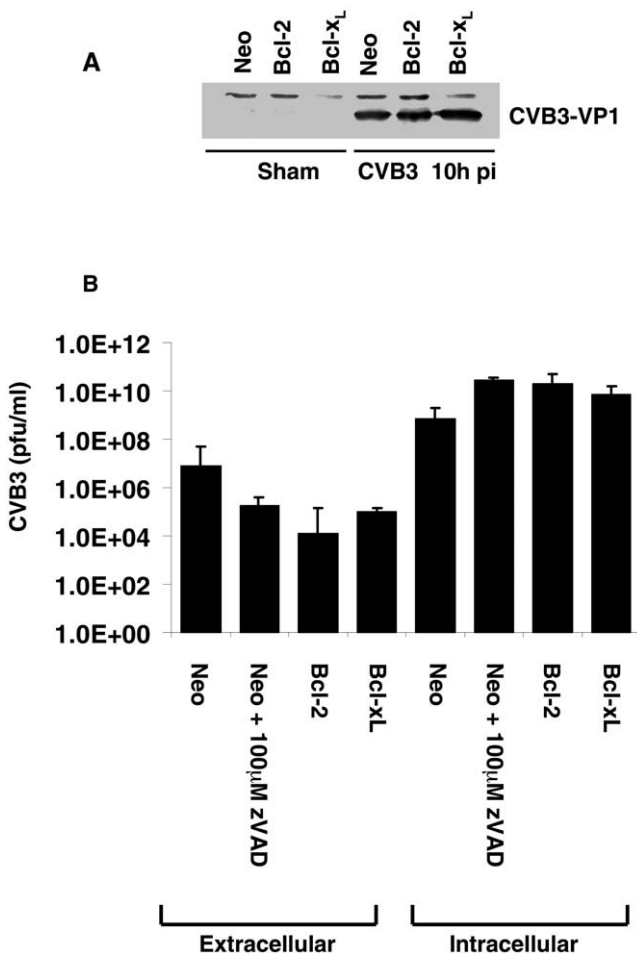


Fig. 5. Bcl-2 and Bcl-xL overexpression and zVAD.fmk prevents viral release. Neo cells, neo cells treated with zVAD.fmk, Bcl-2-overexpressing HeLa cells, and Bcl-xL overexpressing HeLa cells were infected with CVB3 (m.o.i. 10). At 10 h postinfection, (A) immunoblot analysis was performed using an antibody directed against the viral capsid protein VP1 and (B) plaque assay was utilized for determination of intracellular and extracellular virus titers.

cellular homeostasis due to direct viral protease cleavage of structural proteins likely also contribute to the observed apoptosis phenotype. Indeed, it has been shown that picornaviral proteases cleave structural proteins early during infection (Badorff et al., 1999; Seipelt et al., 2000). Protease 2A cleavage of dystrophin disconnects the cytoskeleton from the extracellular matrix anchor, completely disrupting the cytoskeletal architecture (Badorff et al., 2000a, 2000b). Recently, Xiong et al., (2002) showed that such disruption facilitates virus release from target cells which may occur through membrane destabilization. This does not mean that all morphological changes seen during picornavirus infection are independent of caspases. Many structural changes noted during electron microscopic evaluation of picornavirus infected cells are characteristic of apoptosis (McManus et al., 1993).

Overexpression of Bcl-2 and Bcl-xL or caspase inhibition had no effect on virus replication as there was no

change in expression of viral capsid protein VP1. However, they did block virus release from infected cells as shown by a decrease in virus release and a simultaneous increase in intracellular virus as compared to infection of Neo cells alone. This data suggest that host-cell apoptosis does not affect virus replication per se but is necessary for efficient progeny virus egress. Since activated caspases can upset cellular architecture, host apoptosis and associated cytoskeletal and membranous disruption is likely a viral mechanism to facilitate maximum virus spread.

In conclusion, we present evidence that mitochondrial disruption and caspases play a role in loss of functionality and viability of cells during picornavirus infection, which is inhibited by expression of Bcl-2 and Bcl-xL. It is clearly evident that numerous cellular processes are occurring in a cytopathic cell including activation of apoptotic proteolytic machinery. These data suggest a direct role for activation of caspases in the loss of viability and facilitation of efficient progeny virus release following CVB3 infection.

Materials and methods

Cells, virus, reagents, and antibodies

HeLa cells were obtained from the ATCC. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, 1 mM HEPES buffer, and 10% heat-inactivated fetal bovine serum (FBS). HeLa cells overexpressing Bcl-2 and Bcl-xL were generated as previously described (Vander Heiden et al., 1997). Briefly, Bcl-2 and Bcl-xL inserts were cloned into *EcoRI* site of pSFFV-Neo vector (Fuhlbrigge et al., 1988). Vectors containing inserts, or no insert, were transfected into HeLa cells by electroporation. After selection with 1 µg/ml G418 (Gibco, BRL Life Technologies), cells were cloned by limiting dilution and transfectants screened for Bcl-xL or Bcl-2 by Western blot. Transfected cell lines were maintained in complete DMEM with 1 µg/ml G418.

HL-1 cells (murine atrial cardiomyocyte cells) were a generous gift from Dr. William C. Claycomb (Louisiana State University Medical Center, New Orleans, LA). Cells were plated onto fibronectin and gelatin-coated flasks as described previously (Claycomb et al., 1998) and maintained in Claycomb media from JRH Biosciences (Lenexa, KS) supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, 0.1 mM norepinephrine in ascorbic acid, and 10% FBS. Prior to infection, HL-1 cells were grown to 80% confluency in serum-free media and infected as above.

Stock CVB3 was generously provided by Dr. Charles Gauntt (University of Texas Health Sciences Center, San Antonio, TX). Virus was propagated in HeLa cells and stored at -80°C , and titers were routinely determined at the beginning of all experiments.

The general caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD.fmk) was purchased from BACHEM (Bubendorf, Switzerland) and diluted to a stock concentration of 100 mM in DMSO. All other supplies were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Antibodies to cyt *c*, caspase-2, cleaved caspase-3, and caspase-9 were purchased from Pharmingen (San Diego, CA). Antibodies to caspases-6 and -8 were from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibody to caspase-7 was obtained from Transduction Laboratories (Lexington, KY); caspase-3 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and PARP (mAb C-2-10) was obtained from BIOMOL (Plymouth Meeting, PA). Antibodies to Bcl-2 and Bcl-xL were purchased from Transduction Laboratories and polyclonal CVB3 VP1 antibody was obtained from Accurate Chemicals (Westbury, NY).

Virus infection

HeLa cells were infected at an m.o.i. of 10 with CVB3 or sham-treated with DMEM without FBS for 45 min. Cells were washed with phosphate-buffered saline (PBS) and complete DMEM containing 10% FBS was replaced. HeLa cells were exposed to TRAIL (150 ng/ml) for 24 h. Cell viability was measured using MTT as described below.

Cell lysates, electrophoresis, and Western blotting

Cells were washed twice in cold PBS. For total cell lysates, cell were suspended in 1 ml of cold lysis buffer (20 mM Tris pH 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin) per 75-cm² culture area. After 20 min on ice, supernatant was collected following centrifugation at 10,000 *g*. For cytosolic extracts, cells were washed twice in cold PBS and resuspended in 1 ml of ice cold buffer (250 mM sucrose, 20 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, supplemented with 1 mM PMSF and 10 µg/ml aprotinin) per 75-cm² culture area. Cells were disrupted by 20 strokes with the B pestle of a Kontes dounce homogenizer. The supernatant was collected following centrifugation at 10,000 *g* and further centrifuged at 100,000 *g* for 1 h at 4°C in a Beckman Optima ultracentrifuge using a TL-100 rotor. Cell lysate protein concentration was determined by the BCA method (Pierce Chemical Co., Rockford, IL). Samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred to nitrocellulose (Hybond ECL, Amersham Pharmacia Biotech, Uppsala, Sweden). Following blocking and incubation with primary and secondary antibody, horseradish peroxidase conjugated secondary immunoglobulins were detected using the enhanced chemiluminescence (ECL) method (Amersham Pharmacia Biotech) and exposed to Hyperfilm (Amersham Pharmacia Biotech).

Caspase activation assay

To evaluate DEVDase-like (caspase-3 and -7) cleavage activity following CVB3 infection, total HeLa cell lysates were incubated with a caspase-specific fluorescent substrate as previously described (Granville et al., 1997). Briefly, lysates were incubated with reaction buffer (20 mM Tris pH 7.5, 137 mM NaCl, 1% NP-40, 10% glycerol) containing 100 µM of the caspase substrates DEVD-AMC (Calbiochem, Cambridge, MA). Reaction mixture was incubated at 37°C for 2 h and fluorescence excitation at 380 nm and emission at 460 nm was measured using a CytoFluor 2350 from Perseptive Biosystems (Norwalk, CT).

Flow cytometry

At 10 h following CVB3 infection or 2 h following TRAIL (150 ng/ml) treatment, HeLa cells were washed twice in cold PBS and dispersed into suspension by gentle agitation with 2 mM EDTA–PBS. Cells were permeabilized with 0.1% digitonin in PBS. Cells were exposed to the phycoerythrin (PE)-conjugated mouse IgG1 monoclonal antibody APO2.7 (Beckman-Coulter, Fullerton, CA) in a solution of PBS containing 2% FCS for 30 min on ice. Control cells were treated with a PE-conjugated isotype-matched antibody. Cells were washed twice with the labeling solution and read immediately on a Coulter XL flow cytometer (Coulter Electronics, Inc., Hialeah, FL). Mean channel fluorescence intensity (MCFI) values were determined (5000 cells analyzed per sample).

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay

To determine cell viability, cells were cultured in Falcon 96-well microtiter plates and the MTT assay was performed as previously described (Mosmann, 1983) with modifications. Briefly, following CVB3 infection culture medium was decanted and replaced with complete DMEM containing 10% FBS. At 10 h following CVB3 infection or 24 h following TRAIL (0–500 ng/ml) treatment, culture medium was aspirated and 50 µg of MTT in 100 µl of PBS was added to each well. Cells were incubated for 1 h and the reaction was stopped by the addition of 150 µl of acidified isopropanol. Color development correlating to cell viability was measured at 590 nm with a Dynatech Laboratories microtiter plate reader (Chantilly, VA).

Plaque assay

The amount of CVB3 in cell supernatant or cytoplasmic extracts was determined on monolayers of HeLa cells by agar overlay plaque assay method as previously described (Anderson et al., 1996). Briefly, sample supernatant was serially diluted 10-fold and overlaid on 90–95% confluent monolayers of HeLa cells in six-well plates (Corning

Costar, Corning, NY) and incubated for 1 h (5% CO₂, 37°C). Media containing nonbound virus was removed and warm complete DMEM containing 0.75% agar was overlaid in each well. The plates were incubated 36 to 48 h (5% CO₂, 37°C), fixed with Carnoy's fixative (95% EtOH: acetic acid (3:1)), and stained with 1% crystal violet. The plaques were counted and the viral concentration was calculated as plaque forming units (PFU) per milliliter of culture supernatant.

Morphology

Differential interference contrast (DIC) morphological images were taken with a Nikon Eclipse TE300 inverted scope (Nikon, Tokyo, Japan) and collected using SPOT imaging software (Diagnostic Instruments, Inc.; Sterling Heights, MI).

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