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The Major Apoptotic Pathway Activated and Suppressed by Poliovirus

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Cells respond to poliovirus infection by switching on the apoptotic program, implementation of which is usually suppressed by viral antiapoptotic functions. We show here that poliovirus infection of HeLa cells or derivatives of MCF-7 cells was accompanied by the efflux of cytochrome *c* from mitochondria. This efflux occurred during both abortive infection (e.g., interrupted by guanidine-HCl and ending with apoptosis) and productive infection (leading to cytopathic effect). The former type of infection, but not the latter, was accompanied by truncation of the proapoptotic protein Bid. The virus-triggered cytochrome *c* efflux was suppressed by overexpression of Bcl-2. Both abortive and productive infections also resulted in a decreased level of procaspase-9, as revealed by Western blotting. In the former case, this decrease was accompanied by the accumulation of a protein with the electrophoretic mobility of active caspase-9. In contrast, in the productively infected cells, the latter protein was absent but caspase-9-related polypeptides with altered mobility could be detected. Both caspase-9 and caspase-3 were shown to be essential for the development of such hallmarks of virus-induced apoptosis as chromatin condensation, DNA degradation, and nuclear fragmentation. These and some other results suggest the following scenario. Poliovirus infection activates the apoptotic pathway, involving mitochondrial damage, cytochrome *c* efflux, and consecutive activation of caspase-9 and caspase-3. The apoptotic signal appears to be amplified by a loop which includes secondary processing of Bid. The implementation of the apoptotic program in productively infected cells may be suppressed, however, by the viral antiapoptotic functions, which act at a step(s) downstream of the cytochrome *c* efflux. The suppression appears to be caused, at least in part, by aberrant processing and degradation of procaspase-9.

Poliovirus, a small unenveloped virus containing a 7.4-kb RNA genome (an enterovirus representative of the family *Picornaviridae*), is usually cytocidal. Two types of death of the poliovirus-infected cell have been described. The canonical cytopathic effect (CPE), known for several decades (35), consists of a dramatic change in the cytoplasmic infrastructure with accumulation of numerous membranous vesicles, increased plasma membrane permeability, distortion and displacement of the nuclei, partial condensation of chromatin (pyknosis), rounding up of the infected cells, and eventually their lysis. On the other hand, poliovirus-infected cells may develop an apoptotic response (64) with cytoplasmic blebbing, heavy condensation and fragmentation of chromatin, and degradation of chromosomal DNA. The process is completed with cell fragmentation into small membrane-coated entities, the so-called apoptotic bodies. In HeLa cells, productive infection results primarily in CPE, whereas abortive infection caused by some viral mutations or the presence of some inhibitors may trigger the apoptotic reaction (3, 64). However, in some other cell cultures, e.g., CaCo-2 colon carcinoma cells (5) and a line of promonocytic cells (42), signs of apoptosis could also be observed in a proportion of productively infected cells.

Apoptosis is a result of the implementation of a complex cellular death program evolved to eliminate various forms of damaged or unwanted cells both during normal development (44, 67) and under different pathological conditions, including infectious diseases (19, 23). Apoptosis can be triggered by a broad variety of external and internal stimuli. Schematically, three major primary targets for these stimuli are known: (i) receptors on the cell surface, such as FAS, tumor necrosis factor (TNF) receptors, and so on (37, 70); (ii) mitochondria (2, 17, 57); and (iii) the endoplasmic reticulum (ER) (46, 53). The pathways starting from these primary targets generally include, among other components, a cascade of cysteine proteinases or caspases (12, 14, 21, 62). The upstream components of the cascades, initiator caspases, are specific for each primary target. Thus, the upstream caspase in the receptor-mediated apoptotic pathway is caspase-8, the mitochondrion-related pathway exploits caspase-9 as the initiator caspase, and the pathway triggered by ER stress is based on caspase-12. The above-mentioned pathways converge on downstream, or effector, caspases, exemplified by caspase-3. Each pathway also includes a variety of activating and inhibiting proteins, and there is cross talk between different pathways (for recent reviews, see references 2, 7, 11, 30, and 68). In addition, forms of apoptosis or apoptosis-like death which are accomplished through caspase-independent mechanisms are known (9, 28, 38, 59, 60, 66).

Against the background of this wealth of information about the cellular apoptotic machinery, little is known about the mechanisms of virus-triggered apoptosis in general (48, 55) and of apoptosis induced by poliovirus in particular. Expres-

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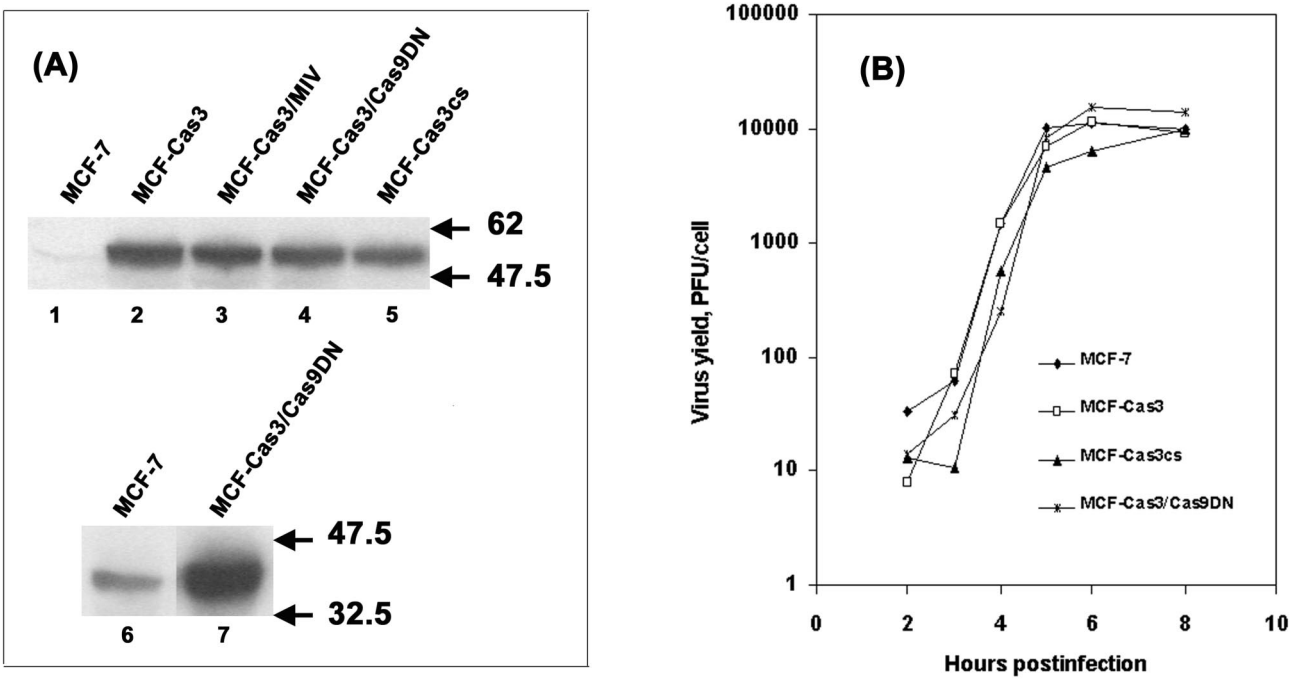

FIG. 1. General properties of MCF-7 cell derivatives. (A) Overexpression of relevant caspase species in the derived cell lines as revealed by Western blotting. Total cell lysates prepared from the respective cells were probed with polyclonal antibodies against procaspase-3 (lanes 1 to 5) and procaspase-9 (lanes 6 and 7). The caspase-3-specific bands, seen in the MCF-7 derivatives, correspond to the proenzyme fused to GFP. (B) Single-cycle growth curves of poliovirus in MCF-7, MCF-Cas3, MCF-Cas3cs, and MCF-Cas3/Cas9DN cells. The infection was carried out as described in Materials and Methods. The harvest was plaque titrated on RD cells.

sion of poliovirus proteases 2A (26) and 3C (6) has been reported to result, under certain conditions, in the apoptotic response. The implementation of poliovirus-induced apoptosis involves activation of caspases and is prevented in the presence of a broad-spectrum caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (zVAD.fmk) (3). However, the nature of the virus-activated apoptotic pathway and the identities of the components involved were not elucidated.

Although poliovirus infection switches on a defensive apoptotic reaction, other viral functions exhibit antiapoptotic activities (64). In fact, the development of CPE rather than apoptosis in certain cells, such as HeLa cells, is caused by the early interruption of implementation of the activated cellular apoptotic program due to the expression of viral antiapoptotic functions (4). At what step and how the apoptotic pathway is interrupted remain unknown.

The major goal of the present study was to identify the nature of the pathway resulting in the apoptotic death of poliovirus-infected cells and to map the step of this pathway that is suppressed by the viral antiapoptotic function. By making use of cells deficient in caspase-3 or caspase-9 activity and by studying some markers of specific apoptotic pathways, we came to the conclusion that the viral infection primarily activates the apoptotic pathway involving consecutive activation of caspase-9 and caspase-3 due to cytochrome *c* release from damaged mitochondria. The results also suggest that productive viral infection suppresses implementation of this apoptotic program downstream of the cytochrome *c* release and that the suppression is caused, at least in part, by aberrant processing and degradation of procaspase-9.

MATERIALS AND METHODS

Cells and virus. A subline of HeLa-S3, HeLa-B (64), and Bcl-2-expressing HeLa-Bcl2 (4) were cultivated in Eagle medium supplemented with 10% bovine serum, while cultivation of derivatives of MCF-7 cells (see below) was carried out in Dulbecco's modified Eagle medium with 10% calf embryonal serum. A derivative of poliovirus type 1 Mahoney strain, Mgs (63), was used.

Derivation of cells with altered properties of caspases. Cells differentially devoid of functional caspase-3 or caspase-9, or both, were derived from MCF-7 human breast carcinoma cells, known to have an inactivating deletion in the gene encoding caspase-3 (34). The genes encoding intact procaspase-3 and procaspase-3 harboring a loss-of-function mutation (24) fused to the gene encoding green fluorescent protein (GFP; Clontech) were cloned into the MaRX-IV-(puro) retroviral vector, while cDNA of caspase 9DN (a dominant-negative mutant of caspase 9 [25]) was cloned into the MaRX-IV(neo) vector (27). The caspase-3-competent MCF-Cas3-GFP and the caspase-3-negative MCF-Cas3cs-GFP cells were obtained from MCF-7 cells after transduction with MaRX-IV/Cas3-GFP and MaRX-IV/Cas3cs-GFP(puro) vectors, respectively. The transduction was performed essentially as described previously (24). The transduced cells were selected in the presence of 1 μg of puromycin/ml for 4 days and were subsequently maintained in the presence of 0.5 μg of puromycin/ml. The fluorescence of GFP in all these cultures was relatively weak and did not interfere with any of our specific assays. For brevity, the suffix GFP will hereafter be omitted from the names of the cultures.

The caspase-3^+ caspase-9^+ MCF-Cas3/MIV cells and caspase-3^+ caspase-9^- MCF-Cas3/Cas9DN cells were similarly obtained by retroviral transduction of MCF-Cas3 cells with MaRX-IV(neo) and Marx-IVCas9DN(neo), respectively. MCF-Cas3cs/Cas9DN cells devoid of both caspase-3 and caspase-9 activities were prepared by using the same procedure after transduction of MCF-Cas3cs cells with the MaRX-IVCas9DN(neo) vector. Cells were subjected to selection with 500 μg of G-418 (Sigma)/ml for approximately 2 weeks, and the resistant clones were pooled. Subsequent cultivation was carried out in the presence of 300 μg of G-418/ml.

The overexpression of relevant caspases in derivatives of MCF-7 was confirmed by Western blotting (Fig. 1A).

Poliovirus infection and single-cycle growth experiments. Aliquots of EDTA-treated cells were plated on 35- or 60-mm-diameter petri dishes (Corning-

Costar) at a density of approximately $10^5/cm^2$ and cultivated overnight under 5% CO_2 at 37°C in Eagle medium with 5% bovine serum (HeLa cells) or Dulbecco's modified Eagle medium with 5% calf embryonal serum (MCF-7 derivatives). The growth medium was discarded, and the virus was added in a volume of 2 ml to provide an effective multiplicity of infection of ~100 (HeLa cells) or ~40 (MCF-7 derivatives) PFU/cell. After a 30-min incubation at 18°C, the cells were washed off with Earle's solution, and 1 or 3 ml (for 35- or 60-mm-diameter dishes, respectively) of serum-free Eagle medium was added. After incubation at 37°C for the appropriate time intervals, the viral harvest was plaque titrated on RD cells.

Apoptosis induction. The protein synthesis inhibitor cycloheximide (CHI; 100 μg/ml) or the transcription inhibitor actinomycin D (ActD; 1 μg/ml) was used as a nonviral inducer of apoptosis. To produce apoptotic response to poliovirus infection, the infected cells were treated with guanidine-HCl (100 μg/ml) at an early step (1.5, 2.0, or 2.5 h postinfection [p.i.]) and fixed for microscopic analysis several hours thereafter. When necessary, the development of apoptosis was prevented by 100 μM zVAD.fmk (Enzyme Systems Products, Dublin, Calif.).

Fluorescence and immunofluorescence microscopy. For observation of the status of chromatin, cells were grown on coverslips placed in petri dishes (see above). The permeable fluorescent dye Hoechst 33342 (Sigma) was added at a final concentration of 5 μg/ml 30 min before cell fixation with Safe Fix (Curtin Matheson Scientific, Houston, Tex.). For the immunofluorescence cytochrome *c* assay, cells were grown on coverslips, fixed with 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS) for 10 min, and permeabilized with 0.2% Triton X-100 in 4% paraformaldehyde in PBS for 10 min. Anti-cytochrome *c* rabbit polyclonal antibodies were from Santa Cruz Biotechnology (catalog no. sc-7159), and anti-rabbit immunoglobulin G fluorescein isothiocyanate-conjugated antibodies were from Sigma (catalog no. F-0382). Three percent nonfat dry milk in PBS was used for blocking nonspecific binding sites and for dilution of primary and secondary antibodies. The DNA dye Hoechst 33342 was added to the blocking solution at a concentration of 5 μg/ml. Subsequent incubations in blocking solution with primary and secondary antibodies were done for 1 h at 37°C. The cells were washed twice for 5 min each time in PBS with agitation at room temperature after incubation with the primary and secondary antibodies. The stained coverslips were placed on a drop of a mixture of glycerol–100 mM Tris, pH 9, and examined with a Leica DMLS epifluorescent microscope equipped with a Leica DC 100 digital camera and with filter cube A or I3 used for the observation of blue (Hoechst) and green (immunostaining) fluorescence, respectively.

TUNEL assay. Cells were grown on coverslips and stained with Hoechst 33342 as described above but were fixed at room temperature with Safe Fix for 30 min. Then, the cells were treated with 96 and 70% ethanol and stored at −20°C. The assay was performed with the kit from Promega according to the manufacturer's instructions. The filter cube I3 was used for the registration of terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling (TUNEL)-positive cells.

DNA fragmentation electrophoretic assay. DNA fragmentation was assayed as described previously (64). EDTA-treated cells suspended in a buffer containing 20 mM EDTA and 10 mM Tris-HCl, pH 7.4, were lysed with 0.5% Triton X-100 for 20 min in an ice bath. The nuclei were pelleted in an Eppendorf 5415C minicentrifuge (12,000 rpm; 15 min; 4°C), and the resulting supernatants were treated with phenol-sodium dodecyl sulfate. After ethanol precipitation, the nucleic acids were dissolved in 10 μl of H_2O and treated with RNase A (10 μg/ml; 37°C; 30 min). Samples were subjected to electrophoresis on 1.5% agarose gels.

Western blotting. Cells were treated with EDTA and collected, together with the detached floating material, by centrifugation in an Eppendorf minicentrifuge at minimal speed for 4 min. The supernatant was discarded, and the pellet was lysed with a buffer containing 2% sodium dodecyl sulfate–35 mM β-mercaptoethanol–50 mM Tris-HCl, pH 6.8, supplemented immediately before use with 1 mM phenylmethylsulfonyl fluoride and a cocktail of protease inhibitors containing chymostatin, pepstatin, leupeptin, and antipain (2 μg of each/ml). Following addition of the lysis buffer, the cells were incubated in a boiling-water bath for 10 min, and the lysates were sonicated to reduce viscosity. The protein concentration was measured using the Bradford protein concentration assay (Bio-Rad) according to the manufacturer's recommendations. The total amount of protein loaded into one well of the discontinuous denaturing polyacrylamide minigel was 30 μg. After electrophoresis, the proteins were transferred onto a 0.45-μm polyvinylidene difluoride membrane (Amersham) using the Bio-Rad minigel transfer system. The membrane was incubated overnight in blocking solution (135 mM NaCl, 17.3 mM Tris-HCl [pH 7.0], 0.1% Tween-20, 5% nonfat dry milk) at 4°C with agitation. This blocking solution was also used for the dilution of primary and secondary antibodies. The following preparations were used as primary antibodies: anti-caspase-9 rabbit polyclonal and anti-caspase-3 mouse monoclonal antibodies (25), anti-Bid rabbit polyclonal antibodies from Pharmingen (catalog no. 550365), anti-cytochrome *c* mouse monoclonal antibodies from Santa Cruz Biotechnology (catalog no. sc-13156), and anti-actin mouse monoclonal antibodies from Sigma (catalog no. A-4700). The secondary antibodies were represented by anti-mouse or anti-rabbit horseradish peroxidase conjugates from Amersham. Incubation with antibodies was performed for 1 h at room temperature, followed by triple washing with the blocking solution without milk. Protein bands were visualized with the ECL+ detection system (Amersham).

Cell fractionation. EDTA-treated cells from 6-cm-diameter petri dishes were pelleted for 5 min by centrifugation in an Eppendorf minicentrifuge at minimal speed. The pellet was resuspended in 200 μl of a hypotonic lysis buffer (20 mM HEPES [pH 7.4], 10 mM KCl, 2 mM EGTA, 1 mM dithiothreitol), supplemented immediately before use with the cocktail of protease inhibitors (see above). The cells were allowed to swell on ice for 15 min and were lysed by 15 strokes of an insulin syringe with a 27.5-gauge needle. The lysis was controlled under a phase-contrast microscope. Organelles, nuclei, and unbroken cells were collected at 15,000 × *g* for 20 min, and the supernatant was used as the soluble cytoplasmic fraction.

RESULTS

Role of caspase-3 in poliovirus-triggered apoptosis. The converging downstream parts of different caspase cascades include effector caspase-3, caspase-6, and caspase-7. Our preliminary experiments demonstrated that MCF-7 human breast carcinoma cells, known to lack functional caspase-3 due to a deletion in the appropriate gene (34), failed to elicit the apoptotic response to poliovirus infection either under permissive conditions or after interruption of productive infection by guanidine-HCl (not shown), a condition known to trigger the apoptotic response in HeLa cells (3, 64). These observations were consistent with a key role of caspase-3 in the implementation of the apoptotic program in poliovirus-infected cells. However, MCF-7 cells are deficient not only in caspase-3 but also in another component(s) of the standard apoptotic machinery (33).

To gain a deeper insight into the role of caspase-3 in poliovirus-triggered apoptosis, we made use of two derivatives of MCF-7 cells. One, MCF-Cas3, was stably transformed with a retroviral vector carrying the intact caspase-3 gene under a cytomegalovirus promoter, while the other, MCF-Cas3cs, was transformed with a similar vector having a loss-of-function mutation in the caspase-3 gene. When tested with a nonviral inducer of apoptosis (the protein synthesis inhibitor CHI), MCF-Cas3 cells, but not MCF-Cas3cs cells, developed a marked apoptotic response, as judged by nuclear staining with a permeable nuclear dye, Hoechst 33342, and electrophoretic assay for DNA degradation (not shown). The CHI-induced signs of apoptosis in MCF-Cas3 cells were essentially prevented in the presence of a 100 μM concentration of a broad caspase inhibitor, zVAD.fmk (not shown). Similar results were obtained with another apoptotic inducer, the transcription inhibitor ActD (not shown). These results demonstrated that the responses of MCF-Cas3 cells to CHI and ActD were similar to those of HeLa cells. On the other hand, MCF-Cas3cs cells proved to be refractory to these apoptotic inducers (not shown).

In single-cycle growth experiments, the efficiency and time course of poliovirus reproduction were essentially the same in the cells with and without caspase-3, with a yield of ~10^4 PFU per cell by 6 h p.i. (Fig. 1B). The productive infection of MCF-Cas3 or MCF-Cas3cs produced a typical pattern of CPE,

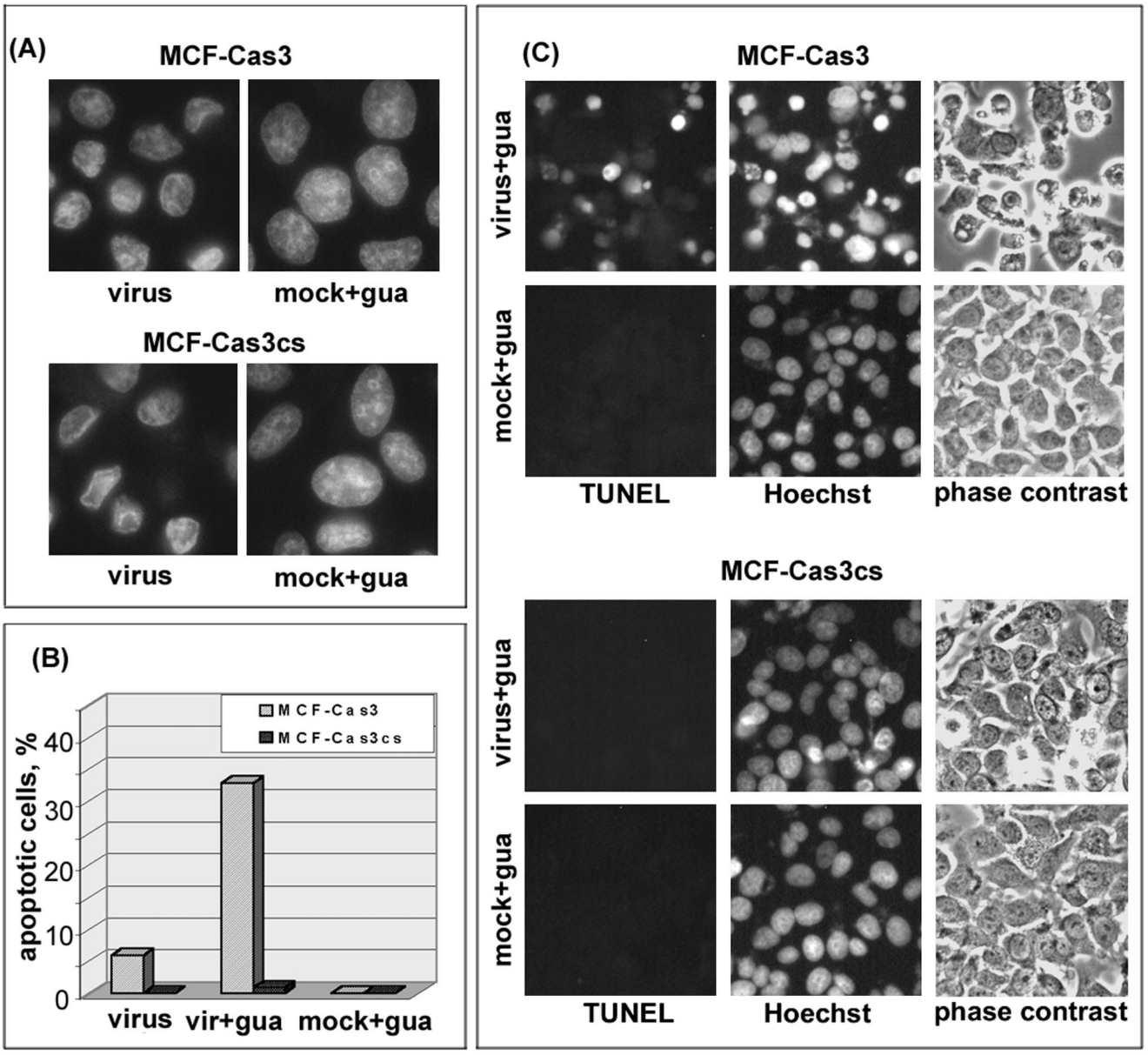

FIG. 2. Responses of MCF-Cas3 and MCF-Cas3cs cells to productive and abortive poliovirus infection. (A) Nuclear CPE in productively infected cells. Hoechst 33342 was added at 5.5 h p.i., and the cells were fixed at 6 h p.i. mock+gua, mock-infected cells with 100 μg of guanidine-HCl/ml added. (B) Percentages of TUNEL-positive infected cells at 6 h p.i. in the absence (virus) and presence (vir+gua) of 100 μg of guanidine-HCl/ml added at 2 h p.i. The data represent the averages of two experiments. (C) Development of apoptosis in MCF-Cas3 but not in MCF-Cas3cs cells upon abortive infection. Guanidine-HCl (100 μg/ml) was added to the virus-infected (virus+gua) and mock-infected (mock+gua) cells at 2 h p.i. Hoechst 33342 was added at 5.5 h p.i. The cells were fixed at 6 h p.i. and processed for the TUNEL assay. Qualitatively similar results were obtained when productive infection was interrupted by guanidine-HCl at 1.5 h p.i. (not shown).

revealed by nuclear staining (nuclear deformation and partial condensation of chromatin) (Fig. 2A) and phase-contrast microscopy (not shown). The electrophoretic assay showed no appreciable DNA degradation in the productively infected MCF-Cas3cs cells (Fig. 3A, lane 7) and only a small accumulation of high-molecular-mass DNA in MCF-Cas3 cells (Fig. 3A, lane 1), which was prevented in the presence of zVAD.fmk (Fig. 3A, lane 2). The TUNEL assay for DNA degradation in productively infected cells revealed only a very small proportion of positive cells, ~6 and 0% for MCF-Cas3 and MCF-Cas3cs cells, respectively (Fig. 2B). Even these small proportions of TUNEL-positive signals corresponded largely to dead cells, as judged by their staining with trypan blue (not shown). Also, a similar proportion of TUNEL-positive infected cells could be revealed in the cultures treated with zVAD.fmk (not shown), suggesting that the degradation of DNA in a few productively infected cells was caused largely by caspase-independent postmortem alterations.

When ongoing poliovirus infection was interrupted by the addition of millimolar concentrations of guanidine-HCl, a

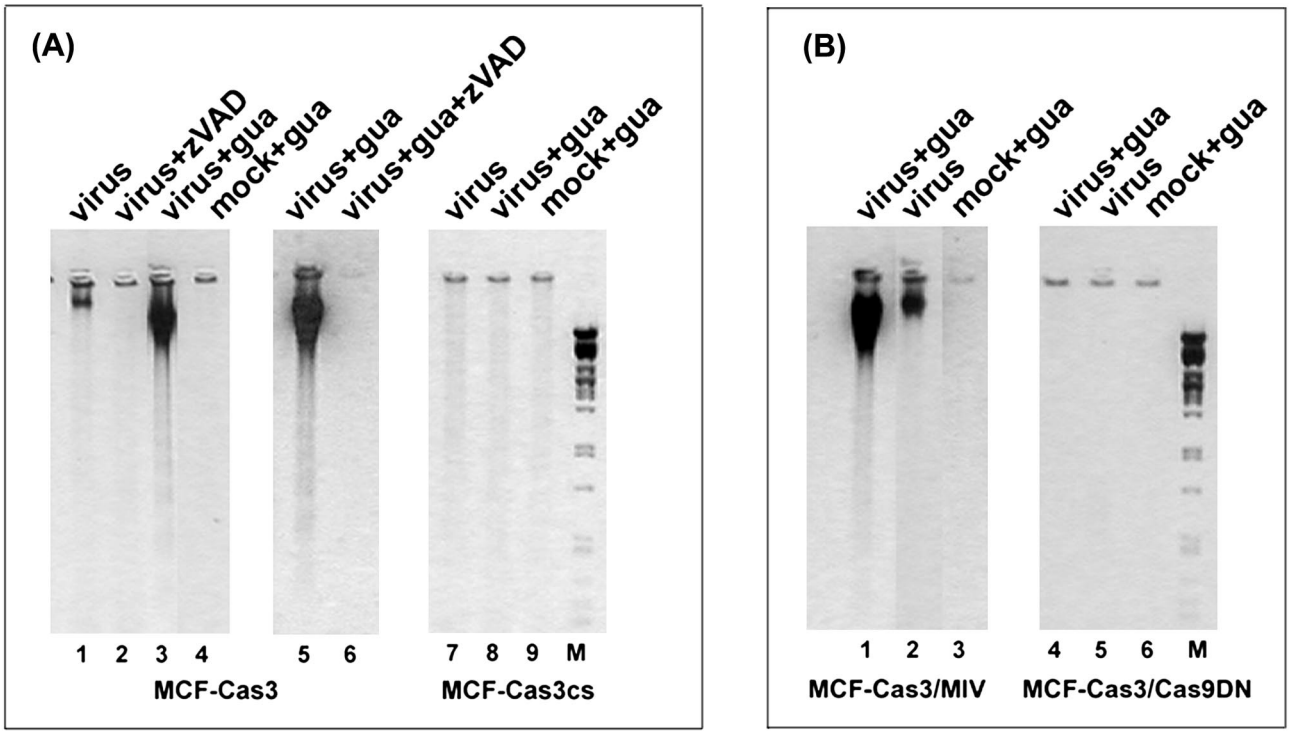

FIG. 3. Electrophoretic assay for DNA degradation upon productive (virus) and abortive (virus+gua) poliovirus infection of MCF-Cas3, MCF-Cas3cs, MCF-Cas3/MIV, and MCF-Cas-3/Cas9DN cells. Guanidine-HCl was added, when appropriate, 2 h after infection (virus+gua) or mock infection (mock+gua). To the sample analyzed in lanes 2 and 6, 100 μM zVAD.fmk (zVAD) was added at the onset of infection. The cells were harvested at 6 h p.i., except for the samples in lanes 5 and 6, where the cells were harvested at 8 h p.i. Lane M shows a *Pst*I digest of λ phage DNA.

marked proportion of MCF-Cas3 cells developed apoptotic reactions, as evidenced by strong condensation of chromatin revealed by Hoechst staining (Fig. 2C), positive TUNEL reactions (Fig. 2B and C), and DNA fragmentation, predominantly into high-molecular-mass species (Fig. 3A, lanes 3 and 5). These effects were suppressed by zVAD.fmk (Fig. 3A, lane 6, and results not shown). Thus, the response to nonpermissive poliovirus infection of the MCF-7 derivative with active caspase-3 was very similar to that of HeLa cells. In contrast, no significant morphological signs of apoptosis or DNA degradation could be observed under these conditions in MCF-Ca3cs cells (Fig. 2B and C and 3A, lane 8).

The above-mentioned facts clearly demonstrate that caspase-3 is an essential component of the apoptotic pathway triggered by abortive poliovirus infection in MCF-Cas3 cells and that the failure of MCF-Cas3cs cells to undergo apoptosis under these conditions is due to the absence of active caspase-3.

Role of caspase-9 in poliovirus-triggered apoptosis. Caspase-3 is an effector caspase which can be activated through specific proteolytic cleavage by upstream components of different apoptotic pathways. One such upstream caspase is caspase-9, which is involved in the mitochondrion-dependent pathway.

To ascertain the role of caspase-9 in poliovirus-triggered apoptosis, we generated a derivative of MCF-Cas3 cells in which caspase-9 was inactivated by stable transfection with a retrovirus vector expressing a dominant-negative mutant of this enzyme with a Cys^{287}-to-Ser substitution (25, 51). These cells, lacking active caspase-9 but possessing caspase-3, were named MCF-Cas3/Cas9DN. As controls, MCF-Cas3 cells transformed with empty vector (MCF-Cas3/MIV cells) were used. In contrast to control cells, the MCF-Cas3/Cas9DN cells failed to develop appreciable apoptosis when treated with nonviral inducers of apoptosis, such as CHI or ActD (not shown).

The absence of caspase-9 did not affect the yield (about 10^4 PFU/cell) or time course of poliovirus reproduction in MCF-Cas3/Cas9DN cells (Fig. 1B), nor could any significant differences be observed in the appearance of nuclear (Fig. 4A) and cytoplasmic (not shown) CPE upon productive infection of these two cultures. At a late stage of infection, only a small proportion (~4%) of productively infected MCF-Cas3/MIV cell appeared to be TUNEL positive (Fig. 4B), and only limited DNA degradation to high-molecular-mass species could be detected in appropriate samples upon electrophoresis (Fig. 3B, lane 2). As indicated above, a limited DNA degradation in productively infected MCF-Cas3 cells was not necessarily apoptosis related.

A significant proportion (20 to 30%) of control MCF-Cas3/MIV cells, which contained functional caspase-9 and caspase-3, efficiently responded by apoptosis to abortive poliovirus infection (i.e., when reproduction was interrupted at an early stage by guanidine-HCl), as judged by strong chromatin condensation revealed by Hoechst staining (Fig. 4C), as well as DNA degradation seen with both the TUNEL assay (Fig. 4B and C) and gel electrophoresis (Fig. 3B, lane 1). On the other

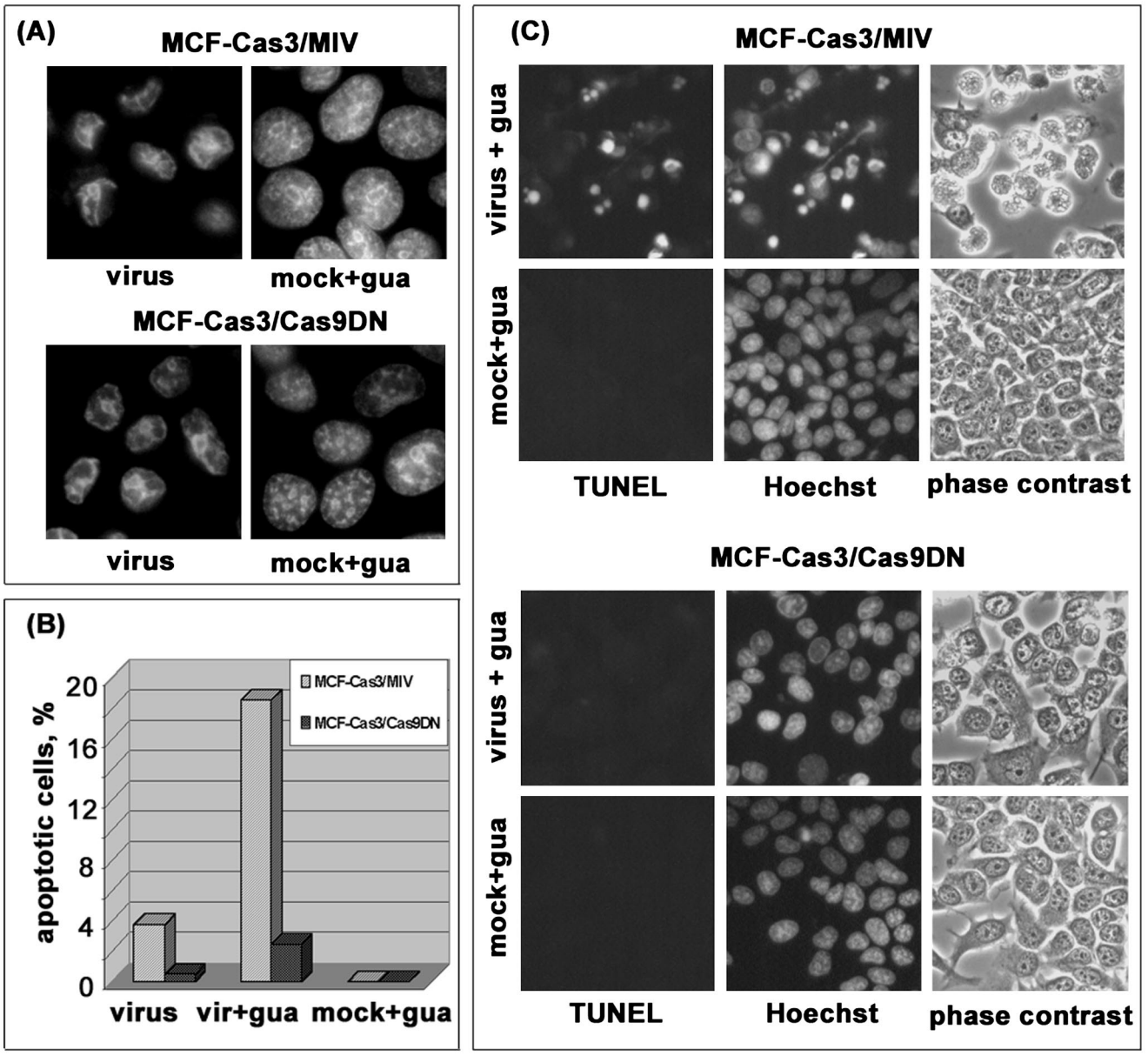

FIG. 4. Responses of MCF-Cas3/MIV and MCF-Cas3/Cas9DN cells to productive and abortive poliovirus infections. (A) Nuclear CPE in productively infected cells. Hoechst 33342 was added at 7.5 h p.i., and the cells were fixed at 8 h p.i. mock+gua, mock-infected cells with 100 μg of guanidine-HCl/ml added. (B) Percentages of TUNEL-positive infected cells at 8 h p.i. in the absence (virus) and presence (vir+gua) of 100 μg of guanidine-HCl/ml added at 2 h p.i. The data represent the averages of two experiments. (C) Development of apoptosis in MCF-Cas3/MIV but not MCF-Cas3/Cas9DN cells. Guanidine-HCl (100 μg/ml) was added to the virus-infected (virus + gua) and mock-infected (mock+gua) cells at 2 h p.i. Hoechst 33342 was added at 7.5 h p.i. The cells were fixed at 8 h p.i. and processed for the TUNEL assay. Qualitatively similar results were obtained when productive infection was interrupted by guanidine-HCl at 1.5 h p.i. (not shown).

hand, MCF-Cas3/Cas9DN cells lacking caspase-9 failed to develop apoptosis upon nonpermissive poliovirus infection, judging by morphological and cytochemical criteria (Fig. 4B and C) and DNA electrophoresis (Fig. 3B, lane 4).

We concluded that caspase-9 activity is essential for efficient apoptotic response to poliovirus infection.

Cytochrome *c* release from mitochondria caused by poliovirus infection. A major mechanism of caspase-9 activation involves its autoproteolysis after recruitment into the apoptosome, a complex also containing Apaf-1 and cytochrome *c* (25, 40). Formation of this complex requires release of the latter from mitochondria damaged by more upstream apoptotic signals (2, 16). Therefore, the distribution of cytochrome *c* in cells undergoing permissive and abortive (apoptogenic) infection with poliovirus was studied by immunostaining. In intact or mock-infected cells, this technique revealed punctate entities, obviously corresponding to mitochondria, concentrated predominantly in the perinuclear region (Fig. 5A and B). The efflux of cytochrome *c* from mitochondria was seen as a diffuse staining of the entire cytoplasm in a significant proportion of

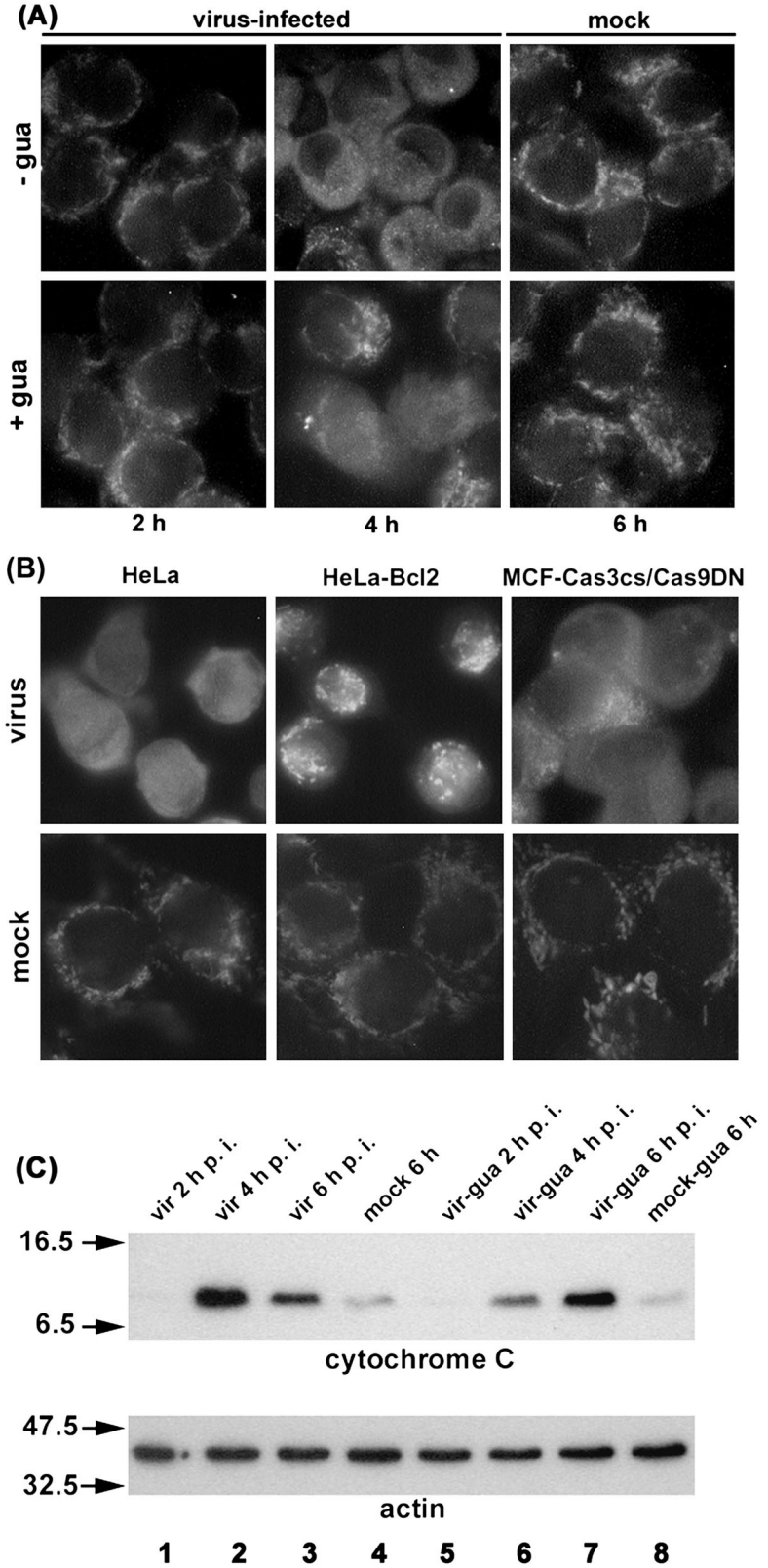

FIG. 5. Cytochrome *c* efflux upon productive and abortive poliovirus infection. (A) Virus-infected and mock-infected HeLa cells were incubated for different time intervals in the absence (− gua) and presence (+ gua) of 100 μg of guanidine-HCl/ml (added at 1.5 h p.i.). (B) Productively infected and mock-infected HeLa, HeLa-Bcl2, and MCF-Cas3/Cas9DN cells were incubated for 6 h. Immunostaining for cytochrome *c* was performed as described in Materials and Methods. (C) Western blot analysis of the soluble cytoplasmic fraction (see Materials and Methods) from HeLa cells productively (vir) and abortively (vir-gua) infected as for panel A. A decrease in the content of cytochrome *c* in the 6-h sample (lane 3) compared to the 4-h sample (lane 2) could be explained by the lysis of a proportion of cells due to CPE caused by productive infection.

HeLa cells, not only when they were undergoing abortive infection (resulting in apoptosis) but, surprisingly, also upon productive infection (resulting in CPE-type death) (Fig. 5A). The cytoplasmic extramitochondrial accumulation of cytochrome *c* in the abortively and productively infected cells could also be registered by Western blotting (Fig. 5C), being clearly observed by 4 h p.i. under both conditions and developing even faster in the case of productive infection (Fig. 5C, compare lanes 2 and 6 or lanes 3 and 7). Similarly, efflux of cytochrome *c* from mitochondria could be registered in MCF-Cas3 cells (i.e., cells containing both caspase-3 and caspase-9) destined for either CPE or apoptosis (not shown). The cytochrome *c* efflux from the mitochondria of infected HeLa cells was markedly suppressed by overexpression of the antiapoptotic Bcl-2 protein (Fig. 5B), a condition shown previously to postpone the development of apoptosis in abortively infected cells but exerting no appreciable effect on either the time course of virus reproduction or CPE development (4). The absence of both caspase-9 and caspase-3 in MCF-Cas3cs/Cas9DN cells did not significantly affect either the time course or the extent of cytochrome *c* release (Fig. 5B), in line with the notion that this release was an upstream event.

We concluded that poliovirus infection is accompanied by mitochondrial damage and exit of cytochrome *c*, which may or may not lead to development of apoptosis, depending on the conditions of infection.

Processing of Bid upon abortive poliovirus infection. Different factors may result in the mitochondrial damage that leads to cytochrome *c* efflux. Among them is Bid, a proapoptotic protein of the Bcl-2 family, the truncated form of which, together with another Bcl-2-related protein, Bax, induces permeabilization of the outer mitochondrial membrane (1, 10, 31). When the content of the unprocessed form of Bid was assayed in extracts from productively and abortively infected HeLa cells by Western blotting, a marked decrease was detected only in the latter, e.g., when the infection was interrupted by the early addition of guanidine at 1.5 h p.i. (Fig. 6, lane 6). Under these conditions, the processing of Bid was even more efficient than in the cells in which apoptosis was triggered by the addition of CHI (Fig. 6, lane 5). On the other hand, no appreciable processing of Bid was detected under productive infection (Fig. 6, lanes 1 to 3). If guanidine was added at 3 h p.i., that is, when the viral antiapoptotic function was expressed sufficiently to suppress the cellular apoptotic response to infection (4), processing of Bid was also markedly suppressed (Fig. 6, lane 7).

Since cytochrome *c* release from mitochondria was observed in both types of infection while truncation of Bid occurred only upon abortive infection, we concluded that processing of Bid was not the primary cause of the permeabilization of mitochondrial membranes in poliovirus-infected cells but rather was involved in the amplification of the apoptotic signal in the cells destined for apoptosis.

Aberrant caspase-9 processing upon productive infection. As was shown above, productive infection of HeLa or MCF-Cas3 cells resulted in the efflux of cytochrome *c* into the cytoplasm, but this event, generally associated with the induction of apoptosis, failed to trigger such a response under these conditions. This failure obviously reflected the interruption of the implementation of the activated apoptotic program by antiapoptotic viral functions (4). We decided to ascertain whether the block in the implementation of the apoptotic program took place upstream or downstream of caspase-9. This enzyme is normally stored in inactive proenzyme form (procaspase-9), and its activation is accomplished by autoproteolysis within the apoptosome, a structure composed of cytochrome *c*, Apaf-1, and procaspase-9. The amount of procaspase-9 diminished under conditions of both productive (destined for CPE) (Fig. 6, lanes 2 and 3) and abortive (destined for apoptosis) infection of HeLa cells (Fig. 6, lane 6). However, in abortive infection, the decrease in procaspase-9 was accompanied by the accumulation of a 35-kDa protein band identical to that generated upon CHI-triggered apoptosis (Fig. 6, compare the bands marked with the arrowhead in lanes 5 and 6) and apparently

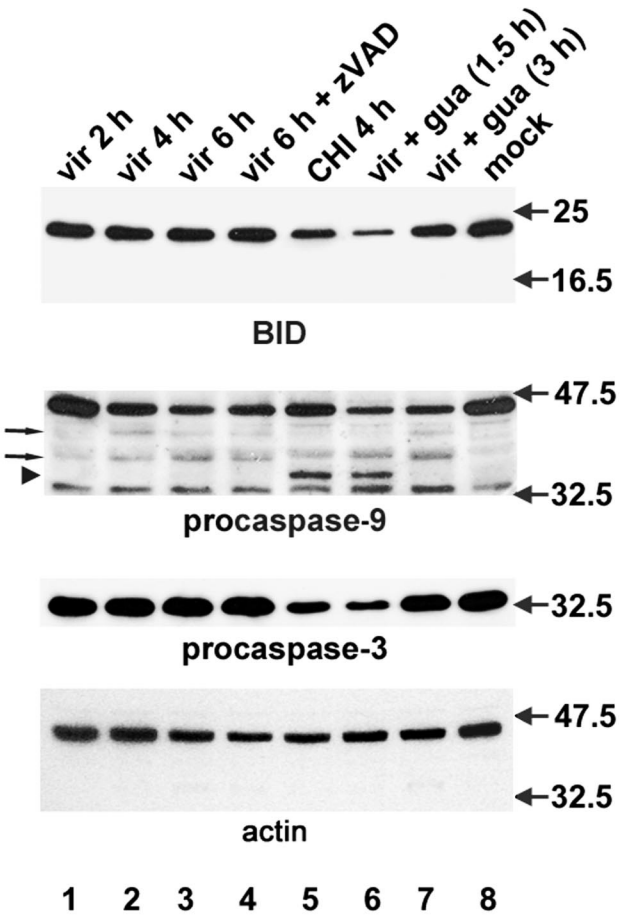

FIG. 6. Processing of Bid, procaspase-9, and procaspase-3 in virus-infected and uninfected HeLa cells. Productively infected cells (vir) were incubated for 2, 4, and 6 h p.i. (lanes 1 to 3). Infection in the sample in lane 4 was carried out in the presence of 100 μM zVAD.fmk (+ zVAD). In the samples in lanes 6 and 7, productive infection was interrupted by the addition of 100 μg of guanidine-HCl/ml (+ gua) at 1.5 and 3 h p.i., respectively, and the cells were harvested at 6 h p.i. In the sample in lane 5, uninfected cells were incubated in the presence of 100 μg of CHI/ml for 4 h. The sample in lane 8 represents mock-infected cells. Western blot analysis of a cellular extract was performed as described in Materials and Methods. The positions of normally and aberrantly processed caspase-9 are marked by the arrowhead and arrows, respectively. The positions of protein markers are also indicated.

corresponding to the active enzyme, while this band was virtually absent upon productive infection (Fig. 6, lanes 2 and 3). Under these conditions, the appearance of caspase-9-related protein species with aberrant electrophoretic mobility could be detected instead. The processing of procaspase-9 and the appearance of the aberrant bands were not prevented by zVAD.fmk (Fig. 6, lane 4), suggesting that other caspases, at least those sensitive to zVAD.fmk, could hardly be involved.

A similar assay with antibodies against procaspase-3 demonstrated that processing of this enzyme occurred upon abortive infection (Fig. 6, lane 6), as expected (4, 42), but no appreciable loss of procaspase-3 could be detected in productively infected HeLa cells (Fig. 6, lanes 2 and 3) or when guanidine was added too late to suppress the viral antiapoptotic activity (Fig. 6, lane 7).

DISCUSSION

Viruses can trigger the defensive apoptotic response of the infected host cell by a variety of mechanisms, but many viruses possess different tools to overcome this defense (48, 55, 65). The results of this study allowed us to identify the major apoptotic pathway activated upon poliovirus infection and to ascertain which part of this pathway is targeted by the viral antiapoptotic activity.

The efflux of cytochrome *c* from mitochondria and the degradation of procaspase-9 in the poliovirus-infected cells were the most upstream of the identified apoptosis-related events studied here. These effects became prominent between 2 and 4 h p.i. (Fig. 5 and 6). Remarkably, switching on of the apoptotic program in a significant proportion of infected cells could be detected as early as 1.5 to 2 h p.i. (4). The upstream reactions occurring at the very early stages of infection prior to the mitochondrial damage have yet to be elucidated. Since several poliovirus proteins, i.e., 2A (26), 3C (6), and possibly some others, exhibit apoptosis-inducing activity, more than one mechanism of the poliovirus-triggered mitochondrial damage may conceivably be involved. Theoretically, the virus-activated pathway leading to cytochrome *c* detachment from cardiolipin and its subsequent efflux into the cytoplasm through an altered outer mitochondrial membrane (2, 17, 43, 49, 71) may include activation of some proapoptotic members of the Bcl-2 family of proteins, such as Bid (1, 10, 31); direct interaction of the viral protein and mitochondria (22); some other indirect effects; or a combination of these factors. Processing of Bid was indeed detected in abortively infected HeLa cells destined for apoptosis. However, since permeabilization of the outer mitochondrial membrane occurred upon productive infection as well, when no appreciable Bid processing was observed, we concluded that Bid was not the primary inducer of mitochondrial damage. More likely, processing of Bid was accomplished by an already activated effector caspase, e.g., caspase-3 (61), and represented a component of the loop involved in the amplification of the apoptotic signal. Caspase-2, which was recently demonstrated to be a key component in the mitochondrial-damage pathway activated by certain apoptotic inducers (26a, 37a), does not appear to play such a role in MCF-7 cells (37a) and therefore could hardly be responsible for the poliovirus-triggered apoptosis in the derivatives of these cells.

It is perhaps appropriate to note that alterations in the oxidative functions of mitochondria have been reported to occur early in poliovirus infection (36).

Although they were not studied here, mitochondrial alterations may also be accompanied by the release of other components of the apoptotic machinery, such as the mitochondrial stores of some procaspases (e.g., procaspase-9 and procaspase-3) (43, 71) and the Smac/Diablo protein acting as an inhibitor of the cellular antiapoptotic IAP proteins (18, 20, 69), as well as proteins capable of killing cells in a caspase-independent way, such as the apoptosis-inducing factor AIF (59), endonuclease G (39, 66), and Omi/HtrA2 serine protease (28). The fact that in the absence of caspase-9 and caspase-3 the virus-triggered apoptotic response was strongly suppressed indicated that mechanisms independent of these caspases (e.g., AIF mediated), if involved, hardly played a decisive role in this response.

It may be noted that apoptosis induced by proteins of some other viruses, e.g., human immunodeficiency virus (32) or chicken anemia virus (16), may also involve the mitochondrion-dependent pathway.

Once cytochrome *c* is released, the energy-dependent generation of high-molecular-mass complexes, the apoptosomes, also involving Apaf-1 and procaspase-9, is expected to occur, ensuring limited autoproteolysis of the latter and the appearance of active caspase-9 (40, 54). In poliovirus-infected cells destined for apoptosis, the proper procaspase-9 cleavage was documented here by Western blotting, and the key role of caspase-9 in the poliovirus-triggered apoptotic pathway was demonstrated by a dramatic suppression of the development of apoptosis-related nuclear damage in cells lacking appropriate activity (i.e., MCF-Cas3/C9DN).

The next downstream essential component of the poliovirus-induced apoptotic pathway is caspase-3. Previously, activation of Asp-Glu-Val-Asp (DEVD)-specific caspases (4) and cleavage of procaspase-3 (42) have been observed in poliovirus-infected cells undergoing apoptosis. As shown here, the lack of caspase-3 in MCF-Cas3cs cells resulted in the nearly complete absence of key signs of apoptosis. Thus, caspase-3 is an essential component of the poliovirus-triggered apoptotic pathway, while other known effector caspases, e.g., caspase-6 and caspase-7, appear to be unable to efficiently replace caspase-3 in this process.

One may speculate that the activation of caspase-3 in abortively infected cells might be accomplished not only by the mitochondrion-dependent pathway but also by some other mechanisms. For example, the involvement of caspase-12 known to be activated in response to ER stress (45, 46, 53) cannot be ruled out, since drastic ER alterations accompany poliovirus infection (56). As shown recently, this enzyme is able to activate caspase-9 through a mitochondrion-independent mechanism (44a, 52a). The ER-dependent pathway was reported to be activated, for example, upon infection with respiratory syncytial virus (8). Although the potential role of this and some other mechanisms in poliovirus-induced apoptosis has yet to be explored, the marked antiapoptotic activity of Bcl-2 argues for a mitochondrion-mediated mode. Thus, our results suggest that the major poliovirus-triggered apoptotic pathway in the cells studied here includes consecutive activation of caspase-9 and caspase-3 and appears to be caused by cytochrome *c* release (Fig. 7).

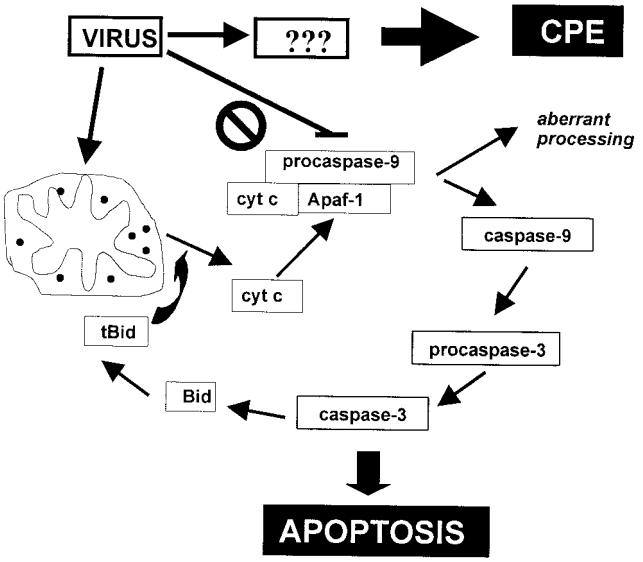

FIG. 7. Schematic representation of the major poliovirus-triggered apoptotic pathway as characterized in the present study. Poliovirus infection results in mitochondrial damage and efflux of cytochrome *c* (cyt c) into the cytoplasm. Together with Apaf-1 and cytochrome *c*, procaspase-9 forms an apoptosome in a dATP-dependent reaction, followed by generation of active caspase-9. The latter cleaves procaspase-3, with generation of active caspase-3. This effector protease destroys various important substrates and activates other hydrolases, e.g., DNases, and eventually leads to apoptotic death. The apoptotic signal appears to be amplified through limited proteolysis of Bid by caspase-3. The truncated form of Bid (tBid), in cooperation with some proapoptotic factors, causes further mitochondrial damage. Concurrently, a viral antiapoptotic function promotes aberrant processing of procaspase-9, hindering its activation and resulting in the development of CPE. The step at which this aberrant processing occurs has yet to be defined.

Concurrently with turning on the apoptotic program and despite the potential availability of all of the necessary components of the major apoptotic pathway, the overwhelming majority of productively infected HeLa and MCF-Cas3 cells develop CPE rather than typical apoptosis. This is due to the antiapoptotic function(s) of poliovirus capable of interrupting implementation of the ongoing apoptotic program (4, 64). Since cytochrome *c* release proceeds upon productive infection essentially as efficiently as upon abortive infection, we conclude that the major target of the putative viral antiapoptotic function(s) is downstream of the mitochondrial damage. In this respect, the antiapoptotic functions of poliovirus differ from those of several other viruses, e.g., some herpesviruses and adenoviruses, which encode proteins able to stabilize mitochondria and thus prevent the release of cytochrome *c* (22).

The molecular basis of poliovirus interference with the activation of caspase-9 is still unclear. The appearance of caspase-9-related proteins with unexpected electrophoretic mobility in productively infected cells may suggest that the viral antiapoptotic function involves aberrant processing, e.g., alternative proteolysis, of procaspase-9. The amino acid sequence of human procaspase-9 contains a YG dipeptide, a canonical cleavage site for the poliovirus 2A protease (50). The putative cleavage should generate peptides of 251 and 165 amino acid residues, respectively. Neither of them could be related to the observed aberrant polypeptide bands. Since the specificity of poliovirus 2A protease, especially its *trans*-acting activity, is highly dependent on the context of the cleavage site (29), it is not surprising that the YG bond in procaspase-9 does not appear to be the target for this protease. No canonical cleavage site (QG [50]) for the poliovirus protease 3C is present in procaspase-9. Thus, either a viral protease cleaves the proenzyme at suboptimal sites or an infection-stimulated cellular proteolytic activity is involved. Interestingly, a Ca-dependent protease, calpain, is known to degrade and inactivate procaspase-9, as well as some other caspases (15). In this regard, it may be noted that a misbalance of ions (Ca^{2+} included) caused by alterations in the permeability of cellular membranes in poliovirus-infected cells is a well-documented phenomenon (13).

If the aberrant cleavage of procaspase-9 occurs closer to its N terminus, it may be accompanied by the removal or inactivation of the caspase recruitment domain (CARD), preventing recruitment of the proenzyme into the apoptosome (72). It may be appropriate to note that the liberated CARD itself may perform an antiapoptotic function by activating NF-κB (58).

The identification of aberrant processing of procaspase-9 as a putative component of the poliovirus antiapoptotic activity does not necessarily rule out the existence of additional relevant mechanisms. A variety of factors are known to impair the recruitment of caspase-9 into apoptosomes or its subsequent activation (7, 41, 52, 68), and they may be regarded as potential targets for viral antiapoptotic functions.

Although the major apoptotic pathway operating in poliovirus-infected cells is mitochondrion mediated, other pathways may potentially be involved under some settings. As already mentioned, the ER stress-mediated pathway also potentially contributes to the cellular response to poliovirus infection. The infection may modulate the extrinsic, receptor-mediated apoptotic pathway as well. For example, expression of poliovirus protein 2A renders the cells more sensitive to the apoptosis-inducing effect of TNF (47). On the other hand, viral infection and, in particular, expression of the viral protein 3A results in depletion of TNF receptor (47) and receptors for some other cytokines (47a) from the plasma membrane, decreasing the sensitivity of the cells to these cytokines.

Thus, although we did not study it, the fate of the infected cell depends on a complex network of interacting cellular and viral proteins and is host specific as well as virus specific. The results reported here contribute to a deeper understanding of this network. The type of death of infected cells is a significant factor in the pathogenesis of viral diseases. CPE, but not apoptosis, is generally accompanied by an inflammatory reaction, and the presence or absence of this reaction should markedly affect the clinical patterns of viral diseases.

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