JOURNAL OF VIROLOGY, Apr. 1999, p. 3219–3226 Vol. 73, No. 4
0022-538X/99/$04.00+0

Herpes Simplex Virus 1 Blocks Caspase-3-Independent and Caspase-Dependent Pathways to Cell Death

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Received 11 November 1998/Accepted 23 December 1998

Earlier reports have shown that herpes simplex virus 1 (HSV-1) mutants induce programmed cell death and that wild-type HSV blocks the execution of the cell death program triggered by viral gene products, by the effectors of the immune system such as the Fas and tumor necrosis factor pathways, or by nonspecific stress agents such as either osmotic shock induced by sorbitol or thermal shock. A report from this laboratory showed that caspase inhibitors do not block DNA fragmentation induced by infection with the HSV-1 *d*120 mutant. To identify the events in programmed cell death induced and blocked by HSV-1, we examined cells infected with wild-type virus or the *d*120 mutant or cells infected and exposed to sorbitol. We report that: (i) the HSV-1 *d*120 mutant induced apoptosis by a caspase-3-independent pathway inasmuch as caspase 3 was not activated and DNA fragmentation was not blocked by caspase inhibitors even though the virus caused cytochrome *c* release and depolarization of the inner mitochondrial membrane. (ii) Cells infected with wild-type HSV-1 exhibited none of the manifestations associated with programmed cell death assayed in these studies. (iii) Uninfected cells exposed to osmotic shock succumbed to caspase-dependent apoptosis inasmuch as cytochrome *c* was released, the inner mitochondrial potential was lost, caspase-3 was activated, and chromosomal DNA was fragmented. (iv) Although caspase-3 was activated in cells infected with wild-type HSV-1 and exposed to sorbitol, cytochrome *c* outflow, depolarization of the inner mitochondrial membrane, and DNA fragmentation were blocked. We conclude that although *d*120 induces apoptosis by a caspase-3-independent pathway, the wild-type virus blocks apoptosis induced by this pathway and also blocks the caspase-dependent pathway induced by osmotic shock. The block in the caspase-dependent pathway may occur downstream of caspase-3 activation.

The studies described here are based on three series of observations. First, in earlier studies this laboratory reported that herpes simplex virus 1 (HSV-1) mutants induced degradation of cellular DNA and morphologic changes characteristic of programmed cell death (15). Specifically, cells infected with a mutant (*d*120) lacking the major regulatory gene coding infected cell protein 4 (ICP4) and exhibiting a defect in the viral protein kinase encoded by the U_s3 gene induced DNA fragmentation in all cell lines tested to date. Another mutant, *ts*B7, induced apoptosis in Vero cells but not in SK-N-SH cells incubated at the nonpermissive temperature (10). Under these conditions, *ts*B7 attaches and penetrates cells. Although the capsid is transported to the nuclear pore, the DNA is not released and viral gene expression does not ensue (2, 3). Both mutants, *d*120 and *ts*B7, are blocked at very early, but distinct, stages in infection. In Vero cells infected with *ts*B7 and maintained at the nonpermissive temperature, the events prior to release of viral DNA into the nucleus are sufficient to induce apoptosis; in both Vero and SK-N-SH cells infected with *d*120, transcription and translation of a subset of proteins that included the α proteins was required. These observations led to the conclusion that HSV-1 can trigger apoptosis at multiple steps in infection and that the virus encodes functions that can block the execution of the death program induced by its presence in the infected cell.

The second observation reported from this and other laboratories was that HSV-1 blocks programmed cell death induced by the effector functions of the immune system such as the Fas and tumor necrosis factor (TNF)-mediated pathways, as well as nonspecific inducers of apoptosis such as thermal or osmotic shock (10–12). Last, this laboratory reported the surprising finding that caspase inhibitors failed to block the fragmentation of cellular DNA induced by *d*120 and raised the possibility that the virus can induce caspase-independent programmed cell death (10). The objectives of the studies reported here were to verify the observation that HSV-1 *d*120 induced caspase-independent programmed cell death and to identify the steps in programmed cell death blocked by wild-type HSV-1. Relevant to this report are the following.

(i) The pathways of programmed cell death comprise at least two effector branches that converge upon the activation of the downstream effector caspases such as caspase-3, -6, and -7 to cause the degradation of specific cytoplasmic and nuclear proteins, the fragmentation of chromosomal DNA, and ultimately the organized breakdown of the cell (reviewed in references 4 and 26). Activation of caspases, a family of cysteine proteases, is central to this process and occurs through the proteolytic cleavage of the proenzymes into their active, catalytic forms. The zymogens of several different caspases have been shown to be substrates for other members of the family and to be able to cleave their own precursors. Thus, upon receipt of a death signal, a cascade of proteolytic cleavages results in activation of preexisting inactive caspases that ultimately destroy the cell (reviewed in reference 5). For example, ligand-induced clustering of death receptors, such as Fas and TNF-R, initiate the execution of one of these pathways, in which the caspase machinery is directly engaged through the activation of caspase-8 (21, 22). Active caspase-8 cleaves and activates the downstream caspase effectors caspase-3, -6, and -7 (25). On the other hand, stress-inducing stimuli (e.g., γ or UV light irradiation, inhibitors of RNA polymerase, or the withdrawal of

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growth factors) have been shown to initiate another converging pathway that is regulated by the Bcl-2 family of proteins (1). This pathway involves mitochondrial events, such as the release of cytochrome *c* into the cytosol and the dissipation of the voltage gradient across the inner mitochondrial membrane. The presence of cytochrome *c* in the cytoplasm results in the formation of a complex of cytochrome *c*, Apaf-1, and procaspase-9. This complex activates caspase-9, which in turn cleaves and activates caspase-3 (30). Activated caspase-3 recognizes and cleaves ICAD/CAD DNA fragmentation factor 45 (DFF45/ICAD), the inhibitory partner of CAD or DNA fragmentation factor 40 (CAD/DFF40), respectively (24, 18). CAD has a DNase activity responsible for the degradation of chromosomal DNA (24). Upon cleavage of ICAD, CAD catalyzes the cleavage of chromosomal DNA into nucleosomal fragments. Cleavage of DFF45 releases DFF40, which has a low intrinsic DNase activity but is thought to activate a nuclear DNase (18). Fragmentation of DNA is not a requirement for cell death since inhibitors of caspase activity block all caspase-dependent events, including the fragmentation of DNA, but do not block the death of the cell (20, 29).

(ii) The death receptor-induced pathway may bypass the mitochondrial regulation and directly engage the downstream effector, caspase-3. Cytochrome *c* release, however, has recently been shown to amplify the effect of caspase-8 on the activation of downstream caspases (14, 19, 17). Studies of caspase $3^{-/-}$ mice have shown that caspase-3 is required for programmed cell death in the central nervous system and for the nuclear events in apoptosis, chromatin condensation, and DNA fragmentation (13, 28).

In the studies described here, we examined cells infected with wild type or *d*120 mutant and cells infected and subjected to osmotic shock for manifestations of activation of programmed cell death. The events we examined were the outflow of cytochrome *c* from the mitochondrial compartment, the activation of caspase-3, and the oligonucleosomal degradation of DNA. To induce osmotic shock, cells were exposed to high osmolar concentrations of sorbitol. Osmotic shock activates multiple signalling pathways (23) and induces a rapid response that allows for the examination of different events in the execution of the cell death program independently of the particular timing and kinetics of a specific pathway. We report that *d*120 induces the release of cytochrome into the cytosol and the depolarization of the inner mitochondrial membrane. However, the fragmentation of the DNA that marks the completion of the apoptotic process in *d*120-infected cells appears to be a caspase-3-independent process consistent with the earlier finding that caspase inhibitors do not block *d*120-induced DNA fragmentation. Wild-type HSV-1 appears to express functions that block multiple events in the execution of the cell death program. This block is sufficient to preclude apoptosis triggered both by expression of viral genes and by a caspase-dependent pathway triggered by osmotic shock-induced stress.

MATERIALS AND METHODS

Cells. SK-N-SH and HEp-2 cell lines were obtained from American Type Culture Collection and were grown in Dulbecco's modification of Eagle minimal essential medium (DMEM) containing 5% (HEp-2) or 10% fetal bovine serum (SK-N-SH).

Viruses. HSV-1(F) is the prototype HSV-1 strain used in this laboratory (8). The HSV-1 mutant *d*120 (a kind gift of N. DeLuca) carries a deletion in both copies of the α4 gene and was grown in a Vero cell line (E5) expressing the α4 gene (6). It also carries a defective U_S3 gene encoding a viral protein kinase (16).

Induction of apoptosis. Osmotic shock was induced by exposing SK-N-SH and HEp-2 cells that were mock infected or were infected with 10 PFU of HSV-1(F) per cell either to 0.5 M sorbitol for 2 h or to 1.5 M sorbitol for 1 h, followed by 5 h of incubation at 37°C in DMEM containing 1% newborn calf serum.

Measurement of caspase-3 activity. Cellular extracts were assayed for caspase-3 activity with a tetrapeptide conjugated to phenylnitroaniline (DEVD-pNA). Briefly, 10^6 SK-N-SH cells were either mock infected or were infected with 10 PFU of HSV-1(F) or the *d*120 mutant per cell. At the indicated times after infection, they were harvested, rinsed in phosphate-buffered saline (PBS), resuspended in lysis buffer, and stored on wet ice for 5 min. The lysates were then centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant fluids were tested for caspase-3 activity as recommended by the manufacturer by using the BIOMOL Quantizyme assay system. The released chromophore was measured by determining the absorbance at 405 nm. The endpoint reaction values shown correspond to the absorbance values obtained at 280 min after the addition of the substrate.

Immunoblot assays. Infected or uninfected cell lysates, each containing approximately 60 μg of protein, were electrophoretically separated in a 10% denaturing polyacrylamide gel, electrically transferred onto a nitrocellulose sheet, and reacted with an anti-poly(ADP-ribose) polymerase antibody (Santa Cruz Biotechnology) at a concentration of 1 μg/ml. The protein bands reacting with the antibody were visualized by using an enhanced chemiluminescence (ECL) system (Pierce, Rockford, Ill.) as described for the localization of cytochrome *c*.

Subcellular fractionation. Cells (4×10^6) were either mock infected or were infected with 10 PFU of HSV-1(F) or the HSV-1*d*120 mutant per cell. At the indicated times after infection they were collected and resuspended in 0.8 ml of ice-cold buffer A (20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 250 mM sucrose, 0.1 mM TLCK [*N*α-*p*-tosyl-L-lysine chloromethyl ketone], 0.1 mM TPCK [tolylsulfonyl phenylalanyl chloromethyl ketone]). After 15 min on ice, cells were homogenized in a Dounce homogenizer and then centrifuged for 10 min at 750 × *g* in order to remove unlysed cells and nuclei. The supernatant fluids were transferred to new tubes and centrifuged again at 10,000 × *g* per 20 min. Supernatant fluids from the second centrifugation represent the cytosolic fractions, whereas the pellets, resuspended in buffer A, represent the mitochondrial fractions.

Localization of cytochrome *c*. The protein concentration in mitochondrial and cytosolic fractions was determined by using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, Calif.), according to the directions provided by the manufacturer. Equivalent amounts of mitochondrial and cytosolic fractions were subjected to electrophoresis in denaturing polyacrylamide gels, transferred to nitrocellulose membranes, blocked in PBS (0.14 M NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4) containing 5% skim milk for 1 h at room temperature or overnight at 4°C, rinsed three times in PBS, and then reacted with the primary antibody against cytochrome *c*, clone 7H8.2C12, (PharMingen, San Diego, Calif.), in a solution of PBS containing 1 μg of the antibody per ml. The membranes were then rinsed three times with PBS, reacted with a goat anti-mouse antibody conjugated to peroxidase (Sigma, St. Louis, Mo.), rinsed again, and visualized with the ECL system according to the protocols provided by the manufacturers. Finally, filters were exposed to X-Omat AR films (Kodak, Rochester, N.Y.) for the detection of specific bands.

Mitochondrial membrane potential measurement and flow cytometry. Subconfluent, uninfected cells or cells exposed to 10 PFU of HSV-1(F) or the *d*120 mutant per cell were collected, rinsed in PBS, resuspended in 5 nM rhodamine-123 (Molecular Probes, Eugene, Oreg.), and incubated at room temperature for 20 min. Cells were then analyzed with a FACScan flow cytometer (Becton Dickinson) as described previously (7) to detect changes in inner mitochondrial membrane potential. Data from 2×10^4 cells were collected, stored, and analyzed by using Cellquest software (Becton Dickinson). Ungated data are shown.

DNA fragmentation assay. Subconfluent SK-N-SH cells were mock infected or exposed to 10 PFU of HSV-1(F) or the *d*120 mutant per cell. Mock- or HSV-1(F)-infected cells were either left untreated or exposed to 1 M sorbitol for 1 h; cells were then incubated in DMEM containing 1% newborn calf serum for 5 h. Samples containing 2×10^6 cells were collected and processed as described earlier (10).

RESULTS

Infection does not induce activation of caspase-3. The purpose of the three series of experiments described below was to test the hypothesis that infection of cells with the *d*120 mutant of HSV-1 does not induce activation of caspase-3. The objective of the first series of experiments was to determine whether the cells were capable of inducing the activation of caspase-3 and to determine the minimum amount of caspase-3 activity that correlates with activation of the cell death program.

In the first series of experiments, SK-N-SH cells were exposed to increasing concentrations of sorbitol in medium containing 1% fetal bovine serum for 5 h. The lysates were assayed for specific DEVDase activity in colorimetric reactions as described in Materials and Methods. In preliminary studies it was determined that exposure of cells to 0.25 M sorbitol was sufficient to induce apoptosis as determined by DNA fragmenta-

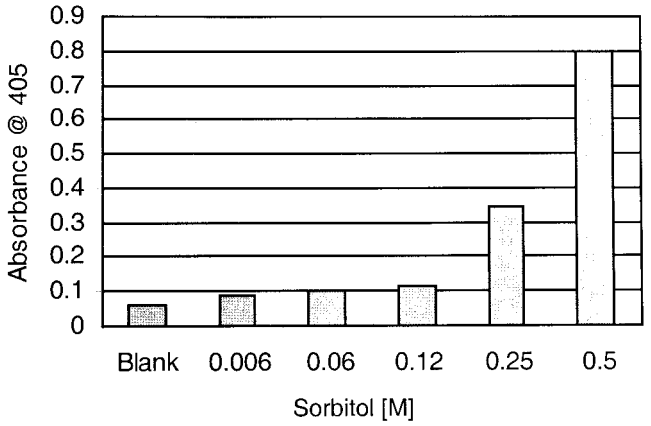

FIG. 1. DEVDase activity in cells exposed to various concentrations of sorbitol. SK-N-SH cells were exposed to various concentrations of sorbitol as shown for 2 h and then incubated for 5 h in DMEM containing 1% fetal bovine serum. Cell extracts were assayed for DEVDase activity as described in Materials and Methods. The colorimetric reaction was monitored by measuring the absorbance at 405 nm. DNA fragmentation was observed only in cells treated with the two highest concentrations tested (data not shown).

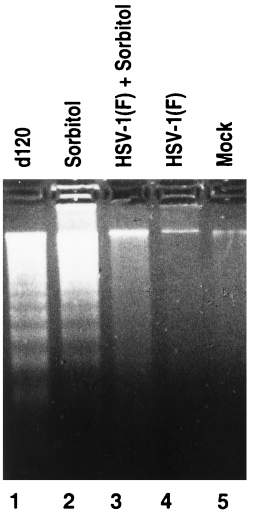

FIG. 2. Photograph of agarose gel containing electrophoretically separated low-molecular-weight DNA fragments. SK-N-SH cells were mock infected, exposed to sorbitol, infected with HSV-1(F) or d120 mutant, or infected with HSV-1(F) and exposed to sorbitol as described in the text. Cultures were mock infected or infected with 10 PFU of HSV-1(F) or the *d*120 mutant per cell and collected 6 h after infection. At 1 h after mock infection or infection with 10 PFU of HSV-1(F) per cell, cells were exposed to 1 M sorbitol for 1 h and incubated in DMEM containing 1% fetal bovine serum for 5 h. Cell lysates were assayed for the presence of oligonucleosomal DNA fragments as described in Materials and Methods.

tion, whereas exposure to 0.12 M sorbitol was insufficient (data not shown). DEVD-pNA cleavage assays showed that cytoplasmic lysates from cells exposed to 0.25 M sorbitol exhibited 0.35 absorbance units at 405 nm, whereas the absorbance of lysates from cells exposed to the lower concentration of sorbitol could not be differentiated from that of the blank sample (Fig. 1).

The purpose of the second series of experiments was to compare the events occurring in cells exposed to sorbitol with those occurring in cells infected with the *d*120 mutant. SK-N-SH cells were mock infected, infected with 10 PFU of the *d*120 mutant per cell and incubated for 12 h, or exposed to 0.25 M sorbitol for 5 h. At the same time after infection, lysates from cells infected by the *d*120 mutant (Fig. 2, lane 1) or exposed to sorbitol (Fig. 2, lane 2) showed extensive DNA fragmentation, whereas cells exposed to wild-type virus alone or to both virus and sorbitol showed little or no degradation of DNA (Fig. 2, lanes 3 to 5). The lysates collected in parallel were tested for caspase-3 activity as described above. The DEVD-pNA cleavage assays yielded endpoint absorbance values for lysates from cells infected with the *d*120 mutant that were not significantly higher than the values obtained for the blank sample. In contrast, lysates of cells exposed to sorbitol yielded DEVD-ase activities comparable to those obtained by the addition of purified recombinant caspase-3 to lysates from untreated cells. This activity was specific, as it could be completely inhibited by the simultaneous addition of the specific tetrapeptide aldehyde inhibitor, Ac-DEVD-CHO (Fig. 3).

The third series of experiments was designed to test the hypothesis that different inducers of apoptosis may trigger the activation of caspase-3 with different kinetics, and thus a peak of DEVDase activity could be transient and no longer present at the time it was measured in lysates of cells infected with the *d*120 mutant. We therefore sought to precisely determine the timing of the appearance of caspase-3 activity in cells exposed to sorbitol or in cells infected with *d*120. A time course analysis was done in which cells were simultaneously infected and exposed to sorbitol or overlaid with 0.5 M sorbitol and then collected at 2, 3, 7, 8, 12, or 20 h after infection or after exposure to sorbitol. No caspase-3 activity, as measured by cleavage of the colorimetric substrate, was detected in mock-infected cells or in cells infected with wild-type or mutant HSV-1. In contrast, a peak of DEVDase activity was observed in cells exposed to osmotic shock at 2 to 3 h after treatment. The DEVDase activity induced by osmotic shock was completely inhibited by the simultaneous addition of the inhibitor Ac-DEVD-CHO (Fig. 4).

Infection does not block the cleavage of poly(ADP-ribose) polymerase induced by osmotic shock. To verify the absence of caspase-3 activity, we examined the integrity of poly(ADP-ribose) polymerase, one of its substrates. Caspase-3 is known to

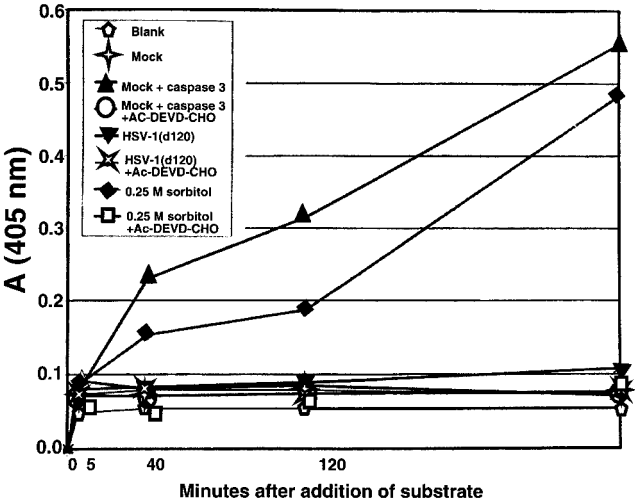

FIG. 3. Time course of DEVDase activity in cell extracts after the addition of substrate. Extracts from infected or uninfected SK-N-SH cells or from SK-N-SH cells exposed to sorbitol were assayed for DEVDase activity at the indicated times after the addition of the colorimetric substrate as described in Materials and Methods. The colorimetric reaction was monitored by measuring the absorbance at 405 nm.

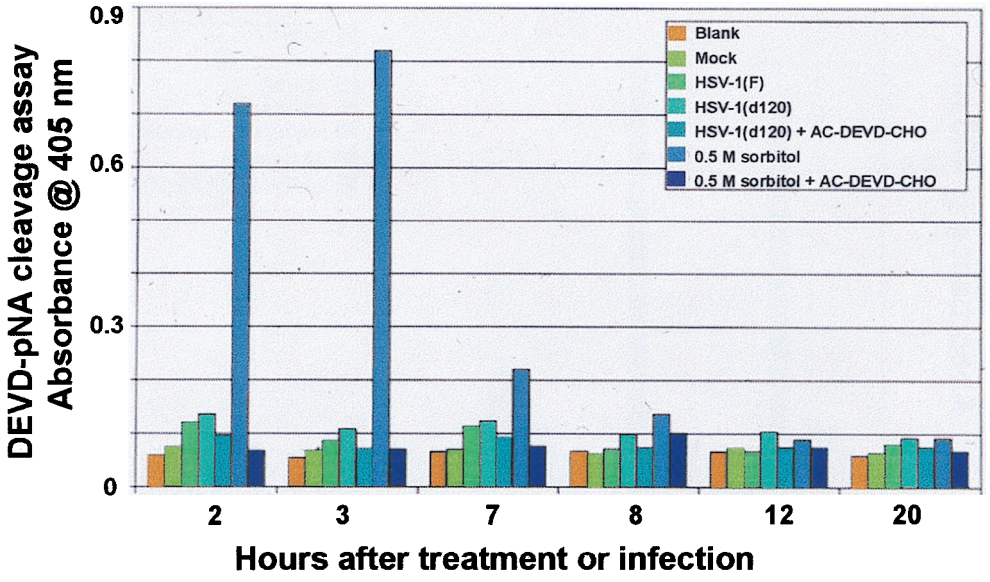

FIG. 4. Time course of DEVDase activity after exposure to sorbitol, wild-type virus, or *d*120 mutant. SK-N-SH cells, either mock infected or infected with HSV-1(F) or the *d*120 mutant or exposed to sorbitol in the presence or absence of the caspase inhibitor Ac-DEVD-CHO were harvested at the times indicated in the figure and assayed for DEVDase activity as described in Materials and Methods. The colorimetric reaction was monitored by measuring the absorbance at 405 nm. Endpoint values of absorbance are shown.

cleave the M_r 110,000 poly(ADP-ribose) polymerase to yield a truncated polypeptide with an M_r of 85,000. In these experiments HEp-2 cells were infected with *d*120 (10 PFU/cell) and incubated for 12 h. Replicate cultures were exposed to 0.25 M sorbitol at 1 h after mock infection or after infection with wild-type or mutant viruses and were maintained for an additional 2 or 5 h at 37°C. The results were as follows.

(i) The M_r-85,000 cleavage product of the poly(ADP-ribose) polymerase was detected in lysates of uninfected cells exposed to sorbitol for 2 or 5 h (Fig. 5A, lanes 4 and 6).

(ii) Lysates of cells infected with either wild-type virus or *d*120 exhibited trace amounts of M_r-85,000 cleavage product (Fig. 5A, lane 3, and 5B, lane 2). The significance of the presence of trace amounts of cleaved poly(ADP-ribose) polymerase is unclear; it could reflect a small fraction of the cells in caspase-3 which was activated as a consequence of an abortive infection. The key finding is that in cells infected with either virus the vast majority of the poly(ADP-ribose) polymerase was intact, a finding consistent with those presented above and indicating that infection with the *d*120 mutant does not result in caspase-3 activation significantly different from that of the wild-type virus. At the same time after infection, as noted above, cells infected with the *d*120 mutant exhibit extensive DNA fragmentation (Fig. 2, lane 1).

(iii) Poly(ADP-ribose) polymerase was cleaved in cells exposed to sorbitol at 1 h after infection with wild-type virus (Fig. 5A, lanes 5 and 7). It is noteworthy that cleavage was apparent as early as 2 h after exposure of cells to sorbitol.

To determine whether a function expressed later in infection could block the activation of caspase-3, we repeated the same experiment but exposed the infected cells to sorbitol at 6, 12, and 18 h after infection. As shown in Fig. 5B, lanes 3 to 6, HSV-1 infection did not preclude the cleavage of poly(ADP-ribose) polymerase induced by osmotic shock at any of the times tested. In contrast, DNA fragmentation was effectively blocked by wild-type HSV-1 in cultures exposed to sorbitol by as early as 1 h after infection (Fig. 3 and Fig. 2, lanes 2 and 3).

We should note that in the two experiments we detected larger amounts of the poly(ADP-ribose) polymerase and of its

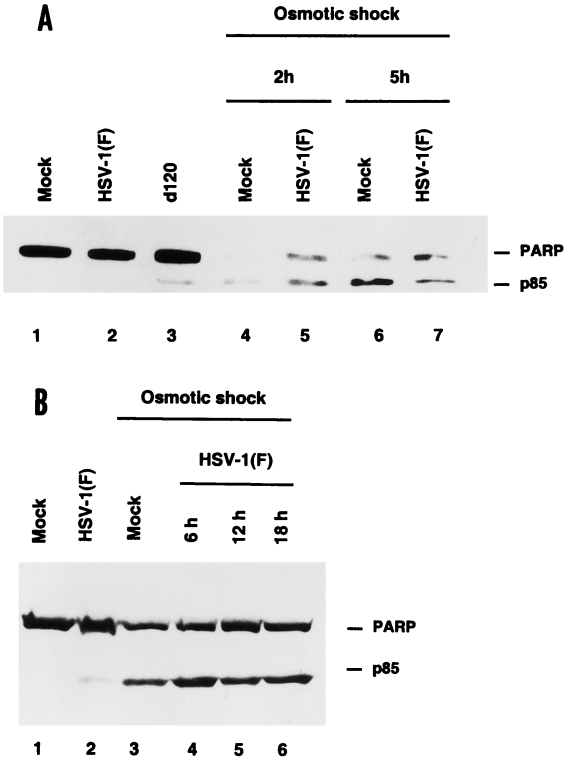

FIG. 5. Photograph of electrophoretically separated cell lysates reacted with an antibody to poly(ADP-ribose) polymerase. Lysates from infected or uninfected HEp-2 cells either left untreated or exposed to sorbitol were electrophoretically separated in a polyacrylamide gel, transferred to a nitrocellulose sheet, and reacted with antibody against poly(ADP-ribose) polymerase (PARP). (A) Cells were treated with sorbitol at 1 h after infection and collected at 2 or 5 h after the addition of sorbitol. (B) Cells were treated at the indicated times after infection and collected at 5 h after the exposure to sorbitol.

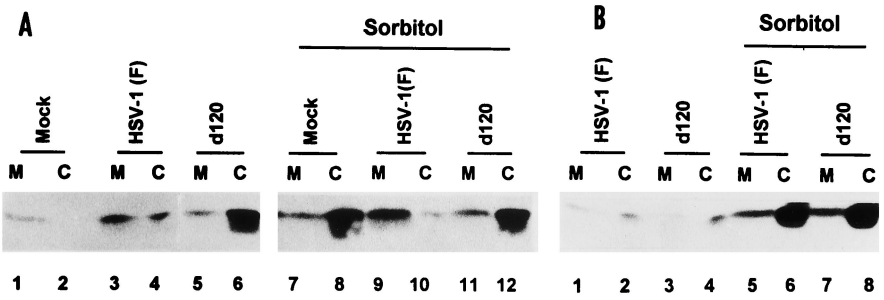

FIG. 6. Immunoblot showing cytochrome *c* distribution in mock-infected, infected, and sorbitol-treated HEp-2 cells. (A) HEp-2 cells were harvested at 16.5 h after mock infection or after infection with viruses as indicated. Cells were exposed to sorbitol for 1.5 h. (B) HEp-2 cells were harvested at 4 h after mock infection or after infection with viruses as indicated. Cells were exposed to sorbitol for 1.5 h. The procedures were as described in Materials and Methods. M, mitochondrial fraction; C, cytosol.

cleavage product in the experiment illustrated in Fig. 5B than in the one illustrated in Fig. 5A. The relative amounts of intact and cleaved product, however, were comparable in both experiments.

Outflow of cytochrome *c* from mitochondria in infected cells. In some pathways of apoptosis, the release of cytochrome *c* precedes the activation of caspase-3 through the formation of a complex that results in the activation of caspase-9, which can in turn cleave and activate caspase-3. The absence of caspase-3 activity in cells undergoing apoptosis induced by infection could be due to a block in the release of cytochrome *c* into the cytoplasm. To test this hypothesis, two series of experiments were done. In the first, replicate HEp-2 cell cultures were mock infected or were infected with wild-type virus [HSV-1 (F)] or *d*120 mutant. At 16.5 h after infection, one set of cells was exposed to 0.5 M sorbitol in DMEM containing 5% newborn calf serum for 1.5 h at 37°C. The cells were then harvested, fractionated, and analyzed to determine the subcellular distribution of cytochrome *c* as detailed in Materials and Methods. The results (Fig. 6A) were as follows. In cells mock infected or in cells infected with HSV-1(F), cytochrome *c* was present in trace amounts and was cofractionated mostly with the mitochondrial fraction (Fig. 6, lanes 1 to 4). In cells infected with the mutant *d*120, cytochrome *c* increased in amount and was fractionated predominantly with the cytosolic fraction (Fig. 6, lanes 5 and 6). As expected, in mock-infected cells exposed to sorbitol, cytochrome *c* increased in amount and was found predominantly in the cytosol (Fig. 6, lanes 7 and 8). Virtually the same increase in cytochrome *c* and prevalent localization in cytosol was observed in cells infected with *d*120 mutant (Fig. 6, lanes 11 and 12). In contrast, in cells infected with wild-type virus and exposed to sorbitol, cytochrome *c* was localized in the mitochondria (Fig. 6, lanes 8 and 9), indicating that a viral function can block the release of cytochrome *c* into the cytoplasm resulting from the induction of apoptosis by osmotic shock.

In the second series of experiments, replicate cultures of HEp-2 cells were infected with HSV-1(F) or with the *d*120 mutant. At 2.5 h after infection, the medium in one set of cells was replaced with a medium containing 0.5 M sorbitol, and the incubation was continued for another 1.5 h at 37°C. The cells were harvested and analyzed as described above. The results, presented in Fig. 6B, were as follows. The total amount of cytochrome *c* detected in both wild-type and mutant-infected cells was significantly lower than the amount detected at 18 h after infection (compare Fig. 6A with Fig. 6B) and, moreover, the cytochrome that was detected was associated with the mitochondrial fraction. The significant finding was that infection with wild-type virus did not block the release of cytochrome *c* in the cytosol of cells exposed to sorbitol.

The studies shown in Fig. 6 indicate that the viral function which blocks the release of cytochrome *c* from the mitochon-

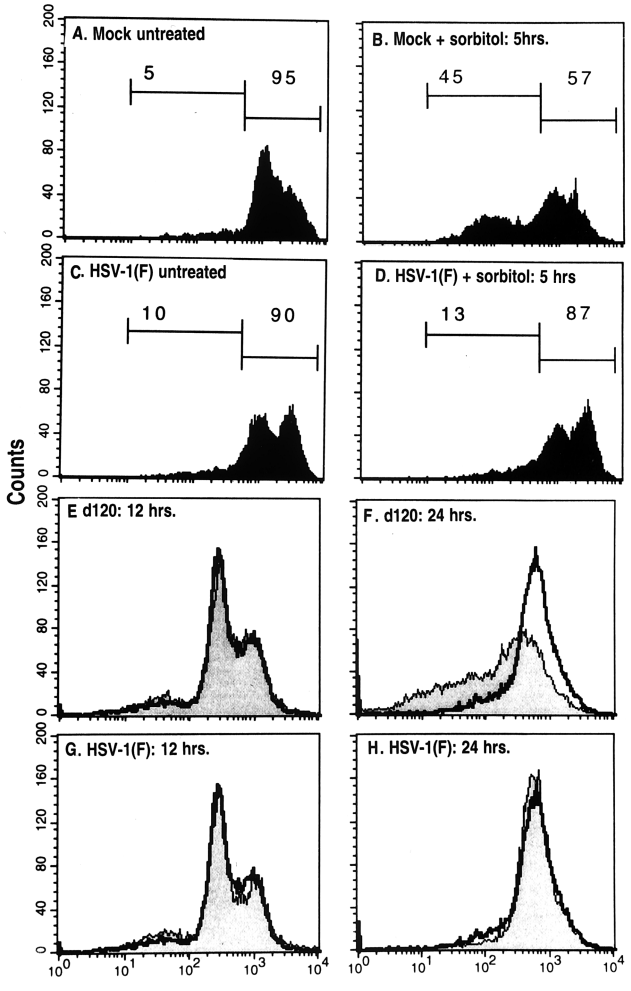

FIG. 7. Distribution of cells according to their Rh-123 fluorescence intensities. (A) Rh-123 fluorescence profiles of mock-infected or HSV-1(F)-infected cells either left untreated or exposed to sorbitol. (B) Rh-123 fluorescence profiles of HSV-1(F)-infected or *d*120-infected HEp-2 cells at 12 and 24 h after infection. The thicker line corresponds to the profile of uninfected cells and is shown as a reference.

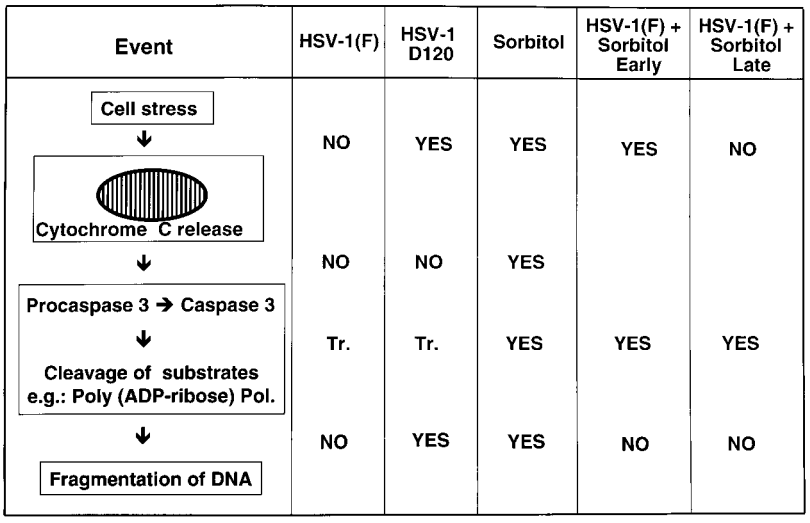

FIG. 8. Schematic representation of the manifestations of the programmed cell death assayed in these studies. "Yes" and "no" refer to detection of or failure to detect, respectively, manifestations of intracellular events associated with programmed cell death.

dria is expressed between 1.5 and 16.5 h after infection. Other studies (data not shown) indicated that this function is expressed before 12 h postinfection.

Two comments should be made concerning the results presented in this experiment. First, the increase in the amount of cytochrome *c* detected in cells infected with wild-type virus and the even greater increase detected in cells undergoing programmed cell death (e.g., mock-infected cells exposed to sorbitol or cells infected with the *d*120 mutant) was reproducible. The data suggest that either a fraction of cytochrome *c* in unstressed cells is unavailable by the method of fractionation or that stress induces the synthesis of cytochrome *c* concomitantly with the activation of the cell death program, which would then be blocked in cells infected with wild-type virus. Second, in the experiment shown in Fig. 6, trace amounts of cytochrome *c* could be detected in the cytoplasmic fraction of cells infected with wild-type HSV-1 (lane 4), even though no fragmentation of DNA can be detected in any cell type tested so far. The presence of trace amounts of cytochrome *c* in the cytoplasm of wild-type-infected cells also suggests the possibility that infection activates and then blocks the execution of the cell death program.

HSV-1(F) infection prevents the depolarization of the inner mitochondrial membrane induced by osmotic shock. The ability of wild-type HSV-1 to prevent redistribution of cytochrome *c* resulting from activation of the cell death program by osmotic shock suggested that it could also be actively regulating other aspects of mitochondrial function. We therefore investigated whether infection could prevent the depolarization of the inner mitochondrial membrane, an event that appears to be associated with some forms of programmed cell death (reviewed in reference 22). In these experiments, we compared the profile of rhodamine-123 (Rh-123) fluorescence of mock-infected or HSV-1(F)-infected cells that were either left untreated or were exposed to sorbitol. Approximately 45% of the mock-infected cells exposed to sorbitol showed a decreased mitochondrial membrane potential compared to untreated or HSV-1(F)-infected cells (Fig. 7, compare panel B with panels A and C). In contrast, HSV-1(F)-infected cells treated by osmotic shock showed no detectable increase in the percentage of depolarized cells (Fig. 7D). Infection therefore prevents the loss of inner mitochondrial membrane potential resulting from induction of apoptosis by osmotic shock.

Depolarization of the inner mitochondrial membrane in *d*120-infected cells. The data presented above showed that the *d*120 mutant induced the release of cytochrome *c* into cytosol. To determine whether *d*120 infection could also induce the loss of inner mitochondrial potential, we examined the Rh-123 fluorescence profiles of cells at 12 and 24 h after infection with either HSV-1(F) or the *d*120 mutant. At 12 h after infection, neither HSV-1(F)- nor *d*120-infected cells showed any detectable decrease in Rh-123 fluorescence (Fig. 7E and G). At 24 h after infection, however, cells infected with the *d*120 mutant show a marked decrease in Rh-123 fluorescence intensity, indicating a loss in their inner mitochondrial membrane potential (Fig. 7F).

DISCUSSION

The current model of programmed cell death delineates two major pathways connecting different apoptotic stimuli with the activation of a family of cysteine proteases called caspases that mediate the organized dismemberment of the cell (reviewed in reference 4). In the case of signalling through death receptors, the proteolytic machinery is engaged directly; in the case of pathways activated by stress, the signal to activate the proteolytic cascade is regulated at the level of mitochondria by members of the Bcl-2 family of proteins (reviewed in reference 1). Both pathways converge in the activation of downstream effector caspase-3, -6, and -7, which are responsible for the morphological features of apoptosis such as membrane blebbing, chromatin condensation, and DNA fragmentation. In particular, it was shown that caspase-3 is directly linked to the activation of the endonuclease that mediates the fragmentation of chromosomal DNA (24, 28). Our interest in programmed cell death in relation to HSV-1 infection stemmed from two observations: (i) that mutants of HSV-1 induce apoptosis and (ii) that infection with the *d*120 mutant causes DNA fragmentation that cannot be blocked by caspase inhibitors. The experimental objectives of the studies described in this study were to identify the manifestations of the programmed cell death blocked by wild-type virus. We examined

cells that were infected with wild-type virus or *d*120 mutant, exposed to sorbitol, or both infected and exposed to sorbitol for the activation of caspase-3, the release of cytochrome *c* into cytosol, the depolarization of the inner mitochondrial membrane, and the fragmentation of DNA. The salient features of the results summarized in Fig. 8 were as follows.

(i) No caspase-3 activity could be detected in cells infected with the *d*120 mutant, even though *d*120 induced the release of cytochrome *c* into cytosol, depolarization of the inner mitochondrial membrane, and extensive fragmentation of chromosomal DNA. Consistent with these results, as reported earlier, caspase inhibitors failed to block the fragmentation of DNA induced by infection with the *d*120 mutant. We conclude that the pathway of programmed cell death induced by the *d*120 mutant of HSV-1 is caspase 3-independent.

(ii) As expected, osmotic shock resulting from the exposure of uninfected cells to sorbitol induced cytochrome *c* release, activation of caspase-3, depolarization of the inner mitochondrial membrane, and degradation of chromosomal DNA. The activation of caspase-3 and DNA fragmentation were blocked by caspase inhibitors.

(iii) In cells infected with wild-type HSV-1 and exposed to sorbitol, cytochrome *c* release, depolarization of the inner mitochondrial membrane, and fragmentation of DNA were blocked.

These results lead to several significant conclusions. First, the evidence unambiguously indicates that cells infected with *d*120 succumbed to caspase-3-independent programmed cell death. The evidence that no manifestations of apoptosis were seen in cells infected with wild-type HSV-1 indicate that the wild-type virus can express functions that block this pathway.

Second, the evidence that cells exposed to sorbitol succumbed to a caspase-3-dependent programmed cell death that is blocked by the wild-type virus indicates that the virus expresses functions that can block this pathway.

Third, the evidence that in infected cells exposed to sorbitol caspase-3 was not blocked has two significant implications. Foremost, HSV-1 may not have evolved functions that block caspase-3 but rather has functions that block a subsequent step, that is, the fragmentation of DNA. Implicit in this conclusion is that the virus evolved a mechanism to block specifically DNA fragmentation and that none of the substrates of caspase-3 are of vital importance to the virus. Of equal significance, the evidence that HSV-1 infection precluded cytochrome *c* release and DNA fragmentation but not caspase-3 activation indicates that the virus blocks the cascade of events leading to programmed cell death at several steps independently.

The studies described here were done with HEp-2 and SK-N-SH cells. The response of these two cell lines to infection or sorbitol and, conversely, the effectiveness of viral gene expression in response to apoptotic events initiated in these cell lines were equivalent. In an earlier publication from this laboratory (10), it has been shown that the response may be cell type dependent. For example, SK-N-SH cells were protected by wild-type virus from the effects of a variety of exogenous inducers of apoptosis, whereas HeLa cells treated in the same fashion were not. Similarly, *ts*B7 at the nonpermissive temperature induced apoptosis in Vero cells but not in SK-N-SH cells. The possibility that Vero cells may respond to wild-type infection in a manner different from that of HEp-2 cells is suggested by the report that mitochondrial RNA polymerase could be found in the cytosolic fraction of Vero cells infected with wild-type virus (27).

The model that emerges from this and preceding studies is that HSV replication occurs in a very hostile environment in which the cells are programmed to respond to stimuli from the invading virus and from exogenous sources (e.g., the immune system) to commit suicide in order to preclude the invader from taking hold. The apoptotic stimuli from the invading virus appear to occur at several steps in its reproductive cycle, most probably from the moment of its interaction with the host cell. Pari passu, the virus expresses functions necessary to block the activation of the programmed cell death to ensure that the infected cells are totally subservient to its needs and to maximize viral progeny. Coevolution of the virus and the host may have led to the acquisition of functions that block not only the apoptotic pathway induced by the virus but also those induced by external activators such as the immune system of the host. Identification of the various functions expressed by the virus to block these pathways is a major objective for three reasons. First, this will identify the viral biochemical events that could lead to programmed cell death. Second, it may yield clues to interventions that nullify viral blocks to apoptosis in order to curtail infection at a critical step. Finally, identification of viral functions that block individual steps in programmed cell death may lead to applications in areas in which blockage of apoptosis is therapeutically desirable.

ACKNOWLEDGMENTS

We thank Julie Auger for assistance with flow cytometry.

These studies were aided by grants from the National Cancer Institute (CA47451, CA71933, and CA78766). The Flow Cytometry Core Facility is supported by grants CA-14599 and DK49799 from the NCI and NIDDK. V.G. is a Markey postdoctoral fellow.

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