Herpes Simplex Virus Type 1 Blocks the Apoptotic Host Cell Defense Mechanisms That Target Bcl-2 and Manipulates Activation of p38 Mitogen-Activated Protein Kinase To Improve Viral Replication

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Wild-type (wt) herpes simplex virus type 1 (HSV-1) suppresses cell death. We investigated the apoptotic pathways triggered during infection with mutant viruses tk and 27lacZ (which lack functional ICP4 and ICP27 viral proteins, respectively) and examined the mechanisms used by wt HSV-1 to protect against programmed cell death induced by the DNA-damaging compound cisplatin. In our studies, we used BHK and HeLa cells, with similar results. We suggest that a decrease in the levels of Bcl-2 protein is a key event during apoptosis induced by the mutant viruses and that Bcl-2 levels are targeted by (i) a decrease of bcl-2 RNA, (ii) caspase-related proteolysis, and (iii) p38 mitogen-activated protein kinase (p38MAPK)-dependent destabilization of Bcl-2 protein. We show that wt HSV-1, but not the mutant viruses, maintains bcl-2 RNA and protein levels during infection and protects from the cisplatin-induced decrease in bcl-2 RNA; our data suggest that both ICP27 and ICP4 are required for this function. Additionally, wt HSV-1 evades but does not actively block activation of caspases. Although wt HSV-1 induces p38MAPK activation during infection, it prevents p38MAPK-dependent destabilization of Bcl-2 and exploits p38MAPK stimulation to enhance transcription of specific viral gene promoters to increase viral yields.

The ability of cells to commit suicide is critical for development, tissue homeostasis, and protection of the organism against pathogens, including viruses. Cells kill themselves in a controlled process known as programmed cell death or apoptosis (reviewed in reference 34). Generally, movement of phosphatidylserine from the inner to the outer surface of the cell membrane, activation of cysteine proteases (caspases), and modifications of key regulatory proteins, such as P53 or members of the Bcl-2 family, are early apoptotic events. Degradation of nuclear DNA, leakage of DNA to the cytoplasm due to disruption of the nuclear envelope, cytoplasmic blebbing, and shrinkage of the cell are late apoptotic markers.

The Bcl-2 family contains about 15 protein members in mammalian cells (reviewed in references 1 and 13). The family includes prosurvival proteins (Bcl-2 and Bcl-XL) and proapoptotic proteins that induce apoptosis either directly (Bax and Bid) or by heterodimerization and titration of the function of prosurvival members (Bax, Bad, and Bcl-XL). A well-established role for prosurvival members is to maintain mitochondrial integrity, and Bcl-2 prevents release of cytochrome c to the cytoplasm. Cytochrome c facilitates a change in the structure of the adapter molecule Apaf-1 to allow pro-caspase-9 recruitment and activation (1, 13). Apart from heterodimerization with proapoptotic proteins, transcriptional control of the gene, phosphorylation, proteolysis, or induction of conformational changes may inactivate Bcl-2 (reviewed in reference 8).

Caspases exist as latent zymogens and are activated by cleavage of their N-terminal prodomains. They are classified as apoptotic initiators (caspase-9), apoptotic executioners (caspase-1,-3,-4, -6, and -7), and cytokine processors (caspase-1). Once activated by apoptotic signals, initiators cleave and activate apoptotic executioners that target pro- and antiapoptotic structural and homeostatic proteins to systematically dismantle the cell (reviewed in reference 44).

In the case of virus-infected cells, induction of early cell death would severely limit virus replication, and many viruses have evolved strategies to avoid or delay apoptosis. In addition, some viruses actively induce apoptosis during the late stages of infection to facilitate progeny spread (reviewed in references 20 and 42). Herpes simplex virus type 1 (HSV-1) is a DNA virus and a ubiquitous human pathogen (reviewed in reference 6). During the lytic cycle, viral gene expression can be divided into three temporal stages. Transcription of the five immediate-early (IE) genes is initiated by the virion tegument protein VP16 (Vmw65) in the absence of de novo protein synthesis. IE genes Vmw175 (ICP4), Vmw63 (ICP27), Vmw110 (ICP0), and Vmw68 act to orchestrate the expression of early and late genes. ICP27 and ICP4 are essential proteins, and elimination of their respective genes blocks the viral replication cycle at early stages of infection in tissue culture. ICP4 transactivates viral gene expression through DNA binding (2), and ICP27 is a multifunctional protein involved in the export, 3’ processing, and poly(A) usage of viral RNAs (24, 25, 37). Early gene products are detectable by 4 to 5 h postinfection and are mostly
enzymes involved in DNA synthesis and replication. Late genes are efficiently expressed after 6 to 7 h postinfection and mostly encode structural proteins.

Wild-type (wt) HSV-1 suppresses apoptotic DNA fragmentation and cell death (19), and early events during wt HSV-1 infection are required for protection against apoptosis (4). Infection of cells prior to treatment with a variety of apoptotic stimuli protects cells from apoptosis, and the effect has some cell type dependency (9). The host cell apoptotic mechanism is activated during HSV-1 infection, but the virus has evolved mechanisms to suppress it (9). Certain HSV-1 proteins were recently proposed to have antiapoptotic functions: viral protein kinase US3 and glycoprotein J (17), IE proteins ICP4 (21), and ICP27 (3), and late protein γ3,4.5 (14). With the exception of γ3,4,5, which blocks interferon-induced protein synthesis shut-off by stimulating dephosphorylation of eukaryotic initiation factor 2, the antiapoptotic functions of these proteins are poorly understood. Significantly, loss of ICP4 was linked to mitochondrial dysfunction and DNA fragmentation (10), and loss of functional ICP27 was associated with activation of caspase-3 and DNA damage (4).

We report results from studies that were performed to investigate in detail the antiapoptotic role of wt HSV-1 and its ICP4 and ICP27 proteins. We used the replication-defective mutant viruses 27lacZ, which lacks ICP27 (39), and ts k, which expresses an inactive form of ICP4 at the nonpermissive temperature of 38.5°C (33), to dissect the apoptotic events triggered by the cell during mutant virus invasion and to identify the mechanisms used in wt virus infection to subvert these events. Two approaches were used. First, we examined the cellular apoptotic pathways triggered during infection of cells with the mutant viruses and compared these to the pathways triggered in mock- and wt HSV-1-infected cells. Second, we examined how preinfection of cells with wt HSV-1 rescues cells from apoptosis induced by cisplatin, a DNA-damaging compound that induces stress kinase-related apoptosis (47), and compared this to results with mock- and mutant virus-infected cells treated with the drug. Cisplatin was used as an apoptotic stimulant since, during the course of our studies, we noted many similarities between cisplatin-, ts k-, and 27lacZ-induced cell death. To control for any cell type-specific responses, as virus-induced apoptosis has been reported to have some cell type dependency (9), we performed experiments in BHK cells, which are commonly used for HSV-1 studies, and in human HeLa cells; results were similar for both cell lines.

Apoptotic pathways activated by cisplatin, 27lacZ, and ts k involved decreased Bcl-2 protein levels, activation of caspases, cytoplasmic cytochrome c release, DNA degradation, and cell death, and importantly, since its overexpression is protective, decreased Bcl-2 levels appeared to be a key element in the apoptotic process. Downregulation of Bcl-2 levels during infection with the mutant viruses involved three mechanisms: (i) decreased Bcl-2 RNA levels, (ii) caspase-dependent degradation of Bcl-2, and (iii) decreased half-life of Bcl-2 protein. wt HSV-1 subverted all three of these mechanisms. wt HSV-1 also protected against cisplatin-induced apoptosis by stabilizing bcl-2 RNA and protein levels, and we propose that ICP4 and ICP27 play an antiapoptotic role in this stabilization of bcl-2 RNA.

In a previous study, we observed activation of the stress kinases Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38MAPK) by VP16 at 6 h postinfection of cells with wt HSV-1, 27lacZ, and ts k viruses (46). Activation of the stress kinases has been related to cell death (47) and prevention of apoptosis (35) in a cell type- and stimulus-dependent manner. Moreover, activation of cellular signalling pathways by HSV-1 has been proposed to facilitate virus replication (31, 46), and therefore a further aim of the present study was to investigate the role of p38MAPK activation during HSV-1 infection. We observed that inhibition of p38MAPK activity during infection of cells with 27lacZ and ts k decreased the levels of apoptosis triggered by the viruses and resulted in an elevated half-life of Bcl-2 protein. Furthermore, we showed that activation of p38MAPK during wt HSV-1 infection increased the viral yield and enhanced expression of specific viral genes, without resulting in p38MAPK-dependent destabilization of Bcl-2. We suggest that activation of p38MAPK during HSV-1 infection is exploited to promote viral replication and that wt, but not 27lacZ and ts k, virus prevents the activated p38MAPK from destabilizing Bcl-2 protein.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney (BHK) and HeLa cells were grown as described previously (46). ts k virus expresses an inactive form of ICP4 at the nonpermissive temperature of 38.5°C (33). In the 27lacZ virus, ICP27 is inactivated by insertion of a loxZ cassette (39).

Proteins, plasmids, and antibodies. Purified Jun and ATF-2 proteins were from Insight Biotechnology, Wembley, United Kingdom. Plasmid pSEK-AL, coding for the Ala-220-Leu-224 dominant-negative MKK4 (SKK1) mutant, was a gift from J. R. Woodgett (47). A plasmid encoding for full-length human Bcl-2 cloned as a 5'–XbaI–NotI–3' fragment in pCEP4 and expressed under control of the cytomegavirus promoter was provided by J. Pietenpol. Plasmids pIE1CAT and pIE3CAT contain IE110 and IE175 gene promoters, respectively, upstream of a chloromphenicol acetyltransferase (CAT) reporter gene (7, 40). For expression of ICP27, pCMV63 was made by inserting an EcoRI/BamHI fragment with the full open reading frame into the expression vector pCMV-10 (41). For expression of ICP4, plasmid p175 was provided by R. Everett (32). Polyclonal antibodies against JunD, p38MAPK, pseudopase-1, -3, -4, -6, and -7; FasL; Bad; and Bax and monoclonal antibody against Mdm-2 were from Insight Biotechnology. Anti-p38 and anti-ICP27 were from the Goodwin Institute for Cancer Research, Plantation, Fla. Anti-ICP0 (monoclonal) and anti-ICP4 (polyclonal) antibodies were provided by R. Everett. Monoclonal antibodies against gC and UL42 were from A. McLean, and anti-R1 and R2 polyclonal antibodies are described in reference 5.

Virus infection, transfection, and treatment with apoptotic stimulants and inhibitors. Cell monolayers were infected with wt HSV-1 (strain 17+) or 27lacZ at multiplicity of infection of 10 PFU per cell and grown at 37°C in 5% CO2. In experiments with the ts k virus, infected and mock-infected cells were grown at 38.5°C. BHK cells were grown in 60-mm dishes and transiently transfected using Lipofectamine (Gibco BRL, Paisley, United Kingdom) as instructed by the manufacturer. For induction of apoptosis, cells were incubated with 30 μg of daily made cisplatin [copper(II) diaminedichloride] (Sigma) per ml prior to harvesting. To protect cells from cisplatin-induced apoptosis, cells were infected for 5 h with wt HSV-1 prior to addition of the drug. To block JNK, cells were transfected with plasmid coding for a dominant-negative SKK1 (MKK4) mutant and treated with apoptotic stimuli or infected with wt HSV-1 at 30 h posttransfection. To block p38MAPK, cells were preincubated for 1 h with 30 μM SB203580 (Calbiochem), a specific inhibitor, prior to addition of virus and throughout infection. For induction of caspases, 25 μM Z-VAD-FMK (Calbiochem) was added to cells 1 h prior to and 6 to 4 h after treatment with cisplatin and/or virus.

DNA fragmentation and viability assays. For detection of cellular DNA, 106 cells per sample were analyzed with a Cell Death Detection ELISAkit (Promega, Madison, Wis.) by following the manufacturer's instructions. In brief, cytotoxicity was determined by colorimetric detection of the histone-DNA complex formed during apoptosis.

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(Boehringer Mannheim). For viability assays, 10^5 cells per sample were analyzed by trypan blue exclusion. During infection, cells gradually detached from the monolayers. Viability was determined by pooling floating and adherent cells. Experiments were repeated at least three times.

Immunoprecipitation from total cell extracts. Cells (5 x 10^5) were lysed in 400 μl of ice-cold radioimmunoprecipitation assay buffer (1% phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM phenylmethylsulfate, 100 mM sodium orthovannadate) by passage through a 21-gauge needle. After centrifugation at 900 x g at 4°C for 5 min, extracts were incubated with 2 μg of the desired antibody and 20 μl of protein G (Insight Biotechnology) under rotation at 4°C overnight.

Immunoblotting procedure. Total cell extracts were prepared as described previously (46). Antibodies for caspase-1, -3, -4, -6, and -7 and Bcl-2 were used at dilutions of 1:100. Antibodies against JunD, PARP, Bax, Bad, P53, Mdm-2, and FasL were used at 1:300. Antibodies for IC7P27, IC7P4, R1, R2, g, and UL42 were used at 1:1,000. Anti-IC7P was used at 1:5,000. One hundred micrograms of total protein per sample was used for immunoblotting, with the exception of caspases, PARP, and Bcl-2, for which 300 μg of protein per sample was analyzed.

Localization of cytochrome c. Cells (5 x 10^5 per sample) were resuspended on ice in 150 μl of buffer A (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 250 mM sucrose, 50 mM phenylmethylsulfonyl fluoride) and passed through a 26-gauge needle. Lysates were centrifuged for 10 min at 750 x g at 4°C; supernatants were transferred to new tubes and centrifuged at 10,000 x g for 20 min. The supernatants from the second centrifugation represent the cytosolic fractions, and 100-μg portions of extracts were immunoblotted with anti-cytochrome c antibody (2 μg/ml).

Immunocomplex kinase assay. For the immunocomplex kinase assay, Bcl-2 or p38MAPK proteins were immunoprecipitated. The matrix was washed extensively (20 mM HEPES [pH 7.6], 50 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA) and resuspended in 30 μl of kinase buffer (20 mM HEPES [pH 7.6], 20 mM MgCl2, 20 mM β-glycerophosphate, 0.1 mM NaVO3, 2 mM dithiothreitol, 0.1 μg of okadaic acid per ml, 0.125 mM [γ-32P]ATP) with addition of 1 μg of purified Jun (specific substrate for JNK) or ATF-2 (substrate for both JNK and reporter plasmid, and liquid scintillation counting assay was performed using Insight Biotechnology) under rotation at 4°C overnight.

RESULTS

Cisplatin and 27lacZ and tsk mutant viruses induce DNA degradation, activation of caspases, cytoplasmic release of cytochrome c, decrease in Bcl-2 protein levels, and cell death, and wt HSV-1 protects cells from cisplatin-induced apoptosis without inhibiting activation of caspases. To investigate the antipoptotic role of ICP27 and ICP4 viral proteins, BJH and HeLa cells were infected with mutant viruses 27lacZ (at 37°C) and tsk (at the nonpermissive temperature of 38.5°C) and tested for markers of programmed cell death, including DNA degradation, caspase activation, cytoplasmic cytochrome c release, and alterations in the levels of key regulatory cellular proteins. In addition, the ability of wt HSV-1 and mutant viruses to protect against apoptosis induced by cisplatin was also examined. For protection against cisplatin-induced apoptosis, cells were preinfected with wt or mutant viruses for 5 h prior to addition of the drug, as this was the minimum time required for wt HSV-1 to confer full protection against cisplatin-induced DNA damage and apoptosis (not shown). Results were always compared to those for the relevant mock-infected controls, i.e., cells incubated at 37°C for cisplatin, wt HSV-1, and 27lacZ treatments or cells incubated at 38.5°C for tsk infections. The data presented in Fig. 1 are from BHK cells. Experiments with HeLa cells produced similar results.

Figure 1A shows the amount of fragmented DNA leaked to the cytoplasm, quantified by enzyme-linked immunosorbent assay (ELISA), at several time points after infection or after cisplatin treatment. Cells were mock infected at 37 or 38.5°C, infected with tsk, 27lacZ, or wt HSV-1; treated with 30 μg of cisplatin per ml; or preinfected with mutant viruses or wt HSV-1 prior to treatment with cisplatin. Cisplatin, 27lacZ, and tsk clearly induced DNA damage at 9 h posttreatment, and this continued to increase up to 24 h posttreatment. Cisplatin induced DNA damage more efficiently than the mutant viruses, and degradation of cellular DNA was higher in cells infected with tsk than in cells infected with 27lacZ. wt HSV-1 failed to induce any DNA damage, and, importantly, infection of cells with wt virus 5 h prior to treatment with cisplatin completely abolished drug-induced DNA fragmentation. Preinfection with 27lacZ or tsk mutant viruses failed to protect against cisplatin-induced DNA damage. A small amount of DNA damage was detected in mock-infected cells at 38.5°C at 12 and 24 h compared to that in mock-infected cells at 37°C (a twofold increase), and this was probably due to thermal shock at the higher temperature. However, the levels of DNA damage in mock-infected cells at 38.5°C were approximately 20% of the levels detected at 24 h postinfection with tsk, suggesting that the elevated temperature makes a small contribution to the induction of DNA damage during infections with tsk virus. We conclude that cisplatin, tsk, and 27lacZ induce DNA damage, whereas the virus protects cells from cisplatin-induced DNA degradation.

Activation of caspases during treatment of cells with cisplatin, tsk, 27lacZ, and wt HSV-1 was tested by immunoblotting (Fig 1B). For caspase-3 activation (lanes 1 to 6), cells were mock infected at 37°C (lane 1) or at 38.5°C (lane 6), treated with 30 μg of cisplatin per ml (lane 2), or infected with 27lacZ (lane 3), wt HSV-1 (lane 4), or tsk (lane 5). At 16 h posttreatment, we observed the cleaved (active) form of caspase-3 in cells treated with cisplatin, 27lacZ, and tsk, whereas no activation was detected in either mock-infected or wt HSV-1-infected cells. Activation of caspase-3 was detectable from 6 h posttreatment (not shown). Cleavage of
pro-caspase-6 was observed at 16 h postinfection in cells infected with 27lacZ (Fig. 1B, lane 8) and tsk (lane 9) viruses and in those treated with cisplatin (lane 12) compared to mock-infected cells at 37 and 38.5°C (lanes 10 and 7, respectively). No cleavage of pro-caspase-6 was detected in cells infected with wt HSV-1 (lane 11). The antibody used recognized only the uncleaved precursor form of caspase-6 and not the cleaved active form. Activation of caspase-7, caspase-1, and caspase-4 by the mutant viruses was not observed (data not shown). In conclusion, cisplatin, tsk, and

FIG. 1. Apoptosis triggered by cisplatin (30 μg/ml) and tsk (38.5°C) and 27lacZ viruses includes DNA degradation, activation of caspase-3 and -6, cytoplasmic release of cytochrome c, and decrease in cell viability. Cells (10⁶) were mock infected or treated with these stimuli for 16 h, unless otherwise indicated. When both cisplatin and virus were added, cells were infected 5 h prior to addition of the drug. (A) Detection of cytoplasmic DNA by ELISA at 6 to 24 h posttreatment. Each value represents the mean ± standard deviation from three experiments performed with duplicate samples. (B) Left panel, cleavage of inactive pro-caspase-3 to active caspase-3 was detected by immunoblotting of total cell extracts. Right panel, Immunoblotting for pro-caspase-6. (C) Total cell extracts were immunoblotted for the intact (112-kDa) and cleaved (85-kDa) forms of PARP. (D) Cytosolic extracts were immunoblotted for cytochrome c. (E) Cell viability was measured by trypan blue exclusion in mock-infected cells and in cells treated with cisplatin and/or mutant viruses.
27lacZ activate caspase-3 and -6, whereas the wt HSV-1 does not.

We then examined whether wt HSV-1 inhibited cisplatin-induced activation of caspases by detecting cleavage of the 112-kDa caspase-3-specific substrate PARP, to its characteristic 85-kDa fragment by immunoblotting (Fig. 1C). Cells were mock infected (lane 2) or preinfected with wt HSV-1 (lane 3), tk (lane 4), or 27lacZ (lane 5) for 5 h prior to addition of 30 μg of cisplatin per ml. At 16 h after cisplatin treatment, activation of caspases was determined. Surprisingly, the wt, tk, and 27lacZ viruses all failed to prevent cisplatin-induced PARP cleavage (compare lanes 3, 4, and 5, respectively, to lane 2). As a control, PARP cleavage was also examined in mock-infected cells without cisplatin treatment at 37 and 38.5°C (lanes 1 and 8, respectively) and in cells infected with wt HSV-1 (lane 6) and 27lacZ (lane 7) at 16 hrs postinfection. PARP cleavage was detectable in 27lacZ-infected cells (compare lane 7 to lane 1) but not in wt HSV-1-infected cells or in mock-infected cells at 38.5°C (compare lanes 6 and 8, respectively, to lane 1). We conclude that wt HSV-1 fails to protect cells from cisplatin-induced PARP cleavage.

We then tested cells treated with cisplatin, wt HSV-1, or the mutant viruses for cytoplasmic release of cytochrome c. As shown in Fig. 1D, we performed immunoblotting analysis of cytosolic extracts of cells mock infected at 37 and 38.5°C (lanes 1 and 2, respectively) or treated with 30 μg of cisplatin per ml (lane 3), 27lacZ (lane 4), tk (lane 5), or wt HSV-1 (lane 7), at 16 h posttreatment. Cisplatin, tk, and 27lacZ, but not wt HSV-1, induced cytoplasmic release of cytochrome c. Preinfection of cells with wt HSV-1 for 5 h prior to treatment with cisplatin protected cells from cisplatin-induced cytoplasmic release of cytochrome c (lane 6). Cytoplasmic cytochrome c was also detectable at 6 h posttreatment of cells with cisplatin or mutant viruses (not shown). We conclude that cisplatin, tk, and 27lacZ induce cytoplasmic release of cytochrome c and that wt HSV-1 protects cells from cisplatin-induced cytochrome c release.

Cell viabilities were examined by trypan blue exclusion at 24 h posttreatment with 30 μg of cisplatin per ml or infection with the mutant viruses and were compared with the viabilities of mock-infected cells at 37 and 38.5°C (Fig. 1E). The results showed an 82% ± 9% decrease in cell viability after treatment with cisplatin and 39% ± 6% and 27% ± 7% decreases in cells infected with tk or 27lacZ, respectively. In addition, preinfection of cells with 27lacZ and tk viruses for 5 h prior to treatment with cisplatin failed to improve cell survival. Viability experiments were not performed with wt HSV-1, since discrimination between apoptotic cell death caused by cisplatin and cell lysis caused by the viral lytic cycle would not be possible. The small decrease (10%) in viability in mock-infected cells at 38.5°C compared to cells at 37°C might be due to cell death induced by the higher temperature. In conclusion, cells with cisplatin, tk, and 27lacZ reduces cell viability.

Furthermore, we tested cells treated with the mutant viruses for alterations in the levels of regulatory cellular proteins that are known to participate in apoptotic mechanisms, for example, Bcl-2. The data presented in Fig. 2 are from BHK cells, and experiments with HeLa cells produced similar results. Immunoblotting of total cell extracts for Bcl-2 (Fig. 2A, upper panels) was performed at 16 h posttreatment. Cells were treated with 30 μg of cisplatin per ml (lane 2) or infected with 27lacZ (lane 4), tk (lane 6), or wt HSV-1 (lane 8), and their Bcl-2 levels were compared to those in untreated, mock-infected cells at the appropriate temperatures (lanes 1, 3, 5, and 7, respectively). Decreased levels of Bcl-2 protein were observed during treatment with cisplatin, 27lacZ, or tk. Bcl-2 levels were unaffected during infection with wt HSV-1. Treatment of cells with 30 μg of cisplatin per ml for 16 hrs resulted in reduced levels of Bcl-2 (lane 10) compared to those in mock-infected cells (lane 11), and preinfection with wt HSV-1 completely rescued Bcl-2 levels (lane 9). JunD was used as control to ensure equal protein loading (Fig. 2A, lower panels), since the intracellular amounts of JunD remain stable during infection (46) and cisplatin treatment (not shown). No alterations in the cellular levels of P53, Mdm-2, Bax, and Bad, which are known generally to be related to apoptosis, were observed during programmed cell death induced by the mutant viruses (data not shown). In conclusion, cisplatin, tk, and 27lacZ, but the not the wt virus, induce decreases in Bcl-2 levels, and wt HSV-1 protects against the cisplatin-induced decrease in Bcl-2.

Quantification of the Bcl-2 levels from Fig. 2A is shown in Fig. 2B and confirmed an 85% ± 3% decrease after treatment of cells with cisplatin and 27% ± 10% and 30% ± 8% decreases during infection with the 27lacZ and tk mutant viruses, respectively. No decreases in Bcl-2 levels were detected during infection with wt HSV-1 in the presence or absence of cisplatin. All Bcl-2 values were normalized to their respective JunD controls.

The kinetics of the decrease in Bcl-2 levels during infections with cisplatin and the mutant viruses were examined. A kinetic experiment with cells infected with tk virus (Fig. 2C, upper panel) demonstrated a clear decrease in Bcl-2 levels at 3 and 6 h postinfection (lanes 3 and 4, respectively) compared to an earlier time (2 h) of infection (lane 2) or in mock-infected cells (lane 1). JunD was used to ensure equal protein loading (lower panel). Kinetic experiments were repeated for 27lacZ and cisplatin-treated cells with similar results (not shown).

We were concerned that we were comparing results from virus infections at two different temperatures and that the cellular response to the higher temperature of 38.5°C used for tk infections could affect the host cell apoptotic mechanisms. It was necessary, therefore, to investigate the effects of wt virus at this elevated temperature. Infection of BHK cells with wt virus at 38.5°C did not induce any DNA damage. Preinfection of cells with wt virus at 38.5°C protected cells from cisplatin-induced DNA degradation and reduced DNA damage to the background levels detected with mock-infected cells at 37°C (not shown). This is in accordance with previous observations that demonstrated wt HSV-1 protection against heat shock-induced apoptosis (9). Additionally, wt HSV-1 failed to activate caspase-3, did not result in decreased Bcl-2 levels, and protected against the cisplatin-induced decrease in Bcl-2 at the elevated temperature (not shown). We believe that there were no significant differences in the host cell responses to wt HSV-1 infection at 37 and at 38.5°C and therefore that our results with tk at the elevated temperature are comparable despite the temperature difference.

To summarize, infection of cells with 27lacZ and tk mutant viruses induced activation of caspase-3 and -6, cytoplasmic
cytochrome c release, decreases in Bcl-2 levels, DNA fragmentation, and cell death. wt HSV-1 failed to induce apoptosis, and preinfection of cells with the wt, but not the mutant viruses, protected the cells from cisplatin-induced cytoplasmic cytochrome c release, decreases in Bcl-2 levels, and DNA fragmentation but not from activation of caspase-3, as shown by cleavage of the caspase-3-specific substrate PARP. Bcl-2 is known to be a key regulator of apoptosis, and our observations, showing a correlation between decreased Bcl-2 levels and induction of apoptosis, suggest a role for Bcl-2 in apoptosis induced by the mutant viruses.

A decreased level of Bcl-2 protein is a key parameter during cell death induced by cisplatin, 27lacZ, and tsK, and Bcl-2 protects against apoptosis induced by cisplatin, tsK, and 27lacZ by acting upstream of cytoplasmic cytochrome c release and DNA degradation. To investigate the role of decreased Bcl-2 levels during programmed cell death triggered by cisplatin and 27lacZ and tsK viruses, we tested whether overexpression of Bcl-2 protein rescued cells from apoptosis induced by these stimuli. The data presented in Fig. 3 are from BHK cells, and experiments with HeLa cells produced similar results. Cells were transfected with 6 μg of bcl-2 expression plasmid or empty vector as a control. At 30 h posttransfection cells were treated with 30 μg of cisplatin per ml or infected with 27lacZ, tsK, or wt HSV-1 for an additional 16 h and were tested for DNA damage, cytoplasmic cytochrome c release, and decrease in cell viability (Fig. 3).

DNA damage was measured by ELISA, and the results are shown in Fig. 3A. Overexpression of bcl-2 resulted in decreased levels of DNA damage induced by cisplatin, tsK, or 27lacZ (five-, three-, and twofold, respectively) compared to that in cells transfected with empty vector. Infection with wt HSV-1 did not induce DNA degradation in the presence of vector or bcl-2 expression plasmid compared to that in mock-infected cells. Increased background levels of fragmented DNA were due to cellular stress during transfections and could be detected in mock-infected cells transfected with bcl-2 or vector. In addition, mock-infected cells at 38.5°C showed higher background levels of DNA damage than those at 37°C. The residual DNA damage detectable in cisplatin-, tsK-, or 27lacZ-treated cells in the presence of the bcl-2 expression plasmid, compared to that in mock-infected cells at the appropriate temperature, may be due to limited efficiency of the transient-transfection method (about 40%). In conclusion, overexpression of bcl-2 reduces DNA damage triggered during treatment of cells with cisplatin or the mutant viruses.

We then tested whether overexpression of Bcl-2 affected cytoplasmic cytochrome c release induced by cisplatin and the...
FIG. 3. Overexpression of Bcl-2 protects cells from DNA damage, cytoplasmic cytochrome c release, and cell death induced by cisplatin (30 µg/ml) and tsk (38.5°C) and 27lacZ viruses. (A) Cells (5 × 10⁶) were transfected with 6 µg of bcl-2 plasmid or empty vector for 30 h, and apoptosis was induced with the stimuli for additional 16 h. Detection of cytoplasmic DNA by ELISA in cells mock infected or treated with wt HSV-1, tsk, 27lacZ, or cisplatin in the presence of bcl-2 plasmid or empty vector is shown. (B) Cells (5 × 10⁶) were transfected with 0 to 6 µg of bcl-2 plasmid, and the amount of transfected DNA was normalized to 6 µg with empty vector. At 30 h posttransfection, cells were mock infected, treated with cisplatin, or infected with tsk or 27lacZ for 16 h. Upper panel, cytosolic preparations were immunoblotted for cytochrome c. Lower panel, Total cell extracts were immunostained for Bcl-2. (C) Cell viability was measured by trypan blue exclusion in cells transfected with Bcl-2 or vector as for panel A and infected for 12 to 24 h with tsk (left panel) and 27lacZ (right panel).
mutant viruses (Fig. 3B, upper panels). Immunoblotting analysis of cytosolic extracts for cytochrome c was performed at 16 h postinfection. Cells were mock infected (38.5°C) in the presence of 6 μg of transfected vector (lane 1) or infected with tsK (lanes 2 to 4) in the presence of 6 μg of empty vector (lane 2) or 3 or 6 μg of bcl-2 coding plasmid (lanes 3 and 4, respectively). Overexpression of Bcl-2 resulted in decreased levels of cytoplasmic cytochrome c (lanes 3 and 4 compared to lane 2). Similar experiments were performed with mock-infected cells (37°C) transfected with 6 μg of empty vector (lanes 5 and 8), with cells treated with 27lacZ (lane 6) or cisplatin (lane 9) in the presence of 6 μg of empty vector and with cells treated with 27lacZ (lane 7) or cisplatin (lane 10) in the presence of 6 μg of bcl-2 expression plasmid. The results showed that transfection of the bcl-2 expression plasmid resulted in decreased cytosolic release of cytochrome c during infection of cells with 27lacZ (compare lane 7 to 6) and treatment with cisplatin (compare lane 10 to 9). Total cell extracts from duplicate preparations were immunoblotted for Bcl-2 to confirm that Bcl-2 was overexpressed after transfection with the expression plasmid (Fig. 3B, lower panels). Cells were mock infected or treated in the presence of 6 μg of empty vector (lanes 1, 5, and 8) or infected with tsK, 27lacZ, or cisplatin in the presence of 6 μg of empty vector (lanes 2, 6, and 9 respectively) or 3 to 6 μg of Bcl-2 expression plasmid (lanes 3, 4, 7, and 10). Treatment of cells with tsK (lane 2), 27lacZ (lane 6), or cisplatin (lane 9) in the presence of empty vector resulted in decreased Bcl-2 levels compared to those in their mock-infected or treated controls (lanes 1, 5, and 8, respectively). In the presence of the bcl-2 expression plasmid (lanes 3, 4, 7, and 10), Bcl-2 protein levels were restored to approximately those of controls (lanes 1, 5, and 8). Overexpression of bcl-2 failed to reduce activation of caspase-3 during infection of cells with tsK and 27lacZ (as shown by detection of cleaved, active caspase-3 in immunoblotting), suggesting that caspase-3 acts either upstream of Bcl-2, or in a distinct pathway (not shown). We conclude that overexpression of bcl-2 protects against cytoplasmic release of cytochrome c induced by cisplatin, tsK, and 27lacZ.

Finally, the effect of overexpression of bcl-2 on cell viability was tested at 12 to 24 h postinfection of cells with tsK (Fig. 3C, left panel) or 27lacZ (Fig. 3C, right panel). In both cases, overexpression of Bcl-2 resulted in increased cell viabilities compared to those for cells transfected with the empty vector. The levels of Bcl-2 protein were constant between 12 and 36 h postinfection as determined by Western blotting of total cell extracts and started to decrease after 36 h (data not shown). Overexpression of Bcl-2 also increased cell viability in cells treated with cisplatin (not shown).

In summary, overexpression of Bcl-2 protects cells from DNA damage, cytoplasmic cytochrome c release, and cell death induced by cisplatin and 27lacZ and tsK viruses. Our results also suggest that Bcl-2 protects against apoptosis induced by cisplatin, 27lacZ, and tsK by acting upstream of cytoplasmic cytochrome c release and DNA degradation and that the decrease in the levels of Bcl-2 protein is a key event during induction of programmed cell death by these stimuli. We then investigated the cellular mechanisms that lead to decreased Bcl-2 levels during infection of cells with the mutant viruses. We examined three possible mechanisms, i.e., decrease of bcl-2 RNA, caspase-dependent degradation of Bcl-2 protein, and destabilization of Bcl-2 protein, all of which have previously been implicated in affecting Bcl-2 levels during other apoptotic insults (8).

Possible mechanisms for decreased Bcl-2 levels during infection with the mutant viruses. (i) Decreased bcl-2 RNA levels during infections with tsK and 27lacZ viruses and a role for ICP4 and ICP27 in maintaining bcl-2 RNA levels. Regulation of bcl-2 expression is a well-established mechanism to control Bcl-2 protein levels (8). We examined whether downregulation of Bcl-2 protein during treatment of cells with cisplatin, tsK, and 27lacZ is caused by a decrease in bcl-2 RNA. The data presented in Fig. 4 are from HeLa cells, and experiments with BHK cells produced similar results.

In Fig. 4A, upper panels the levels of bcl-2 RNA were analyzed by Northern blotting in mock-infected cells (lanes 1, 5, and 6) and in cells infected with wt HSV-1 (lane 2), 27lacZ (lane 3), or tsK (lane 4) or treated with 30 μg of cisplatin per ml (lane 7), at 16 h after infection or cisplatin treatment. Bcl-2 RNA levels were unaffected during wt HSV-1 infection compared to those in mock-infected cells (lanes 2 and 1, respectively). Infection with 27lacZ (lane 3) and tsK (lane 4) viruses resulted in 27% ± 7% and 53% ± 5% decreases in bcl-2 RNA, respectively, compared to those in their mock-infected controls (lanes 1 and 5, respectively). Treatment of cells with cisplatin (lane 7) resulted in a 70% ± 3% decrease in bcl-2 RNA compared to that in untreated cells (lane 6). Preinfection of cells for 5 h prior to cisplatin treatment with tsK (lane 8) or 27lacZ (lane 10) failed to protect against cisplatin-induced decreases in bcl-2 RNA (compared lanes 8 and 10 to lane 7). However, preinfection of cells with wt HSV-1 (lane 9) restored bcl-2 RNA to the level observed in the untreated control (compare lane 9 to lane 6). γ-Actin was probed to ensure equal loading (Fig. 4A, lower panel). Quantification of bcl-2 RNA is shown in Fig. 4B, and each value was normalized to its appropriate γ-actin control. The small decrease in the amount of Bcl-2 RNA in mock-infected cells at 38.5 compared to 37°C is in line with our observations in Fig. 1, which showed induction of low levels of cell death at the higher temperature. Reduction of bcl-2 RNA was detectable after 3 h postinfection (not shown), which is in line with the data for Bcl-2 protein levels presented in Fig. 2. Our results show that downregulation of bcl-2 RNA is a mechanism of decreasing Bcl-2 protein levels during infection of cells with 27lacZ and tsK viruses and treatment with cisplatin. Moreover, wt HSV-1, but not the mutant viruses, is capable of maintaining bcl-2 RNA levels during cisplatin-induced apoptosis, thus suggesting a role for ICP27 and ICP4 in regulation of bcl-2 RNA levels during HSV-1 infection.

To investigate the role of ICP27 and ICP4 in maintaining bcl-2 RNA levels in the absence of other viral proteins, cells were transfected with 6 μg of empty vector (Fig. 4C, upper panel, lanes 1 and 2) or cotransfected with 3 μg of each expression plasmid coding for ICP27 and ICP4 (lane 3). At 30 h posttransfection, cells were mock treated (lane 1) or treated with 30 μg of cisplatin per ml for an additional 16 h (lanes 2 and 3), and bcl-2 RNA levels were analyzed by Northern blotting and visualized with a phosphorimager. In the presence of ICP27 and ICP4, we observed a 70% increase in bcl-2 RNA levels compared to those detected in cells treated with cisplatin in the presence of empty vector (compare lane 3 to lane 2).
This effect was also seen with Bel-2 protein levels (not shown). γ-Actin was probed to ensure equal loading (Fig. 4C, lower panel). Quantification of bcl-2 RNA (Fig. 4D) was normalized to the appropriate γ-actin controls. Failure to restore fully bcl-2 RNA during treatment of cells with cisplatin in the presence of transfected ICP4 and ICP27 may be explained by limited transfection efficiencies (approximately 40%). However, we cannot rule out the possibility that additional viral proteins are required to reproduce fully the phenotype observed with the wt virus. In conclusion, cotransfection of ICP4 and ICP27 protected in part against cisplatin-induced downregulation of bcl-2 RNA.

Viability assays, performed at 6 to 24 h posttreatment with cisplatin, showed that cotransfection of ICP4 and ICP27 increased cell survival by up to 70% ± 10% compared to cells treated with cisplatin in the presence of empty vector (not shown).

We suggest that both ICP4 and ICP27 contribute to the antiapoptotic effect of wt HSV-1 and are necessary and, at least in part, capable of maintaining bcl-2 RNA levels during HSV-1 infection and cisplatin treatment of cells.

(ii) Infections with tsk and 27lacZ viruses cause caspase-dependent degradation of Bel-2, and caspases act upstream of Bel-2. Regulation of Bel-2 levels by caspase-dependent degradation was previously reported (8). To investigate whether activated caspases act upstream of Bel-2 downregulation and whether caspase-dependent degradation of Bel-2 occurs during apoptosis induced by the mutant viruses, we examined the effects, on the apoptotic pathway and on Bel-2 levels, of blocking caspase activation using the inhibitor Z-VAD-FMK. Z-VAD-FMK is an inhibitor of caspase-1, -3, -4, and -7, and since no activation of caspase-1, -4, and -7 was observed during apoptosis induced by the mutant viruses, the effects observed using Z-VAD-FMK are most probably caspase-3 dependent. However, we cannot exclude the possibility that other caspases were inhibited. The data presented in Fig. 5 are from BHK cells. Experiments with HeLa cells produced similar results.

Figure 5A shows the effects of caspase inhibition on Bel-2 protein levels during infection with mutant viruses. Cells were mock infected at the appropriate temperature (lanes 1 and 4) or infected for 16 h with 27lacZ (lanes 2 and 3) or tsk (lanes 5 and 6) viruses. For inhibition of caspases, 25 μM Z-VAD-FMK was added 1 h prior to and 6 h after infection (lanes 3 and 6). The cellular amounts of Bel-2 were determined by immunoblotting (upper panels); Z-VAD-FMK partially restored Bel-2 levels (compare lane 3 to lane 2 and lane 6 to lane 5). JunD was probed to ensure equal protein loading (lower panels). Z-VAD-FMK partially restored Bel-2 levels (compare lane 3 to lane 2 and lane 6 to lane 5). JunD was probed to ensure equal protein loading (lower panels). Quantiﬁcation of Bel-2 levels (Fig. 5B) showed that inhibition of caspases resulted in 17% ± 5% (27lacZ) and 28% ± 3% (tsk) increases in Bel-2. Quantification of Bel-2 levels was normalized to the appropriate JunD control for differences in protein loading. Similar results were obtained with cisplatin (not shown). The failure to restore fully Bel-2 levels in the presence of the inhibitor (compare lane 3 to lane 1 and lane 6 to lane 4) may be explained by the fact that other mechanisms of bel-2 downregulation (for example, at the RNA level) are also operating. Treatment of cells with Z-VAD-FMK had no effect on the cisplatin- or mutant virus-induced decrease in Bel-2 RNA.
levels (not shown). We suggest that inhibition of caspases using Z-VAD-FMK in part protects against decreases in Bcl-2 protein levels during infections with tsk and 27lacZ viruses.

Furthermore, we examined the effects of inhibition of caspases by Z-VAD-FMK in the apoptotic events downstream of Bcl-2 (i.e., cytoplasmic release of cytochrome c, DNA damage, and cell viability) during infections with tsk and 27lacZ. For cytoplastic release of cytochrome c (Fig. 5C), cytosolic extracts from mock-infected cells (lane 1) or cells infected with tsk for 16 h (lanes 2 and 3), in the absence (lane 2) or presence (lane 3) of 25 μM Z-VAD-FMK were analysed by immunoblotting. Infection of cells with tsk resulted in cytoplasmic release of cytochrome c (lane 2) compared to that in mock-infected cells (lane 1), and cytoplasmic levels of cytochrome c were reduced in the presence of Z-VAD-FMK (lane 3 compared to lane 2). Similar results were obtained for 27lacZ infection (not shown). We suggest that treatment of cells with the caspase inhibitor Z-VAD-FMK reduces cytoplasmic release of cytochrome c during infections with the mutant viruses.

The effects of caspase inhibition on induction of DNA damage were examined by ELISA in mock-infected cells and in cells infected with wt HSV-1, 27lacZ, or tsk or treated with 30 μg of cisplatin per ml (Fig. 5D). In the presence of Z-VAD-FMK, we observed a 40% ± 10% decrease in the levels of cytoplasmic DNA at 16 h posttreatment with cisplatin and 40% ± 6% and 24% ± 6% decreases for tsk and 27lacZ, respectively. Only background DNA damage was observed in mock-infected cells at 37 and 38.5°C and in wt HSV-1-infected cells in the presence or absence of Z-VAD-FMK. We suggest that Z-VAD-FMK reduces DNA damage during treatment of cells with cisplatin, tsk, and 27lacZ.

Finally, the viability of cells at 16 h posttreatment with cisplatin, tsk, and 27lacZ was increased in the presence of Z-VAD-FMK by 30% ± 5%, 20% ± 5%, and 20% ± 5%, respectively (not shown).

In conclusion, our results suggest that caspase-dependent degradation of Bcl-2 contributes to decreasing Bcl-2 protein levels during apoptosis induced by the tsk and 27lacZ viruses. In addition, we suggest that activation of caspases during infection of cells with the mutant viruses functions upstream of Bcl-2 downregulation, cytochrome c release, and DNA degradation, as Z-VAD-FMK in part protected cells from all of these apoptotic events.

(iii) Infections with tsk and 27lacZ viruses cause a p38MAPK-dependent decrease in the half-life of Bcl-2 protein. Destabilization of Bcl-2 via phosphorylation has been identified as a further mechanism of Bcl-2 regulation following specific stimuli (8). In addition, the p38MAPK stress kinase is reported to phosphorylate Bcl-2 in vitro (23), and p38MAPK is activated during infection of cells with 27lacZ and tsk viruses (46). We therefore examined whether activation of p38MAPK was
FIG. 6. Infection of cells with 27lacZ but not with the wt virus induces a p38MAPK-dependent decrease in Bcl-2 half-life, and p38MAPK kinase activity communoprecipitates with Bcl-2 in cells infected with tsk and 27lacZ. (A) Left panel [35S]methionine pulse-chase analysis of Bcl-2 degradation rates during infection with wt HSV-1. Cells (5 × 10⁶) were infected with wt HSV-1 and at 7 h post-infection cells were labeled with [35S]methionine and either lysed (0 h) or cultured in medium containing unlabeled methionine prior to immunoprecipitation for Bcl-2. The gel was analyzed by autoradiography. Right panel, [35S]methionine pulse-chase analysis of Bcl-2 degradation rates during infection with 27lacZ. Cells (5 × 10⁶) were infected with 27lacZ, and at 7 h postinfection cells were analyzed as described for panel A. (B) Quantification of Bcl-2 levels from [35S]methionine pulse-chase analysis during infection with wt HSV-1 and 27lacZ as described for panel A. (C) Left panel, [35S]methionine pulse-chase analysis of Bcl-2 degradation rates during infection with 27lacZ in the presence of DMSO. Cells (5 × 10⁶) were treated with 30 μM DMSO for 1 h and infected with 27lacZ in the continuous presence of DMSO. At 7 h postinfection cells were labeled with [35S]methionine, and
linked to destabilization of Bcl-2 protein and whether Bcl-2 interacted with a p38MAPK-associated kinase during infections with the mutant viruses. The data presented in Fig. 6 are from BHK cells, and experiments with HELa cells produced similar results. Cisplatin treatment failed to activate p38MAPK (not shown) and was not used in these experiments.

The half-life of Bcl-2 protein was measured in cells infected with wt HSV-1 and 27lacZ virus (Fig. 6A). At 7 h postinfection, cells were metabolically labeled with [35S]methionine for 2 h and either lysed immediately (0 h) (lanes 1 and 6) or cultured in medium containing unlabeled methionine for the times indicated (lanes 2 to 5 and 7 to 9). Bcl-2 was immunoprecipitated, and gels were analyzed by autoradiography. In cells infected with wt HSV-1 (Fig. 6A, left panel), degradation of Bcl-2 protein was minimal at 1 h postlabeling (lane 2) compared to the levels of Bcl-2 at the start of the radioactive chase (lane 1). Decreased Bcl-2 levels were observed at 2 h postlabeling (lane 3), the protein was still detectable at 4 h postlabeling (lane 4), and no Bcl-2 was detectable at 5 h postlabeling (lane 5). In contrast, during infection of cells with the 27lacZ virus (Fig. 6A, right panel), decreased Bcl-2 levels were detectable as early as 0.5 h postlabeling (lane 7) compared to the levels of Bcl-2 at the start of the radioactive chase (lane 6). At 1 h postlabeling, Bcl-2 was detectable in greatly reduced amounts (lane 3), and at 2 h postlabeling, no Bcl-2 protein was detected (lane 4). Quantification of Bcl-2 protein levels is presented in Fig. 6B. In cells infected with wt HSV-1, Bcl-2 showed a half-life of approximately 3 h. Infection with 27lacZ resulted in a fourfold decrease in the Bcl-2 half-life (to 45 min). The Bcl-2 half-life in mock-infected cells, at both 37 and 38.5°C, was the same as in HSV-1-infected cells (3 h). In cells infected with tsK, the Bcl-2 half-life was the same as in 27lacZ-infected cells (approximately 45 min) (data not shown). Our results show that infection of cells with the mutant viruses resulted in approximately a fourfold decrease in Bcl-2 half-life compared to that in cells infected with wt HSV-1 or mock-infected cells.

To examine whether destabilization of Bcl-2 during infection with the mutant viruses was p38MAPK-dependent, cells were treated for 1 h with dimethyl sulfoxide (DMSO) (control) or with 30 μM of the specific p38MAPK inhibitor SB203580 and then infected with tsK (not shown) or 27lacZ (Fig 6C) viruses in the continuous presence of the DMSO or inhibitor. At 7 h postinfection, cells were metabolically labeled with [35S]methionine for 2 h and either lysed immediately (0 h) (lanes 1, 6, and 8), or cultured in medium containing unlabeled methionine for the times indicated (lanes 2 to 5 and 9 to 12). Bcl-2 was immunoprecipitated, and gels were analyzed with a phosphor-imager. In cells infected with 27lacZ in the presence of DMSO (Fig. 6C, left panel), decreased Bcl-2 levels were detectable as early as 0.5 h postlabeling (lane 2) compared to the levels of Bcl-2 at the start of the radioactive chase (lane 1). At 1 h postlabeling only small amounts of protein were detectable (lane 3), and at 2 or 3 h postlabeling no Bcl-2 protein was detected (lanes 4 and 5, respectively). In cells infected with 27lacZ in the presence of SB203580 (Fig. 6C, right panel), degradation of Bcl-2 protein was undetectable at 0.5 h postlabeling (lane 9) compared to the levels of Bcl-2 at the start of the radioactive chase (lane 8). Decreased Bcl-2 levels were detected at 2 h postlabeling (lane 10). Bcl-2 protein was still detectable at 3 h postlabeling (lane 11), and no Bcl-2 was detectable at 5 h postlabeling (lane 12). Immunoprecipitation at 0 h postlabeling with preimmune serum confirmed the specificity of the antibody; no Bcl-2 band was observed (lane 7) compared to when the Bcl-2 antibody was used (lane 6). Quantification of Bcl-2 levels (Fig. 6D) showed that the half-life of Bcl-2 protein in cells infected with 27lacZ in the presence of DMSO was approximately 45 min, as in 27lacZ-infected cells. However, in the presence of the p38MAPK inhibitor, the half-life of Bcl-2 protein in cells infected with 27lacZ increased by threefold and was approximately 2.4 h. Similar results were obtained with the tsK virus. Our results show that inhibition of p38MAPK using the SB203580 inhibitor during infection of cells with 27lacZ increases the half-life of bcl-2 protein by threefold, almost to the levels detected in mock-infected cells. We therefore suggest that the decrease in Bcl-2 half-life during infection of cells with the mutant viruses is p38MAPK dependent.

If the effect of p38MAPK on Bcl-2 was via p38MAPK-dependent phosphorylation of Bcl-2, we might expect p38MAPK to coimmunoprecipitate with Bcl-2 protein. This possibility was tested, and the results are shown in Fig. 6E (tsK) and F (27lacZ). In Fig. 6E, cells were treated with a 30 μM concentration of the specific p38MAPK inhibitor SB203580 (lanes 5 and 6) or DMSO (lanes 1 to 4) for 1 h prior to and throughout infection with tsK (lanes 3 to 6) or were mock infected (lanes 1 and 2); duplicate results are shown in lanes 1 and 2, 3 and 4, and 5 and 6. At 8 h postinfection, Bcl-2 was immunoprecipitated and immunocomplex kinase assays were performed with 1 μg of purified ATF-2 protein, which is a substrate for JNK and p38MAPK, to detect associated p38MAPK activity (Fig. 6E, upper panel). SB203580 was included to assess the contribution of p38MAPK to phosphorylation of ATF-2. Infection with tsK resulted in increased phosphorylation of ATF-2 (lanes 3 and 4) compared to that in mock-infected cells (lanes 1 and 2). Substrate phosphorylation during tsK infection was mark-
edly reduced in cells treated with the p38MAPK inhibitor (lanes 5 and 6), suggesting that the kinase coimmunoprecipitating with Bcl-2 was p38MAPK related. To verify that immunoprecipitation had worked, duplicate immunoprecipitations, similar to those analyzed in immunocomplex assays, were immunoblotted for Bcl-2 (Fig. 6E, lower panel). In the presence of DMSO the amount of Bcl-2 immunoprecipitated was decreased in cells infected with ts[k (lanes 3 and 4) compared to control cells (lanes 1 and 2). In ts[k-infected cells in the presence of SB203580 (lanes 5 and 6) the amount of Bcl-2 immunoprecipitated was increased compared to that in infected cells treated with DMSO (lanes 3 and 4). (This is in line with data presented in Fig. 7, showing an increase in Bcl-2 levels in the presence of the SB203580 in cells infected with the mutant viruses [see below].)

Immunocomplex kinase assays were repeated in mock-infected cells (Fig. 6F, upper panel, lanes 1 and 2) and 27lacZ-infected cells (lanes 3 to 6) in the presence of DMSO (lanes 1 to 4) or 30 μM SB203580 (lanes 5 and 6). The results showed that increased kinase activity immunoprecipitated with Bcl-2 (lanes 3 and 4 compared to lanes 1, 2), and this activity was p38MAPK dependent (lanes 5 and 6). To verify that immunoprecipitation had worked, duplicate immunoprecipitations were immunoblotted for Bcl-2 (Fig. 6F, lower panel), and the results were similar to those obtained for ts[k-infected cells. Although p38MAPK is activated during wt HSV-1 infection (46), no kinase activity coimmunoprecipitated with Bcl-2 in cell extracts infected with the wt virus (data not shown); possible explanations are discussed below.

We conclude that p38MAPK-associated kinase activity coimmunoprecipitates with Bcl-2 during infection of cells with ts[k and 27lacZ viruses, thus suggesting interaction of Bcl-2 with a p38MAPK-associated kinase.

JNK is also activated during infection of cells with wt HSV-1 and with ts[k and 27lacZ mutant viruses (46). Immunocomplex kinase assays using Jun protein, which is a JNK-specific substrate, from extracts of cells transfected with or without the JNK-inhibitory plasmid SEK-AL suggested that JNK activity coimmunoprecipitates with Bcl-2 in cells infected with ts[k and 27lacZ but not in mock-infected or wt HSV-1-infected cells (data not shown). SEK-AL expresses a dominant-negative MKK4 mutant, and MKK4 was shown to be the only upstream activator of JNK during HSV-1 infection (46). The significance of these observations is currently under further investigation.

In summary, we propose that p38MAPK-dependent destabilization of Bcl-2 protein contributes to decreasing Bcl-2 levels during apoptosis induced by infection of cells with ts[k and 27lacZ viruses. We also suggest that the effect of p38MAPK on Bcl-2 is mediated by interaction of Bcl-2 with a p38MAPK-related kinase.

Inhibition of p38MAPK increases Bcl-2 levels and rescues cells from DNA damage during infection with the mutant viruses. To determine whether p38MAPK-dependent destabilization of Bcl-2 had any effect on the levels of apoptosis...
induced by the mutant viruses, we examined the results of inhibiting p38 MAPK activity on downregulation of Bcl-2 levels and induction of DNA damage (Fig. 7). The data presented are from BHK cells. Experiments with HeLa cells produced similar results.

To examine the effect of inhibition of p38 MAPK on Bcl-2 levels during apoptosis induced by the mutant viruses (Fig. 7A), cells were treated with a 30 μM concentration of the p38 MAPK inhibitor SB203580 (lanes 3, 6, and 9) or DMSO (lanes 1, 2, 4, 5, 7, and 8) for 1 h prior to and throughout infection with 27lacZ (lanes 2 and 3), tsk (lanes 5 and 6), or wt HSV-1 (lanes 8 and 9). Extracts were immunoblotted for Bcl-2 (lanes 1, 2, 4, 5, 7, and 8) for 1 h prior to and throughout infection with p38 MAPK inhibitor SB203580 (lanes 3, 6, and 9) or DMSO (lanes 1, 2, 4, 5, 7, and 8) for 1 h prior to and throughout infection with 27lacZ (lanes 2 and 3), tsk (lanes 5 and 6), or wt HSV-1 (lanes 8 and 9). Extracts were immunoblotted for Bcl-2 (upper panels) at 16 h postinfection. Bcl-2 levels were compared to those in mock-infected cells at the appropriate temperature (lanes 1, 4, and 7). Treatment with SB203580 increased Bcl-2 levels in cells infected with 27lacZ (lane 3) or tsk (lane 6) compared to infected cells treated with DMSO (lanes 2 and 5, respectively). In cells infected with wt HSV-1 (lanes 8 and 9), Bcl-2 levels remained constant in the presence of DMSO (lane 8) or SB203580 (lane 9) and at the level in mock-infected cells (lane 7). JunD was used as a protein loading control (Fig. 7A, lower panels). SB203580 did not affect the levels of Bcl-2 in mock-infected cells compared to DMSO-treated cells (not shown). Quantification of Bcl-2 levels were normalized to the appropriate control JunD levels for differences in protein loading and showed that inhibition of p38 MAPK during infections with 27lacZ and tsk increased Bcl-2 levels by 40% ± 10%. In cells infected with the mutant viruses, bcl-2 RNA levels were unaffected by the increase in Bcl-2 protein in the presence of SB203580 compared to those in DMSO-treated cells (not shown), showing that stabilization of bcl-2 RNA by Bcl-2 protein was unlikely to occur. Our results suggest that inhibition of p38 MAPK increases the levels of Bcl-2 protein during infection of cells with tsk and 27lacZ viruses.

To determine the effect of inhibition of p38 MAPK on induction of DNA damage, cytoplasmic DNA in mock-infected cells or cells infected with tsk, 27lacZ, or wt HSV-1 at 16 h postinfection, in the presence of DMSO or 30 μM SB203580, was analyzed by ELISA (Fig. 7B). During tsk and 27lacZ infection we detected 30% ± 8% and 38% ± 8% decreases in DNA damage, respectively, in the presence of the p38 MAPK inhibitor compared to that in infected cells treated with DMSO. Background levels of DNA damage were observed in mock-infected cells at 37°C and 38.5°C and in wt HSV-infected cells in the presence of DMSO or SB203580. We suggest that inhibition of p38 MAPK activity reduced DNA damage induced during infection of cells with the mutant viruses.

Cell survival was increased by 28% ± 5% or 34% ± 4% in cells infected with tsk or 27lacZ viruses, respectively, in the presence of SB203580 compared to that in infected cells treated with DMSO. SB203580 did not affect cell survival for mock-infected cells (not shown).

Thus, we conclude that activation of p38 MAPK is involved in the decrease of Bcl-2 levels and induction of DNA damage during infection of cells with tsk and 27lacZ.

**Activation of p38 MAPK is independent of caspases.** Both p38 MAPK and caspases act upstream of Bcl-2 during apoptosis induced by the mutant viruses. We tested whether caspase activation and stimulation of p38 MAPK were interdependent events.

In Fig. 7C, we examined whether inhibition of caspases using Z-VAD-FMK affected activation of p38 MAPK during infection with tsk. Cells were mock infected at 38.5°C (lanes 1 and 2) or infected with tsk (lanes 3 to 6) in the absence (lanes 3 and 4) or presence (lanes 5 and 6) of a 25 μM concentration of the caspase inhibitor Z-VAD-FMK. At 8 h postinfection, p38 MAPK was immunoprecipitated and the kinase activity was measured in immunocomplex kinase assays, using purified ATF-2 as a substrate. Duplicate results are shown in lanes 1 and 2, 3 and 4, and 5 and 6. p38 MAPK was activated in tsk-infected cells (lanes 3 and 4) compared to mock-infected cells (lanes 1 and 2), and this activation was not affected by Z-VAD-FMK (lanes 5 and 6 compared to lanes 3 and 4). Similar results were obtained during 27lacZ infections (not shown).

Further, we examined whether inhibition of p38 MAPK activity during tsk infections affected activation of caspase-3. Figure 7D shows immunoblotting for caspase-3 activation in cells mock infected at 38.5°C (lane 1) or infected with tsk (lanes 2 and 3) in the presence of 30 μM DMSO (lane 2) or 30 μM SB203580 (lane 3). Inhibition of p38 MAPK did not affect cleavage of pro-caspase-3 to active caspase-3 during tsk infection (compare lane 3 to lane 2). Similar results were obtained for 27lacZ (not shown).

Thus, we suggest that p38 MAPK is activated independently of caspases during apoptosis induced by tsk and 27lacZ.

### Inhibition of p38 MAPK kinase during wt HSV-1 infection decreases viral yield

Our results suggest that activation of the stress kinase p38 MAPK during infection with the mutant viruses is part of an antiviral apoptotic response. However, we wished to determine whether activation of p38 MAPK and of the closely related JNK stress kinases by wt HSV-1 (46) also serves some beneficial purpose for the virus. To test this hypothesis, viral yields were measured at 0–32 h postinfection of control BHK cells with wt HSV-1. BHK cells were preincubated for 1 h with either 30 μM DMSO (control) or SB203580 (p38 MAPK inhibitor) and then infected with 10 PFU of wt HSV-1 per cell in the continuous presence of DMSO or SB203580. The yields at 18, 24, 28, and 32 h postinfection are given in Table 1. The titer of the virus increased in both experiments between 18 and 32 h, reaching a maximum by 28 h postinfection. At 32 h postinfection, the virus titer dropped slightly, presumably because most of the cells were lysed and some degradation of the virus occurred. Inhibition of p38 MAPK resulted in decreases of 33% ± 5% in the yield of wt HSV-1 virus at 24 h postinfection, 58% ± 10% at 28 h postinfection, and 55% ± 6% at 32 h postinfection compared to untreated controls.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Viral yield (PFU, 10^6) at:</th>
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<tr>
<td></td>
<td>18 h</td>
</tr>
<tr>
<td>DMSO</td>
<td>6.5 ± 0.3</td>
</tr>
<tr>
<td>SB203580</td>
<td>6.0 ± 0.3</td>
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* Cells (5 × 10^3) were pretreated with 30 μM SB203580 or DMSO for 1 h and infected with wt HSV-1 in the continuous presence of SB203580 or DMSO. Viral yields were determined at 18, 24, 28, and 32 h postinfection. Each value represents the mean ± standard deviation from three independent experiments, and in each experiment triplicate determinations were performed.
to that for the control wt HSV-1-infected cells treated with DMSO, and all of these differences were statistically significant as confirmed by the $\chi^2$ test ($P < 0.05$).

To examine the effects of inhibiting JNK on the HSV-1 yield, cells were transfected with 5 $\mu$g of the plasmid SEK-AL, coding for a dominant-negative MKK4 mutant, or with empty vector as a control. At 30 h posttransfection cells were infected with wt HSV-1. Inhibition of the JNK pathway resulted in a 20%–65% decrease in viral yield at 30 h postinfection compared to that for control cells. The minimal effect of JNK inhibition on virus replication may be due to the limited transfection efficiency of the SEK-AL plasmid (around 30%).

In conclusion, our results suggest that activation of p38MAPK during wt HSV-1 infection increased viral yields.

Activation of p38MAPK during wt HSV-1 infection enhances expression of specific viral gene promoters. Decreased viral yields in the presence of the SB203580 inhibitor might have resulted from inhibition of the release of assembled viral particles. Alternatively, inhibition of p38MAPK might reduce expression of all or specific viral proteins during infection with wt HSV-1. To test the first possibility, virus release assays were performed at 18, 24, 28, and 32 h postinfection of cells with wt HSV-1 in the presence of SB203580 or DMSO. No difference in virus release was observed at any of these time points (not shown).

To examine whether activation of p38MAPK enhanced the levels of viral proteins during wt HSV-1 infection, we tested the effects of inhibition of p38MAPK on the production of several viral IE, early, or late proteins by immunoblotting. The data presented in Fig. 8A and B are from BHK cells, and experiments with HeLa cells produced similar results. Cells were incubated with 30 $\mu$M DMSO or SB203580 for 1 h prior to and throughout infection with wt HSV-1, and the levels of viral proteins were examined by immunoblotting at 6, 9, and 12 h post-infection. In Fig. 8A, upper panel, accumulation of the IE protein ICP0 was tested at 6 h (lanes 1 and 2), 9 h (lanes 3 and 4) and 12 h (lanes 5 and 6) postinfection in the presence of DMSO as a control (lanes 1, 3, and 5) or SB203580 (lanes 2, 4, and 6). In the presence of SB203580, the levels of ICP0 were decreased compared to those in infected cells treated with DMSO (compare lane 2 to 1, lane 4 to 3, and lane 6 to 5). Densitometric analysis showed a consistent 30% ± 10% decrease in ICP0 levels in the presence of the p38MAPK inhibitor at all time points. The same membrane was probed for the ICP4 IE protein, and, as shown in Fig. 8A, lower panel, the levels of ICP4 remained unaffected in the presence of SB203580 or DMSO. Expression of the essential IE protein ICP27 at 9 h postinfection is shown in Fig. 8B, upper panel, in the presence of DMSO (lane 1) or SB203580 (lane 2), and decreased levels of ICP27 were observed with SB203580 (compare lane 2 to 1). The same filter was probed for the early protein UL42 (Fig. 8B, lower panel) and no alteration in the amounts of UL42 was observed in the presence or absence of SB203580. Furthermore, expression of the R2 subunit (early protein) of the viral ribonucleotide reductase and glycoprotein C (late protein) was decreased in the presence of SB203580, whereas accumulation of the R1 subunit of viral ribonucleotide reductase (which is expressed during IE times) was
not p38MAPK dependent (data not shown). We conclude that inhibition of p38MAPK activity decreased the levels of specific viral proteins during infection with wt HSV-1.

To determine whether the p38MAPK-enhanced accumulation of viral proteins was due to transcriptional activation of their respective genes during wt HSV-1 infection, we used CAT assays to examine the effect of p38MAPK inhibition on the promoter activities of two genes: the IE110 gene (which produces ICP4, whose levels were affected by inhibition of p38MAPK) and the IE175 gene (which produces ICP4, whose levels were unaffected by inhibition of p38MAPK). HeLa cells were transfected with reporter plasmids pIE1CAT and pIE175CAT, which contain the promoters of IE110 and IE175 genes, respectively, upstream of the CAT-coding sequence. At 30 h posttransfection, cells were pretreated for 1 h with either DMSO or SB203580 and infected with wt HSV-1 in the continuous presence of DMSO or SB203580. CAT activity was measured at 16 h postinfection (Fig. 8C). In keeping with the immunoblotting data (Fig. 8A), a twofold decrease in the activity of the IE110 promoter was observed in the presence of the SB203580 inhibitor compared to that in infected cells treated with DMSO, whereas the activity of IE175 promoter remained unchanged in the presence of DMSO or SB203580.

Thus, we propose that activation of p38MAPK during wt HSV-1 infection enhanced accumulation of specific viral proteins by activating expression of the respective viral gene promoters.

DISCUSSION

In the present study, we examined the apoptotic events triggered during infection of BHK and HeLa cells with the mutant viruses tk and 27lacZ, which lack functional ICP4 and ICP27 proteins, respectively. Experiments with both cell lines produced similar data. Our results demonstrated that these mutant viruses induce an apoptotic pathway involving activation of caspases-3 and -6, activation of the p38MAPK stress kinase, decreases in Bcl-2 RNA and protein levels, release of cytochrome c to the cytoplasm, degradation of DNA, and cell death. Bcl-2 acts upstream of cytochrome c and DNA degradation. The caspases and p38MAPK act independently, and both act upstream of Bcl-2 protein.

We also demonstrated that the decrease in Bcl-2 levels is a key element in the apoptotic process and that downregulation of Bcl-2 protein during infection with the mutant viruses was mediated by three distinct mechanisms: (i) decreases in bel-2 RNA levels, (ii) caspase-related degradation of Bcl-2, and (iii) p38MAPK-dependent decreases in the Bcl-2 half-life. wt HSV-1 evades apoptosis during infection by subverting all three of these mechanisms that target Bcl-2. Thus, activation of caspases and a decrease in bel-2 RNA during infection of cells are prevented, and although p38MAPK is activated during wt HSV-1 infection (46), this does not alter the Bcl-2 half-life. Indeed, induction of p38MAPK activity during infection of cells with the wt virus increased viral yields by enhancing transcriptional activation of specific viral gene promoters and accumulation of their respective proteins. Cisplatin induces an apoptotic pathway that has a number of similarities to mutant virus-induced cell death, with decreased Bel-2 RNA and protein levels, activation of caspase-3 and -6, cytoplasmic cytochrome c release, and DNA degradation. wt HSV-1 protected cells from cisplatin-induced apoptosis and stabilized bel-2 RNA and protein levels without preventing activation of caspases.

Both tk and 27lacZ infection failed to prevent cisplatin-induced apoptosis, suggesting a protective role for ICP4 and ICP27 during wt virus infection. Transfection of cells with both proteins was necessary to maintain Bcl-2 RNA and partially prevent cisplatin-induced apoptosis. However, we cannot rule out completely the involvement of other viral proteins. In addition, tk and 27lacZ trigger activation of caspases and destabilization of Bcl-2 by activated p38MAPK during infection. Whether ICP4 and ICP27 play an antiapoptotic role in these two mechanisms is under investigation. It is possible that other viral early or late proteins that are not expressed in the ICP4 and ICP27 viral mutants play a role in evading caspase activation and p38MAPK-dependent destabilization of Bcl-2 during wt HSV-1 infections.

We provide evidence for mechanisms used by wt HSV-1 to circumvent and exploit the apoptotic host cell response to ensure and facilitate viral propagation that occur as early as 6 h postinfection. These are summarized schematically in Fig. 9. The only previously known apoptotic mechanism against HSV-1 involved host cell protein synthesis shutoff and the late viral protein γ34.5 (14).

Infection of cells with 27lacZ or tk mutant viruses triggers an apoptotic response that targets the key regulatory protein Bcl-2 through three cellular pathways (Fig. 9). In the first pathway (pathway I), the bel-2 RNA levels (step Ic) and the Bcl-2 protein levels (step Id) are decreased. wt-HSV-1 blocks step Ic by maintaining cellular bel-2 RNA levels, and viral proteins ICP4 and ICP27 are indispensable for that function. Numerous studies have indicated transcriptional regulation of Bcl-2, for example, by survival factors (28). Viruses commonly exploit the antiapoptotic function of Bcl-2 by encoding proteins that mimic its effect (20, 22) but only Epstein-Barr virus latent membrane protein 1 has been reported to stimulate cellular Bcl-2 expression (15).

The mechanism used by ICP4 and ICP27 to maintain bel-2 RNA is under investigation. ICP4 acts as a transcription factor to transactivate viral genes (2). ICP27 regulates transcription of HSV-1 genes (36), modulates 3′ RNA processing and poly(A) usage (24, 25), and is involved in the export of HSV-1 intronless RNAs (37). Furthermore, ICP27 interacts with ICP4 (30) and with host cell factors, including casein kinase 2 and the multifunctional heterogeneous nuclear ribonucleoprotein K, which is linked to transcription, RNA processing, transport, and translation (43). It is therefore possible that ICP27 and ICP4 regulate bel-2 RNA levels through multiple mechanisms.

Second, the apoptotic mechanisms induced by the mutant viruses involved a caspase-dependent decrease in Bel-2 levels (Fig. 9, pathway II). Infection of cells with 27lacZ or tk viruses resulted in activation of caspase-3 and -6 (step IIc). Inhibition of caspase activity by Z-VAD-FMK during infection with the mutant viruses partially protected against decreased Bel-2 levels (step IIc) and subsequent cytoplasmic cytochrome c release, degradation of nuclear DNA, and cell death, thus suggesting caspase-dependent degradation of Bel-2 protein. Z-VAD-FMK is an inhibitor of caspase-1, -3, -4, and -7 but not of caspase-6. Since caspase-1, -4, and -7 were not activated during
apoptosis triggered by cisplatin and the mutant viruses, it is probable that the Z-VAD-FMK effects are caspase-3 dependent. wt HSV-1 inhibits this apoptotic pathway by blocking the caspase-dependent decrease in Bcl-2 levels (step II2).

Inactivation of the antiapoptotic function of Bcl-2 by caspase-3 cleavage at amino acids 31 and 34 has been reported (12). Cleavage removes the N-terminal BH4 region known to be essential for the death-protective activity of Bcl-2. In our study, we failed to observe characteristic products of Bcl-2 cleavage at these specific residues. It is possible that after specific cleavage of Bcl-2 by caspases, rapid degradation of the protein by noncaspase proteases occurs during infection of cells with the mutant viruses.

Our results are in agreement with previous observations showing that inhibition of caspases failed to protect against apoptosis induced by a virus that lacked functional ICP4 and Us3 genes (9) and that wt HSV-1 protects from apoptosis triggered by osmotic shock and heat without preventing caspase-3 activation (10). Z-VAD-FMK rescues Bcl-2 levels during infection with 27lacZ and tsk viruses by approximately 20 to 30%. This level of protection, although highly reproducible, was considerably lower than that obtained by transfection of cells with bcl-2. This implies that the caspase-dependent decrease in Bcl-2 levels plays a supplementary role in induction of apoptosis during infections with the mutant viruses and may explain why the wt virus does not need to block caspase activation to protect against cisplatin-induced apoptosis. Additional mechanisms could be used by wt HSV-1 to compensate for the caspase-dependent decrease in Bcl-2, for example, by increased translation efficiency of the stabilized bcl-2 RNA.

Activation of caspases during infection with tsk and 27lacZ viruses may result from cytoplasmic release of cytochrome c induced by decreased bcl-2 RNA levels and destabilization of Bcl-2 protein by p38MAPK (see below). Cleavage of Bcl-2 by caspases may induce additional release of cytochrome c and further promote caspase activation as part of a positive feedback loop (18). Alternatively, our results favor activation of caspases through a cytochrome c-independent mechanism, since stabilized expression of Bcl-2 decreased cytosolic cytochrome c but not activation of caspase-3 and wt HSV-1 abolished cytochrome c release caused by cisplatin but not caspase-3-dependent PARP cleavage. Furthermore, stimulation of stress kinases in our model (Fig. 9) is independent of caspase activation, in contrast to other apoptotic systems reported previously (29).

The third apoptotic pathway induced during infection with the mutant viruses resulted in a p38MAPK-dependent destabilization of Bcl-2 protein (Fig. 9, pathway III). The p38MAPK stress kinase is activated during infection with tsk and 27lacZ mutant viruses (46) (step III1). Activated p38MAPK coimmunoprecipitated with Bcl-2, and stimulation of p38MAPK resulted in reduction of the Bcl-2 half-life by approximately 3.5 fold and of Bcl-2 levels by 40% (step III2). The remaining decrease in Bcl-2 protein levels is probably a consequence of the decreased Bcl-2 RNA and of the caspase-related degradation of the protein. Inhibition of p38MAPK destabilization of
Bcl-2 using the SB203580 compound (step III.) reduces DNA damage induced by the mutant viruses by approximately 40% and increased cell survival by approximately 30%. The fact that inhibition of p38MAPK did not completely restore Bcl-2 stability to the levels observed in mock-infected cells or in cells infected with the wt virus suggests that additional pathways contribute to the decreased Bcl-2 half-life. The JNK pathway is also activated by infection with HSV-1 mutants (46), and whether activated JNK acts through a mechanism similar to that for p38MAPK is under investigation. Phosphorylation of Bcl-2 is essential for the anti-apoptotic role of the protein (16) and inactivation of its anti-proliferative function (38). The ability of p38MAPK to phosphorylate Bcl-2 was demonstrated in vitro (23), and a recent report suggested inactivation of Bcl-2 via phosphorylation by the closely related ASK1/JNK pathway, activated at the G2/M phase of the cell cycle (45). Our results propose a role for p38MAPK in destabilizing Bcl-2 and blocking its anti-apoptotic functions during mutant HSV-1 infections. p38MAPK activity co-immunoprecipitates with Bcl-2 during infection of cells with the mutant viruses, suggesting that Bcl-2 interacts with a p38MAPK-related kinase. This interaction may be direct or could be mediated by a third component. It is also possible that p38MAPK activation results in subsequent stimulation of downstream proteins, which interfere with Bcl-2 stability.

Although p38MAPK is also activated during infection of cells with wt HSV-1 (46), no significant p38MAPK activity co-immunoprecipitated with Bcl-2, and the rate of degradation of the protein was the same as in mock-infected cells. Possibly, activation of p38MAPK during infection of cells with HSV-1 resulted in stimulation of two different populations, one involved in apoptotic processes and another that targets transcriptional factors. If the wt virus inhibits activation of the p38MAPK target proteins that lead to destabilization of Bcl-2 and allows only the p38MAPK-dependent activation of transcription factors to occur, that may explain the ability of wt HSV-1 to evade the p38MAPK-dependent decrease in Bcl-2 half-life while simultaneously using activation of p38MAPK to promote viral replication. If Bcl-2 is a direct target of p38MAPK, wt HSV-1 may interfere, for example, with the cellular localization of p38MAPK during infection, thus affecting the availability of the p38MAPK substrates. If interaction of Bcl-2 with p38MAPK is mediated by a third factor, this factor may be absent or inhibited during wt HSV-1 infections. The apoptotic pathway downstream of Bcl-2 includes mitochondrial dysfunction (release of cytochrome c to the cytoplasm), degradation of cellular DNA, and cell death. This is in accordance with observations for mutant virus d120 (lacking functional ICP4), which is reported to induce cytochrome c release and depolarization of the inner mitochondrial membrane (10). Recently published data indicated that mutant d120 induced caspase-dependent apoptosis in HEp-2 cells and that overexpression of Bcl-2 blocked d120-induced apoptosis without inhibiting caspase-3 activity (11). These data strongly support our findings regarding the central role of Bcl-2 in mutant HSV-1-induced apoptosis. In addition, our observations provide evidence for cellular mechanisms that target Bcl-2 during infection and for strategies employed by the wt virus to subvert programmed cell death.

Activation of p38MAPK during infection with wt HSV-1 enhanced viral growth by up to 60% (Fig. 9, pathway IV). Although this twofold increase is modest, it could be very important in vivo for the balance between viral growth and a cellular antiviral response or, at a low multiplicity of infection, when lower numbers of viral particles per cell are present. Furthermore, it is possible that in the presence of the p38MAPK inhibitor, other cellular pathways partially compensate for the loss of p38MAPK function and that a multiplicity of infection of 10 in our experiments masks certain aspects of the virus-host cell interactions. Thus, the contribution of p38MAPK in viral growth could be more important than twofold. A recent study reported that activation of the other stress kinase, JNK, also enhanced HSV-1 replication (26).

Inhibition of p38MAPK during wt HSV-1 infection resulted in decreased levels of specific viral proteins. Although this decrease is again modest, the effects of the p38MAPK inhibitor on HSV-1 protein levels are specific for certain viral proteins and highly reproducible. Activation of transcription of the respective viral gene promoters, presumably through activation of transcription factors downstream of p38MAPK, appears to be a mechanism for p38MAPK-dependent increases in viral protein levels. More direct measures of transcription activity, for example nuclear run-on assays, will be very important for verifying our results from the CAT assays. It is also possible that additional mechanisms, for example, increased stability of viral mRNAs, participate in p38MAPK-dependent upregulation of viral protein levels. Unfortunately, little is known about regulation of HSV-1 gene promoters by cellular transcription factors. Transcription factor AP-1 is a common target of JNK and is activated during HSV-1 infection (46). Infection with HSV-1 also induces translocation of NF-kB to the nucleus, and inhibition of this process reduces viral yield (31). Furthermore, HSV-1 stimulates accumulation and redistribution of E2F (27).

In conclusion, we propose that cellular kinase cascades and regulatory factors are manipulated in the infected cell and that these activities are important for the efficiency of viral proliferation.

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