Protection by Herpes Simplex Virus Glycoprotein D against Fas-mediated Apoptosis

ROLE OF NUCLEAR FACTOR κB*

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Signals involved in protection against apoptosis by herpes simplex virus 1 (HSV-1) were investigated. Using U937 monocytoid cells as an experimental model, we have demonstrated that HSV-1 renders these cells resistant to Fas-induced apoptosis promptly after infection. UV-inactivated virus as well as the envelope glycoprotein D (gD) of HSV-1, by itself, exerted a protective effect on Fas-induced apoptosis. NF-κB was activated by gD, and protection against Fas-mediated apoptosis by gD was abolished in cells stably transfected with a dominant negative mutant IκBα, indicating that NF-κB activation plays a role in the antiapoptotic activity of gD in our experimental model. Moreover, NF-κB-dependent protection against Fas-mediated apoptosis was associated with decreased levels of caspase-8 activity and with the up-regulation of intracellular antiapoptotic proteins.

Interest in the understanding of mechanisms by which viruses belonging to a variety of families regulate cell apoptosis has grown rapidly in recent years (1–3). Herpesviruses, due to the relatively large quantity of information contained in their genomes, seem particularly well equipped to exert a fine control over cell apoptosis (4). This occurs through various interactions among viral and cell products acting at different levels (5).

Among herpesviruses, herpes simplex viruses have been shown to regulate apoptosis of infected cells both positively and negatively, according to the presence or absence of specific genes, experimental conditions, or specificity of target cells (6–21).

Glycoprotein D (gD) is a main component of the external structure of HSV-1, and its function is essential for HSV-1 spread. Interaction between gD and cell receptors allows virion entry into cells to be infected (22–25). At least one of the cell receptors for gD, namely herpesvirus entry mediator A (HveA; also known as HVEM, TNFRSF14), belongs to the family of tumor necrosis factor receptors, which play a central role in mediating signal transduction leading to death receptor-associated apoptosis (26–28). Recent results have shown that gD delivered in trans blocks the apoptotic cascade triggered by HSV-1 mutants lacking the gene encoding gD in SK-N-SH cells (29, 30). Cellular signals involved in the antiapoptotic action exerted by HSV-1-gD remain to be elucidated. Interestingly, overexpression of the gD receptor HveA has been shown to cause activation of the transcription factor, NF-κB (28). Furthermore, it has been reported that engagement with HveA receptor of its natural ligand, LIGHT, can stimulate the activation of NF-κB in different cellular systems (31, 32). This suggests the possibility that also engagement of gD with HveA could lead to NF-κB activation. The transcription factor NF-κB consists of a homodimeric or heterodimeric complex of two subunits belonging to the highly conserved family of Rel-related proteins (33). The most important complex is that formed by two proteins with molecular masses of 50 kDa (p50) and 65 kDa (p65), respectively. This heterodimer is present in the cytoplasm of most cell types as an inactive form due to the inhibitory action of an associated protein, inhibitory κB (IκB). Activation of NF-κB, in response to a variety of stimuli, including viral infections, involves phosphorylation and degradation of IκB (34) and can be considered one of the first lines of defense against infections and cellular stress (33, 35, 36). In addition, it has been shown that NF-κB can act as an important regulator of apoptosis, through the induction of already identified or unidentified apoptosis-controlling genes, which exert either an inducing or a preventing action on cell death (37–40). In fact, it has recently been demonstrated that NF-κB signaling induces protection from anti-Fas-mediated apoptosis (41). Interestingly, it has been shown that HSV-1 infection activates NF-κB (42, 43). Particularly, it has been recently demonstrated that HSV-1 induces persistent activation of NF-κB by activating the IκB kinase in the early phase of infection of HEP-2 permissive cells (44). However, little information is available at the molec-
Protection against Apoptosis by HSV through NF-κB

Experimental Procedures

Cells and Virus—U937 cells, originally obtained from the Istituto Zooprolattifico (Brescia, Italy), were propagated in RPMI 1640 (HyClone, Cramlington, UK) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% FCS (HyClone Europe, Cramlington, UK) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% FCS (HyClone Europe). An F' strain of HSV-1, originally obtained from ATCC was used in these experiments. Virus stocks were produced in Vero cells propagated in minimal essential medium (HyClone Europe), containing 6% FCS, at 37 °C in a CO2 incubator. Virus stocks, collected and stored in aliquots at −80 °C, gave a titers of ~1 × 106 pfu/ml in Vero cells. In experiments requiring inactivated virus, virus suspension was placed in Petri dishes and exposed, for 150 s, to UV light at an intensity of 30 mW from a germicidal lamp situated 10 cm above the sample. UV-inactivated HSV-1 was used at a multiplicity of infection (MOI) of 50 pfu/cell. In cocultivation experiments, 1 × 104 U937 cells were cocultured with gD-transfected or control adherent cells at ~80% confluence, in 6-well plates. After 24 h, nonadherent U937 cells were collected from the supernatants and centrifuged at 800 × g. The pellets were resuspended at 1 × 106 cells/ml in fresh medium containing 1% FCS, and apoptosis was induced with anti-human Fas. In other experiments, conditioned medium from gD-transfected or control adherent cells, routinely maintained in RPMI 1640 with 10% FCS, was collected 18 h after infection and centrifuged at 800 × g to remove cellular debris. U937 cells were then suspended at 1 × 106 cell/ml in conditioned medium before inducing and testing apoptosis.

Experimental Infections—Approximately 6 × 104 U937 cells in 96-well plates were exposed to virus inoculum (HSV-1 or UV-inactivated HSV-1), 24 h after cultures had been split, at 4 °C for either 60 or 90 min. Subsequently, virus inoculum was replaced by fresh growth medium (RPMI 1640 containing 1% FCS), and the infected and control cells were then shifted to 37 °C.

Antibodies and Reagents—Anti-gD monoclonal antibody affinity-purified recombinant forms of gD, gD-I306(I4), or gD-I2(Δ290–299) were kindly provided by Dr. Gerard Cohen and Dr. Roselyn Eisenberg (University of Pennsylvania, Philadelphia, PA) (45). Anti-human XIAP rabbit polyclonal antibody and anti-human survivin were purchased from R&D Systems, Inc. Minneapolis, MN. 4′,6-diamidino-2-phenylindole-conjugated anti-human Fas, clone DX2, was purchased from BD PharMingen (San Diego, CA). Anti-IκBα rabbit polyclonal antibody sc-371, which recognizes an epitope mapping at the carboxyl terminus of human IκBα identical to the corresponding mouse sequence, and anti-human-IκBα rabbit polyclonal antibody H-85 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-human FLIP rabbit polyclonal antibody was purchased from ProfSci Inc. (Powy, CA). Human recombinant tumor necrosis factor-α was purchased from ICN (Costa Mesa, CA). Anti-human Fas antibody, clone CH11, was purchased from Zooprofilattico S.p.A. Brescia, Italy. Cells were infected and were then incubated at 4 °C for 1 h prior to the addition of anti-human Fas. Cultures were incubated for a further 1 h at 4 °C and then shifted to 37 °C.

Evaluation of Apoptosis—Apoptosis was evaluated using various techniques. Morphological analysis of the cells was performed following staining with acridine orange as previously described (11). Briefly, over 600 cells, including those showing typical apoptotic characteristics, were counted using a fluorescence microscope. The identification of apoptotic cells was based on the presence of uniformly stained nuclei showing chromatin condensation and nuclear fragmentation. Flow cytometry analysis of isolated nuclei, following staining with propidium iodide performed on a FACScan (Becton Dickinson) as described (46). Analysis of DNA fragmentation at the single cell level was carried out using the TUNEL technique, as described (11).

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared from either untreated cells or cells treated with supernatants from gD transfected or soluble forms of gD. Briefly, cells were resuspended in buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride), incubated on ice for 15 min, and then homogenized by 15 passages through a 25-gauge needle. The nuclei were collected by centrifugation at 1000 × g for 5 min at 4 °C and resuspended in buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 0.5 mM aprotinin, and 5 μg/ml apronitin). After 15 min of incubation on ice, the nuclear extracts were collected by centrifugation at 10,000 × g for 2 min. Nuclear extracts (5 μg of protein) were subjected to EMSA using a 32P-labeled xB DNA probe (47). The resulting DNA-protein complex was analyzed by nondenaturing 4% polyacrylamide gel electrophoresis. Quantitative evaluation of NF-κB binding was determined by analysis of a phosphorimager (Amer sham Biosciences). Specificity of the protein-DNA complex was verified by immunoactivity with polyclonal antibodies to p65 (Rel A) and, in competition experiments, with an excess of unlabeled oligodeoxynucleotide.

Construction of Stable Transfectants Expressing Constitutively HSV-1-gD and Mutant Murine IκBα—HSV-1-gD open reading frame, containing 14 bp upstream of the AUG codon and 32 bp downstream of the STOP codon, was PCR-amplified from HSV-1 DNA and cloned into the pPRneoCMV vector (48); the resulting plasmid was transfected into NIH3T3 cells. Transfected cells were grown under G418 (Invitrogen) selection, distributed into 24-well plates, and screened by immunofluorescence with HSV-1-gD-specific antibodies. Cells expressing different positive wells were mixed and amplified under G418 to obtain stable transfectants named IκBα. Dominant negative murine IκBα, mutated at serines 32, 36, 283, 288, 293 and at threonines 291 and 296, all substituted by alanine, was generated by polymerase chain reaction-based site-directed mutagenesis and was kindly provided by Dr. In der M. Verma (The Salk Institute, La Jolla, CA) (49). The gene was contained in a pcMX vector and was subcloned in a pcDNA 3.1 Neo vector (Invitrogen). The subcloning was done using the EcoRI sites. The construction was sequenced, and the mutations were verified. To obtain stable expression of mutant mouse IκBα (mIκBα) in the U937 cell line, 2 × 105 cells were plated in 60-mm dishes and grown in RPMI medium with 10% FCS and 1% penicillin-streptomycin. The next day, the cells were harvested, washed in sterile phosphate-buffered saline without calcium and magnesium and resuspended in fresh medium without serum and antibiotics. A volume of 12 μl of FuGene™ 6 transfection reagent (Roche Applied Science) was added to 185 μl of RPMI medium with neither serum nor antibiotics and then mixed with 3 μg of linearized pcDNA-mIκBα or pcDNA3.1(+) constructs, respectively. The DNA mixture was left under the hood at room temperature for 15 min and then added dropwise to each tissue culture dish. Mock-transfected cells were also included as a control. After 48 h, the cells were harvested, washed in phosphate-buffered saline, resuspended in complete RPMI medium containing 400 μg/ml G418, and seeded into 24-well plates at a concentration of 2 × 105 cells/well. G418 was replaced every 3 days for 3 weeks. Resistant cells deriving from three positive wells were mixed and amplified, maintaining constant G418 concentration and then tested for pcDNA-mIκBα or pcDNA3.1(+) expression.

Western Blot Analysis and Caspase-8 Activity Assay—A quantity of 5 × 106 cells was solubilized at 4 °C in lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 7.4, 1% Triton-X, NaCl 150 mM, 0.25% sodium deoxycholate, 1% Nonidet P-40, and, freshly added, 1 mM Na3VO4, 20 mM NaF completed with protease inhibitor mixture (Roche Applied Science) and centrifuged at 10,000 × g. An aliquot of the supernatant was saved for determination of protein concentration. The rest was boiled in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 10 mM dithiothreitol, 2% SDS, 0.1% Phenol blue, 10% glycerol), and then 50 μg of proteins were run on a 10% (mIκBα) or 12.5% (c-IAP2, FLIP, XIAP, and survivin) SDS-polyacrylamide gel. For detection of mIκBα, gels were transferred to a polyvinylidene difluoride membrane (PerkinElmer Life Sciences), whereas for detection of c-IAP2, FLIP, XIAP, and survivin, gels were transferred to a nitrocellulose membrane (Schleicher & Schuell). The membranes were first incubated with an antibody against mIκBα, then washed, and incubated with a peroxidase-conjugated goat anti-rabbit IgG (Calbiochem). Antibody binding was detected using a luminescence staining using the ECL detection kit (PerkinElmer Life Sciences). For presentation, immunoblots were digitally scanned at 600 dpi using Adobe Photoshop 5.0.

Caspase-8 activity was assayed using the ApoAlert colorimetric kit.
RESULTS

HSV-1 Renders U937 Cells Resistant to Fas-induced Apoptosis as an Early Event following Infection—We have previously demonstrated that U937 cells are sensitive to apoptosis induced by wild type HSV-1 (11). Here we show the kinetics of induction of apoptosis by HSV-1 in this cell line (Fig. 1A, bars), paralleled with the kinetics of virus yield released from infected cells in the same representative experiment (Fig. 1A, lines). Apoptosis was detected at remarkable levels only as a late event after infection, whereas it was practically absent during the early phase. Moreover, increasing values of virus yield confirmed that infection by HSV-1 in U937 monocytoid cells was a true productive one. Thus, we attempted to verify whether HSV-1 was able to protect against Fas-induced apoptosis early after infection in our experimental model. To this purpose, target cells were exposed to virus inoculum at an MOI of 50 pfu/cell, and an agonist anti-Fas antibody was added to the cells immediately after infection with virus inoculum (0 h) and at 1.5, 3, and 6 h following the end of exposure to virus. Apoptosis was detected in all cultures 20 h after infection. Results demonstrated that infection with HSV-1 inhibited Fas-mediated apoptosis, in a highly significant way (p < 0.001) in all experimental conditions tested (Fig. 1B).

Treatment with UV of HSV-1 Does Not Abrogate Inhibition of Fas-induced Apoptosis in U937 Cells—In order to understand whether completion of the replicative cycle of HSV-1 was a necessary step in the inhibition of Fas-induced apoptosis by the virus, we repeated our experiments using virus inocula inactivated by UV treatment. The cells were exposed for 1 h to UV-inactivated HSV-1 or, as a control, to UV-treated medium, and anti-Fas antibody was added at various times following the end of exposure. Results demonstrated that infection with HSV-1 inhibited Fas-mediated apoptosis, in a highly significant way (p < 0.01) in all experimental conditions tested (Fig. 1B).

Statistical Analysis—Data analysis was performed using the SPSS statistical software system (version 10.0 for Windows; Chicago, IL). Comparisons of means were carried out using the Student's t test for independent or paired samples and Tukey's honestly significant difference (HSD) test, as a multiple comparison and range test, where appropriate.
in order to verify whether gD, which is present in the virion envelope of HSV-1, could be responsible for the above described phenomena, we cocultured nonadherent U937 cells with adherent stable transfectants expressing gD at surface level for 24 h. At the end of coculture, nonadherent cells were harvested and centrifuged. Pellets were then suspended in fresh medium before inducing and testing, 18 h later, Fas-mediated apoptosis. Expression of gD was verified, prior to harvesting and centrifugation, by immunofluorescence. As controls, U937 cells were either left alone or cocultured with nontransfected Ig6D cells, before anti-Fas addition. Exposure to gD-expressing transfectants caused a highly significant inhibition of Fas-mediated apoptosis in U937 cells, in comparison with results obtained in noncocultured cells (p < 0.001) or in cells cocultured with control, non-gD-expressing cells (p < 0.002; Fig. 2). Moreover, similar results were obtained when U937 cells were exposed to supernatants collected from gD-expressing transfectants, which were found to contain detectable amounts of gD when tested by immunoblot (data not shown). In this case, Fas-mediated apoptosis was detected by flow cytometry analysis of hypodiploid nuclei following staining with propidium iodide. The percentage of hypodiploid nuclei from apoptotic cells was clearly inhibited by preincubation of U937 cells with supernatants collected from gD-expressing transfectants (35%, SUP/gD+, Fig. 3) compared with control cells cultured with medium alone (68%, CTR, Fig. 3) or with cells preincubated with supernatants from non-gD-expressing cultures (65%, SUP/gD−, Fig. 3). Moreover, no significant difference in the expression of surface Fas, between cells exposed to control treatments and cells exposed to gD transfectants or their supernatants, was found by flow cytometry analysis (data not shown).

**Treatment of U937 Cells with Soluble Forms of gD Protects against Fas-induced Apoptosis**—In order to confirm that exposure to gD of HSV-1 was sufficient by itself to protect against Fas-mediated apoptosis, we performed a series of experiments using soluble forms of recombinant gD. U937 cells were incubated for 1 h with a deleted-truncated gD-1(Δ290–299k) or a native-truncated gD-1(306k) form of baculovirus-produced gD (1 μg/ml) and, successively, for 1 h with anti-Fas (200 ng/ml) at 4°C. These recombinant forms of gD, which have been fully characterized, retain key biological properties of natural gD, including the capability to block virus entry and, important to our aims, to bind to HveA (50). Thus, we were interested in comparing their possible activity on modulation of Fas-mediated apoptosis. As a control, in preliminary experiments we used fetuin, a glycoprotein from fetal calf serum showing some similarities with HSV-1 gD (51). Fetuin did not show any protective effect on Fas-induced apoptosis in U937 cells. Fig. 4 shows the results of an experiment in which apoptosis of gD-1(306k)-treated cells stimulated with anti-Fas was evaluated by using the TUNEL technique and flow cytometry analysis following a further 20 h of incubation at 37°C. In this experiment, we obtained 28% positive apoptotic cells from the culture treated with gD-1(306k) plus anti-Fas (Fig. 4C) in comparison with 57% positive cells from a culture treated with anti-Fas only (Fig. 4B) and 2% positive cells from the control untreated culture (Fig. 4A). Similar results were obtained using gD-1(Δ290–299k) (data not shown), indicating a clear protective action of both recombinant forms of gD on Fas-induced apoptosis without any difference in their activity. In other experiments, apoptosis was detected by fluorescence microscopy following only a further 6 h of incubation at 37°C after exposure to soluble gD and anti-Fas treatment. Results of four independent experiments show that pretreatment with both recombinant forms of gD caused a reduction in Fas-mediated apoptosis that was highly significant even after this short time of incubation (Fig. 5).

**Treatment of U937 Cells with Supernatants from gD Stable Transfectants or Soluble Forms of gD Activates NF-κB**—The gD receptor HveA has been shown to transduce signals that can lead to NF-κB activation (28, 31, 32). As a consequence, we hypothesized the possible involvement of NF-κB in the protection by HSV-1-gD of Fas-mediated apoptosis in U937 cells. To assess this hypothesis, first U937 cells were incubated at 37°C with supernatants from gD stable transfectants, Ig6D, or with supernatants from I143tk− cells as a control. At different times after incubation, nuclear extracts were analyzed for NF-κB activation by EMSA. Levels of NF-κB-DNA binding were quantified by phosphorimagery analysis. NF-κB binding activity was rapidly up-regulated after incubation of U937 cells with conditioned medium from Ig6D in comparison with conditioned medium from I143tk− control cells (Fig. 6). An increase in NF-κB activity was seen as early as 30 min following the
cells were cultured for 24 h in the presence of conditioned medium from control, non-gD-expressing, I143tk/H11002 treatment groups, from fluorescence observed in sample thiocyanate-dUTP solution alone without TdT, did not differ, for all emissions, detected in control samples incubated with fluorescein isothiocyanate. Protection against Apoptosis by HSV through NF-κB (C) or medium plus a baculovirus-produced recombinant gD (1 μg/ml) and for another 1 h with the addition of anti-Fas antibody. Apoptosis was detected after a further 18-h incubation in the presence of anti-Fas (100 ng/ml) in cells preincubated with conditioned medium and in untreated, control cells (CTR), by flow cytometry analysis of hypodiploid nuclei isolated and stained with a hypotonic solution containing detergent and propidium iodide. The dashed lines indicate the boundaries among the peaks of negative emotions, detected in hypodiploid nuclei isolated and stained with a hypotonic solution containing detergent and propidium iodide. The dashed lines indicate the boundaries among the peaks of negative apertic nuclei, which were arbitrarily set on control samples and maintained for all other samples. The numbers in the cytograms represent the percentages of hypodiploid, apoptotic nuclei, based on the total number of nuclei from which debris was excluded. Similar results were obtained in four other independent experiments in which apoptosis was evaluated using fluorescence microscopy.

addition of supernatants, reaching a 3-fold induction after 60 min (Fig. 6A). The NF-κB complex was not formed by nuclear extracts derived from U937 cells incubated with either supernatants from I143tk− control cells or medium alone, strongly suggesting the involvement of HSV-1 gD in triggering signaling, leading to NF-κB activation. To confirm the specificity of HSV-1 gD in initiating NF-κB signaling, U937 cells were treated with different concentrations of soluble forms of recombinant gD proteins. Results obtained using the deleted-truncated form of gD after 60 min of incubation are illustrated (Fig. 6B). The recombinant soluble gD was found to stimulate the transcriptional factor at levels above those seen in control cells. The stimulatory effect (2–3-fold increase) was reproducible, clearly detectable, and similar to that observed using supernatants from gD transfectants. Similar effects were obtained when the native truncated form of recombinant gD was utilized (data not shown). From these experiments, we concluded that gD is per se able to trigger NF-κB activation following interac-
tion with target cells, suggesting that protection against Fas-mediated apoptosis by HSV-1 gD could be related to antiapoptotic signals initiated by NF-κB.

**Dominant Negative Inhibition of NF-κB Activity Abolishes Protection against Fas-mediated Apoptosis by UV-inactivated HSV-1 as Well as by Soluble Forms of gD**—In order to confirm the involvement of NF-κB activation in the protective effect of HSV-1 gD against apoptosis initiated by Fas, we stably transfected U937 cells with either the empty vector pcDNA3.1, as a control, or a vector expressing a murine mutant inhibitor-κBα protein, which acts as a dominant negative (DN) for human NF-κB activation. This mutant murine IκBα protein binds to human NF-κB but is not degraded in response to cellular activation. As a consequence, irreversible complexes are formed in the cytoplasm, and translocation of active NF-κB to the nucleus is prevented. Expression of either the pcDNA-mIκBα vector or the empty vector in stably transfected U937 cells was tested and confirmed by reverse transcription-PCR (data not shown). Ectopic, murine protein expression was also confirmed in transfected cells by Western blot analysis (Fig. 7A). In fact, the DN murine IκBα prevented NF-κB activation triggered by structural components of HSV-1, as clearly shown by EMSA binding experiments in parallel on control transfectants and on pcDNA-mIκBα transfectants exposed to UV-inactivated HSV-1 for 1 h (Fig. 7B). Moreover, we tested whether the block in NF-κB signaling rendered our DN transfectants more susceptible to tumor necrosis factor-α-induced apoptosis, as previously demonstrated in a number of cell types expressing a DN IκBα (38). In fact, our pcDNA-mIκBα stable transfectants were highly sensitive to tumor necrosis factor-α-induced apoptosis in comparison with control transfectants, as expected (Fig. 7C). We then asked whether DN inhibition of NF-κB signaling had abolished protection against Fas-mediated apoptosis by UV-inactivated HSV-1 as well as by soluble forms of gD. Control and NF-κB-DN transfectants were either left untreated or exposed for 1 h to UV-inactivated HSV-1 and then cultured for further 18 h with or without the addition of anti-Fas before detection of apoptosis. Expression of mIκBα repressed the ability of UV-inactivated HSV-1 to inhibit Fas-mediated apoptosis detected by flow cytometry analysis of hypodiploid nuclei (mIκBα; Fig. 7D). In contrast, control transfectants maintained their capacity to undergo reduced Fas-mediated apoptosis following exposure to UV-inactivated HSV-1 (pcDNA; Fig. 7D). Similarly, expression of mIκBα, but not of empty vector, completely repressed the ability of the two soluble forms of gD utilized in our experiments to reduce Fas-mediated apoptosis as detected by fluorescence microscopy (Fig. 7E). HveA expression was not altered in DN mIκBα transfectants, as assayed by flow cytometry (data not shown). These experiments indicate that NF-κB activation is required for protection against Fas-mediated apoptosis by gD of HSV-1 in U937 cells.

**NF-κB-dependent Protection against Fas-mediated Apoptosis by Structural Components of HSV-1 Occurs at Caspase-8 Apical Level and Is Associated with Up-regulation of Intracellular Antiapoptotic Proteins**—In order to obtain clues about the molecular mechanisms involved in the NF-κB-dependent anti-apoptotic action of HSV-1-gD, we investigated the role of caspase-8 and of some NF-κB-responsive prosurvival genes.

To ascertain whether inhibition of Fas-mediated apoptosis by structural components of HSV-1 could act at the apical level in the caspase cascade triggered by Fas, we determined the caspase-8 protease activity in control transfectants and in DN mIκBα transfectants either untreated or treated for 1 h with UV-inactivated HSV-1 alone, with anti-Fas alone, and with UV-inactivated-HSV-1 for 1 h followed by anti-Fas. At 18 h after the addition of anti-Fas, a ~5-fold increase in caspase-8 activity, with respect to cells unstimulated with anti-Fas, was observed in control transfectants treated with anti-Fas alone (Fig. 8A). Conversely, no significant change in caspase-8 activity was observed in control transfectants treated with UV-inactivated-HSV-1 alone, whereas an increase of about 50% of that observed in cells treated with anti-Fas alone was detected.

![Diagram](http://www.jbc.org/Downloadedfrom/hp://www.jbc.org/dg)
Fig. 7. Effects of prevention of NF-κB activity by dominant negative mutant murine IκBα expression on inhibition of Fas-mediated apoptosis by UV-inactivated HSV-1 and HSV-1-gD. U937 cells were stably transfected with either an empty, control vector (pcDNA) or a vector containing a dominant negative murine mutant inhibitor-IκBα gene (mIκBα). A, ectopic expression of the mIκBα gene in U937 cells determined by Western blot analysis. Proteins extracted from wild type U937 cells or from mIκBα-transfectants by lysis buffer were separated by SDS-PAGE electrophoresis, transferred to membrane, and immunoblotted with a commercially available antibody that recognizes both human and murine IκBα. Visualization by ECL shows a single band in wild type U937 cells (lane 1) and two bands in mIκBα-transfectants (lane 2), the upper band corresponding to human IκBα and the lower band to murine IκBα, respectively. B, control transfectants and mIκBα-expressing transfectants were exposed to UV-inactivated-HSV-1 and cultured for 60 min before nuclear extracts were prepared and assayed for NF-κB activation by EMSA, as described under "Experimental Procedures." The positions of NF-κB-DNA (NF-κB-DNA) and nonspecific protein-DNA (ns) complexes are indicated. C, control transfectants or mIκBα-expressing transfectants were treated with human recombinant tumor necrosis factor-α (20 ng/ml) and incubated for 18 h in 1% FCS medium. Apoptosis was then evaluated using fluorescence microscopy by calculating the percentage of cells showing nuclear morphology of apoptosis after staining with acridine orange. Results are expressed as mean values ± S.D. obtained from three replicate cultures in one of the two experiments performed with similar results. D, apoptosis, evaluated using flow cytometry analysis of hypodiploid nuclei isolated and stained with a hypotonic solution containing detergent and propidium iodide, in control transfectants or mIκBα-expressing transfectants treated with medium alone (untreated), with UV-inactivated HSV-1 alone (UV-HSV-1), with anti-Fas alone (anti-Fas), or with UV-inactivated HSV-1 plus anti-Fas alone (UV-HSV-1 + anti-Fas). Apoptosis was detected 18 h after the end of exposure for 1 h to UV-inactivated HSV-1 and the addition of anti-Fas (100 ng/ml). The lines designated as M1, M2, and M3 indicate the boundaries among the peaks of diploid (M3) and hypodiploid (M2) nuclei and presumably of debris (M1), which were arbitrarily set on untreated samples and maintained for all other samples. The percentages of hypodiploid, apoptotic nuclei, based on the total number of nuclei from which debris was excluded, were as follows: pcDNA, untreated 12.99%, UV-HSV-1 14.08%, anti-Fas 85.41%, UV-HSV-1 + anti-Fas 38.24%; mIκBα, untreated 11.68%, UV-HSV-1 15.23%, anti-Fas 79.81%, UV-HSV-1 + anti-Fas 72.09%. Similar results were obtained in three independent experiments. E, control transfectants or mIκBα-expressing transfectants were precultured for the first 1 h in the presence of 1% FCS control medium (ctr) or a deleted-truncated form (gDΔ290–299t) or native-truncated form (gDΔ306t) of baculovirus-produced recombinant gD (1 μg/ml) and for another 1 h with the addition of anti-Fas antibody. Apoptosis, evaluated as described for C, was detected following a further 6 h incubation in 1% FCS medium with anti-Fas antibody at 37 °C. Results are expressed as mean values ± S.D. obtained from three replicate cultures in one of the three experiments performed with similar results. Multiple comparisons by the Tukey’s HSD test gave the following results: pcDNA-transfectants, p < 0.001 between both the two gD-treated groups and the control group, not significant between the two gD-treated groups; mIκBα-transfectants, not significant among all groups.
in control transfectants treated with UV-inactivated-HSV-1 plus anti-Fas (pcDNA; Fig. 8A). A similar reduction of anti-Fas-induced caspase-8 activity, following exposure to UV-inactivated-HSV-1, was not observed in DN transfectants (mlkβB; Fig. 8A). These results indicate that the inhibition in apoptotic signaling following Fas engagement exerted by HSV-1 structural components, through NF-κB activation, is already detectable at the caspase-8 level.

The NF-κB family of transcription factors promotes the expression of a variety of genes, including some that exert antiapoptotic effects (52). We then asked whether NF-κB activation by HSV-1 structural components was associated with the up-regulation of some of these NF-κB-responsive survival genes. To answer this question, we determined intracellular levels of c-IAP2, FLIP, XIAP, and survivin proteins by Western blot analysis, in control transfectants and in DN mlkβB transfectants, either mock-treated or treated for 1 h with UV-inactivated-HSV-1. Cells were harvested after a further 30 min or 4 h of incubation. Cellular contents of total proteins were controlled in all samples in order to dispense exactly the same amounts of proteins from mock-treated and UV-HSV-1-treated samples. Western blot analysis revealed a detectable, up-regulation of c-IAP2, FLIP, and survivin, but not of XIAP, in control transfectants treated with UV-inactivated-HSV-1 in comparison with mock-treated cells as early as at 30 min of incubation, after treatment (pcDNA, 30 min; Fig. 8B). The intracellular up-regulation of the antiapoptotic proteins was confirmed at 4 h of incubation, with the induction of FLIP more evident than at 30 min of incubation (pcDNA, 4 h; Fig. 8B). DN inhibition of NF-κB signaling abolished the up-regulation of the prosurvival proteins by UV-inactivated HSV-1 (mlkβB; Fig. 8B). Actually, FLIP was not at all detectable in DN mlkβB transfectants, either mock-treated or treated with UV-inactivated-HSV-1. Similar results were obtained in two independent experiments. From these experiments, we concluded that the interaction of HSV-1 viral particles with the cell is sufficient by itself to promptly trigger some antiapoptotic genes and that NF-κB activation is required for this action.

**DISCUSSION**

Results of several studies by different groups led to the concept that HSV-1 has the ability to both prevent and induce apoptosis in infected cells. This dual effect of the virus can be alternatively detected under particular experimental conditions (6, 8, 9, 10, 11, 13, 14, 17, 21). Here we demonstrate that HSV-1 renders U937 cells resistant to induction of apoptosis through Fas death receptor although transiently and under appropriate experimental conditions. Thus, HSV-1 infection of U937 cells is characterized by an initial phase, during which inhibition of apoptosis is predominant, followed by a successive phase during which induction of apoptosis occurs. A similar, biphasic model for regulation of apoptosis has been proposed by us for the permissive infection caused by bovine herpesvirus 4, a γ-2-herpesvirus (53).

Regarding the viral mechanisms involved in the protection against Fas-mediated apoptosis by HSV-1, results of our experiments using UV-inactivated virions induced us to focus our attention on the structural components of HSV-1. Among these, gD was, by itself, sufficient to reproduce the antiapoptotic action exerted by entire virions. This observation is consistent with recent studies published by other authors using different experimental models. In fact, Zhou et al. (29, 30) found that induction of HSV-1 gD expression in permissive SK-N-SH cells blocked apoptosis in infected cells with either gD (−/−) or gD (−/−) virus, whereas Aubert et al. (19) reported that accumulation of gD was associated with the inhibition of apoptosis by HSV-1. However, no evidence for prevention of apoptosis mediated by death receptors was reported by these authors. Obviously, the fact that gD has a primary role in inhibiting host cell death during the early phase of HSV-1 infection does not exclude that herpes simplex viruses have also selected other mechanisms to prevent or delay apoptosis of infected cells. In fact, it is plausible that following an early antiapoptotic signal triggered by gD, successive HSV-1 gene products could act in a cascade manner to maintain a state of apoptosis prevention. Particularly early and leaky-late proteins as well as cellular antiapoptotic products induced by HSV, as suggested by several experimental results (10, 11, 16, 17, 19, 20), could exert these functions. Moreover, also other glycoproteins of HSV-1 could exert inhibitory actions on Fas-induced apoptosis, as recently reported (54).

Another novel finding reported in the present paper is the identification of gD as an HSV-1 component able to initiate, by itself, NF-κB activation. In the past, several reports have illustrated the capacity of HSV-1 to trigger NF-κB activation following infection (42–44). A possible role for gD as a trigger in NF-κB activation could be predicted, considering that HveA,
which acts as a cell receptor for gD binding, activates NF-κB through interaction with members of the tumor necrosis factor receptor-associated factor family (28) and following the engagement of its natural ligand, LIGHT (31). Here we show that exposure of target cells to UV-inactivated HSV-1 supernatants containing gD or soluble forms of recombinant, purified gD is sufficient to activate NF-κB, presumably through signals transduced by the HSV-1 receptor HveA.

Viruses belonging to various families have been shown to possess gene products able to activate NF-κB. Activation of NF-κB by some of these viruses has been associated with the prevention of apoptosis or the masking of the ability of the virus to mediate apoptosis (55–57). Similarly, the activation of NF-κB seems to be associated with inhibition of apoptosis by HSV-1. In this paper, we have also addressed the issue of how activation of NF-κB transcription factor by HSV-1-gD regulates Fas-mediated apoptosis. We have shown that the inhibition in the apoptotic signaling triggered by Fas, following contacts with UV-inactivated HSV-1, is detectable early in the signal cascade (i.e. at the apical caspase-8 level) and that this is also an NF-κB-dependent action. Moreover, we have shown that UV-inactivated viral particles induce the up-regulation of some well known antiapoptotic proteins, such as FLIP, c-IAP2, and NF-κB cascade (with UV-inactivated HSV-1, is detectable early in the signal Fas-mediated apoptosis. We have shown that the inhibition in the action of antiapoptotic NF-κB, presumably through signals transduced by the HSV-1 receptor HveA.

The definition of this cascade of signals contributes to the understanding of the complex HSV-host interactions. More-
Protections by Herpes Simplex Virus Glycoprotein D against Fas-mediated Apoptosis: ROLE OF NUCLEAR FACTOR κB
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