JOURNAL OF INTERFERON & CYTOKINE RESEARCH 27:365–376 (2007)

DOI: 10.1089/jir.2006.0143

Herpes Simplex Virus Blocks Fas-Mediated Apoptosis Independent of Viral Activation of NF-κB in Human Epithelial HEp-2 Cells

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ABSTRACT

The goal of our study was to characterize the apoptotic response of herpes simplex virus (HSV)-infected, human epithelial HEp-2 cells to extrinsic treatments through the Fas receptor. Initially, we defined the Fas response of these cells. We found the following: (1) Treatment of HEp-2 cells with anti-Fas antibody or Fas ligand (FasL) alone did not induce apoptosis. (2) In addition, these inducers did not activate NF-κB in these cells. (3) The addition of cycloheximide (CHX) during these treatments caused a dramatic increase in programmed cell death. (4) HEp-2 cells infected with HSV for 6 h prior to anti-Fas plus CHX treatment were nonapoptotic, and (5) these cells possessed nuclear NF-κB. (6) HSV blocked anti-Fas or FasL plus CHX-induced apoptosis in HEp-2 cells that stably expressed a dominant-negative form of IκBα. These results indicate that HSV infection can block the process of Fas-mediated apoptosis through a mechanism that is independent of viral activation of NF-κB. Our findings help define the molecular mechanisms involved in HSV evasion of the cytokine-driven, innate immune response in human epithelial cells.

INTRODUCTION

APOPTOSIS IS A HIGHLY REGULATED process of cell suicide induced by death signals received by either the mitochondria (intrinsic pathway) or a cell surface death receptor (extrinsic pathway) (reviewed in ref. 1). Typical examples of extrinsic pathway cell surface death receptors are Fas (APO-1 or CD95) and tumor necrosis factor receptor (TNFR). Fas and TNFR are both members of a superfamily containing type 1 transmembrane proteins.[2,3] Apoptotic signaling through these receptors stimulated by Fas ligand (FasL) or TNF-α, respectively, requires recruitment of such adaptor proteins as FADD or TRADD to induce activation of caspase-8.[4–9] Some typical characteristics of cells undergoing apoptosis include cell rounding and shrinkage, membrane blebbing, condensed chromatin, DNA fragmentation, and cleavage of the DNA repair enzyme, poly(ADP-ribose) polymerase (PARP).[1,10–12]

Herpes simplex virus type 1 (HSV-1) is a large, enveloped, double-stranded DNA (dsDNA) virus with a genome that is approximately 152 kbp in size, encoding over 80 proteins (reviewed in ref. 13). The completion of the HSV-1 replication cycle ultimately leads to morphologic and biochemical changes collectively referred to as cytopathic effect (CPE). These changes include cell rounding, chromatin margination, nucleolar alterations, and a decrease in cellular macromolecular synthesis.[13] However, numerous recent studies have concluded that during HSV-1 infection, the virus first induces and then blocks the process of apoptosis in human cells (reviewed in ref. 14), which contributes to its CPE.[15] During HSV-1 infection, the cellular transcription factor NF-κB becomes activated, participates in apoptosis prevention, and is therefore needed for efficient viral replication.[16–21] In addition, the accumulation of infected cell proteins at late times during infection facilitates the prevention of apoptosis, which is exogenously triggered by certain environmental stimuli, including TNF-α.[16,22–26] This latter effect plays a significant role in viral evasion of the innate immune response (reviewed in ref. 27).

The prototypical cell system for the analysis of HSV-1-dependent apoptosis is human epithelial HEp-2 cells.[19,22,25,26,28–32] Surprisingly, their cellular response through the Fas receptor has not been investigated in detail. Therefore, our initial goal was to define the Fas-dependent response of HEp-2 cells. Next, we characterized the response of HSV-1-infected HEp-2 cells to Fas signaling. We show that treatment

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with either anti-Fas antibody or FasL did not activate NF-κB or induce apoptosis in these cells. However, cotreatment with cycloheximide (CHX) was sufficient to induce cell death. HSV-1-infected cell proteins produced prior to 6 h postinfection (hpi) prevented apoptosis induced by either FasL or anti-Fas plus CHX through a mechanism that was independent of NF-κB activation. Taken together, our results imply that the ability of HSV-1 to block the innate immune response through the Fas receptor is not absolutely dependent on activated NF-κB. These findings have important implications in the design of antiviral therapeutics that target the NF-κB pathway.[17]

MATERIALS AND METHODS

Cell lines and viruses

All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS). Human carcinoma HEp-2[33] and African green monkey kidney Vero cells[34] were obtained from the American Type Culture Collection (Rockville, MD). HSV-1(F), the wild-type strain[35] used in these studies,[22] was cultured and titered on Vero cells. IκBαDN cells are a derivative HEp-2 cell line that stably expresses a dominant-negative form of IκBα under the control of the human Ef1α promoter, as well as a puromycin resistance gene,[16] and were maintained in 5% FBS containing 400 μg/mL puromycin (Sigma, St. Louis, MO). Cell monolayers ($\sim 2.0 \times 10^6$), maintained for at least 24 h in DMEM containing 5% newborn calf serum (5% NCS), were infected at multiplicities of infection (moi) of 10 plaque-forming units (PFU)/cell in 199 medium containing 2% FBS (199V) for 1 h at 37°C. Following viral adsorption, the infected cells were again maintained in 5% NCS for the various times indicated in the text. Unless stated otherwise, all cell culture reagents were obtained from Life Technologies (Gaithersburg, MD).

Treatment with anti-Fas, FasL, and TNF-α

Anti-Fas antibody (anti-Fas), which recognizes the human cell surface receptor Fas,[36] and membrane-bound, vesicular[37] FasL were both purchased from Upstate Biotechnology. Anti-Fas was diluted in phosphate-buffered saline (PBS) containing 50% glycerol to a final concentration of 25 μg/mL and stored at −20°C. Lyophilized FasL was reconstituted in sterile distilled H_2O. Cells were maintained in the presence of treatment agents until the indicated times of harvest. To inhibit *de novo* protein synthesis of anti-Fas-treated and FasL-treated or HSV-1-infected cells, CHX (Sigma) was added to the medium of HEp-2 monolayers at a final concentration of 10 μg/mL. This concentration was shown previously to be sufficient to completely block viral protein synthesis in HSV-1-infected HEp-2 cells.[28] The times selected for CHX treatments of the HSV-1-infected cells, 3 and 6 hpi, correspond to the boundaries of the previously described apoptosis prevention window, within which antiapoptotic infected cell proteins are produced during HSV-1 infection.[22] TNF-α (Sigma) (10 ng/mL) and CHX (10 μg/mL) were added to HEp-2 cells as a positive control for the nuclear translocation of NF-κB and apoptosis induction. Lyophilized TNF-α was dissolved in sterile PBS containing 1% bovine serum albumin (BSA) and added directly to the medium (5% NCS) of HSV-1 infected or uninfected HEp-2 cell monolayers. Cells were maintained in the presence of treatment agents until the times indicated.

Preparation of whole, nuclear, and cytoplasmic cell extracts

Infected or treated cells were harvested by scraping directly into medium. Cellular material was pelleted by low-speed centrifugation (800*g* for 5 min). Pellets were resuspended in 300 μl PBS containing 10 μM concentrations of each protease inhibitor, PBS*, N-tosyl-L-phenylalanine-chloromethylketone (TPCK), phenylmethylsufonyl fluoride (PMSF), and tosyl-L-lysine-chloromethylketone (TLCK), and separated into two 1.5 mL tubes. One tube was designated for whole cell extract preparation and the other for nuclear and cytoplasmic fractionation. Cells were pelleted again by centrifugation at 1000*g* for 3 min. Whole cell pellets were resuspended in 150 μL of a lysis solution (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA), sonicated for 2×10 sec at an output level of 2 with a Branson sonifier, and frozen at −80°C. Cells to be fractionated were resuspended in 150 μL PBS* containing 0.4% NP-40. Tubes were inverted gently, incubated on ice for 10 min, and centrifuged again at 1000*g* for 3 min. Supernatants (cytoplasmic fractions) were transferred to fresh 1.5-mL tubes. Nuclear pellets were resuspended in 150 μL PBS* containing 0.1% NP-40 and centrifuged for 3 min at 1000*g*. Washed pellets were resupended in 150 μL of 0.4% NP-40 in PBS* and sonicated on ice for 3×5 sec. Nuclear and cytoplasmic extracts were stored at −80°C.

Denaturing gel electrophoresis and immunoblotting

Approximately 50 μg of infected or treated cell proteins from whole cell extracts or subcellular fractions were electrophoretically separated in 15% sodium dodecyl sulfate-polyacrylamide gels cross-linked with N,N′-diallyltartardiamide (Sigma) and electrically transferred to nitrocellulose using a tank apparatus (Bio-Rad, Hercules, CA) for 2 h at 100 V, prior to probing with various primary antibodies. Nitrocellulose was obtained from Schleicher & Schuell (Keene, NH). Protein molecular weight markers (not shown in figures) were purchased from Bio-Rad. Blots were incubated at 4°C in primary antibodies (1 h for viral proteins and 12–18 h for cellular proteins). Antimouse, antigoat, or antirabbit secondary antibodies conjugated to alkaline phosphatase were obtained from Southern Biotechnology (Birmingham, AL). Specific proteins were detected following development with x-phosphate/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 4-nitroblue tertrazolium chloride (NBT) (Roche, Nutley, NJ). Antimouse, antirabbit, or antigoat secondary antibodies conjugated to horseradish peroxidase (HRP), as well as reagents for development by chemiluminescence (ECL or Lumi-light) were supplied by Amersham (Arlington Heights, IL). Blots were incubated in secondary antibodies for 45 min to 1 h at 25°C. All immunoblots and autoradiograms were digitized to 300 dots per inch using an AGFA Arcus II scanner linked to a Macintosh PowerPC workstation. Raw digital images were saved as 8-bit gray-scale tagged image files (TIFF) using Adobe Photoshop and organized into figures using Adobe Illustrator.

Immunologic reagents

The following primary antibodies used to detect viral and cellular proteins were diluted in PBS containing 1% BSA: (1) H1114 mouse anti-ICP4 monoclonal antibody (mAb) (Goodwin Institute for Cancer Research, Plantation, FL), 1:1000, (2) mouse anti-PARP mAb (PharMingen, San Diego, CA), which recognizes both the full-length 116,000 molecular weight form and the 85,000 molecular weight cleavage product, 1:2000, (3) mouse anti-α-tubulin mAb (Sigma), used as a control for cytoplasmic fractions, 1:1000, (4) mouse mAb specific for the p65 subunit of NF-κB (Santa Cruz, Santa Cruz, CA), 1:1000, (5) goat antilamin polyclonal antibody (Santa Cruz), used as a control for nuclear fractions, 1:1000, and (6) mouse anti-IκBα mAb (Santa Cruz), 1:500. For indirect immunofluorescence, the anti-NF-κB antibody was used at a dilution of 1:500 in PBS containing 1% BSA, and FITC-conjugated goat antimouse IgG (Boehringer Mannheim, Indianapolis, IN), diluted 1:300, was used as the secondary antibody.

Indirect immunofluorescence and microscopy

Indirect immunofluorescence experiments were performed as previously described.[16,38] Infected or treated cells, initially grown on glass coverslips in 35-mm dishes, were washed with PBS and fixed using 2.5% methanol-free formaldehyde (Polysciences, Warrington, PA) in PBS. After 20 min incubation at 25°C, cells were washed twice with PBS, permeablized with 100% acetone at −20°C for 4 min, and washed twice again in PBS. Infected cells were blocked with PBS containing 1% BSA and 10 μg/mL pooled human immunoglobulin (Sigma) for 1 h at 4°C. Fixed, blocked cells were incubated with primary antibodies, diluted in 1% BSA, for at least 1 h. Cells were washed twice with PBS and incubated for 45 min in secondary antibody in 1% BSA. All incubations with antibodies were conducted in the dark at 25°C. Finally, cells were washed in PBS and mounted onto glass microscope slides using Prolong Antifade reagent as a mounting medium (Molecular Probes, Eugene, OR). Slides were then maintained in the dark at 4°C for at least 24 h. For visualization of chromatin condensation, 0.05 mg/mL of Hoechst 33258 (Sigma) was added to the medium overlying cells 1 h prior to preparation for fluorescence microscopy. Fluorescence was observed with an Olympus IX70/IX-FLA inverted microscope, and images were acquired using a Sony DK-5000 digital photo camera. Percentages of apoptotic cells were determined by dividing the number of cells with condensed chromatin by the total number of cells (at least 300 cells for each condition) multiplied by 100.

RESULTS

The extrinsic activator TNF-α is a cytokine that both induces apoptosis and activates NF-κB. In human epithelial HEp-2 cells, the cytotoxic effects of TNF-α are only manifested during cotreatment with CHX. Because CHX prevents the synthesis of IκBα, NF-κB's inhibitor, NF-κB cannot be retained in the cytoplasm, and it remains in the nucleus. We previously explored the inhibition of TNF-α plus CHX-induced apoptosis by HSV-1 and showed that infected cell proteins produced prior to 6 hpi, which include NFκB,[16] are necessary to prevent this process from killing the infected cell.[22] Unlike TNF-α, FasL and anti-Fas treatments have been reported to initiate a signal transduction cascade that results in the recruitment of the initiator caspase, caspase-8, without activation of NF-κB.[39,40] The goal of these studies is to document to response of prototypical HEp-2 cells to signaling through Fas.

Anti-Fas antibody and FasL do not induce nuclear translocation of NF-κB in HEp-2 cells

Human epithelial HEp-2 cells are used almost exclusively for assessing apoptosis during HSV-1 infection,[22,25,26,28–32] and NF-κB participates in apoptosis prevention in this system.[14,16,19,20] The consequences of Fas receptor activation on cell signaling have been debated and still remain somewhat controversial.[39–43] The first issue we addressed was whether anti-Fas antibody (anti-Fas) and FasL treatments of HEp-2 cells lead to activation of NF-κB in these cells. Two series of experiments were performed. In the first set, HEp-2 cells were treated for 24 h with increasing amounts (25–75 ng) of anti-Fas, nuclear and cytoplasmic fractions were prepared, and immune reactivities using an anti-NF-κB antibody were examined as described in Materials and Methods. Controls included untreated cells and cells exposed to TNF-α plus CHX. In all anti-Fas-treated HEp-2 cells, NF-κB remained predominantly cytoplasmic (Fig. 1A, lanes 5, 7, 9). In contrast, a significant amount of activated nuclear NF-κB was observed in control TNF-α plus CHX-treated cells (lane 3). These results suggest that anti-Fas does not have the same ability to activate NF-κB as does TNF-α in HEp-2 cells.

NF-κB shuttles between the cytoplasm and nucleus.[44,45] In the second set of experiments, we set out to prevent this from happening and to confirm that NF-κB was not being activated. Duplicate sets of HEp-2 cells were treated with anti-Fas or FasL in either the presence or absence of CHX. As controls, cells were either left untreated or treated with TNF-α, CHX, or a combination of the two. In the case of TNF-α, cotreatment with CHX prevents the synthesis of IκBα, and NF-κB remains unbound to this inhibitor so it localizes to the nucleus.[46] For one set of cells (Fig. 1B), nuclear and cytoplasmic extracts were prepared at 20 h, and NF-κB immune reactivities were measured following immunoblotting. The integrities of the subcellular fractions were assessed using antilamin (nuclear marker) and antitubulin (cytoplasmic marker) antibodies. In the other set (Fig. 1C), NF-κB subcellular localizations were visualized by indirect immunofluorescence at 2 h posttreatment as described in Materials and Methods.

In cells treated with TNF-α alone, NF-κB was detected in nuclei only at 2 h and not at 20 h (compare Fig. 1C with Fig. 1B, lane 5 and 6) because of shuttling, as predicted.[46] NF-κB was predominantly cytoplasmic in untreated and CHX-treated cells (Fig. 1B, lanes 1–4, and Fig. 1C). As expected, NF-κB was nuclear in TNF-α plus CHX-treated cells (Fig. 1B, lane 5, 6, and Fig. 1C); the presence of tubulin in the nuclear fractions at 20 h was likely due to the fact that these cells were undergoing cell death.[16] Consistent with the results in Figure 1A, NF-κB predominated in the cytoplasm of anti-Fas-treated cells (Fig. 1B, lanes 9, 10, and Fig. 1C). Little to no nuclear NF-κB was observed in FasL-treated cells (Fig. 1B, lanes 13, 14). Based on these results, we conclude that neither anti-Fas nor FasL

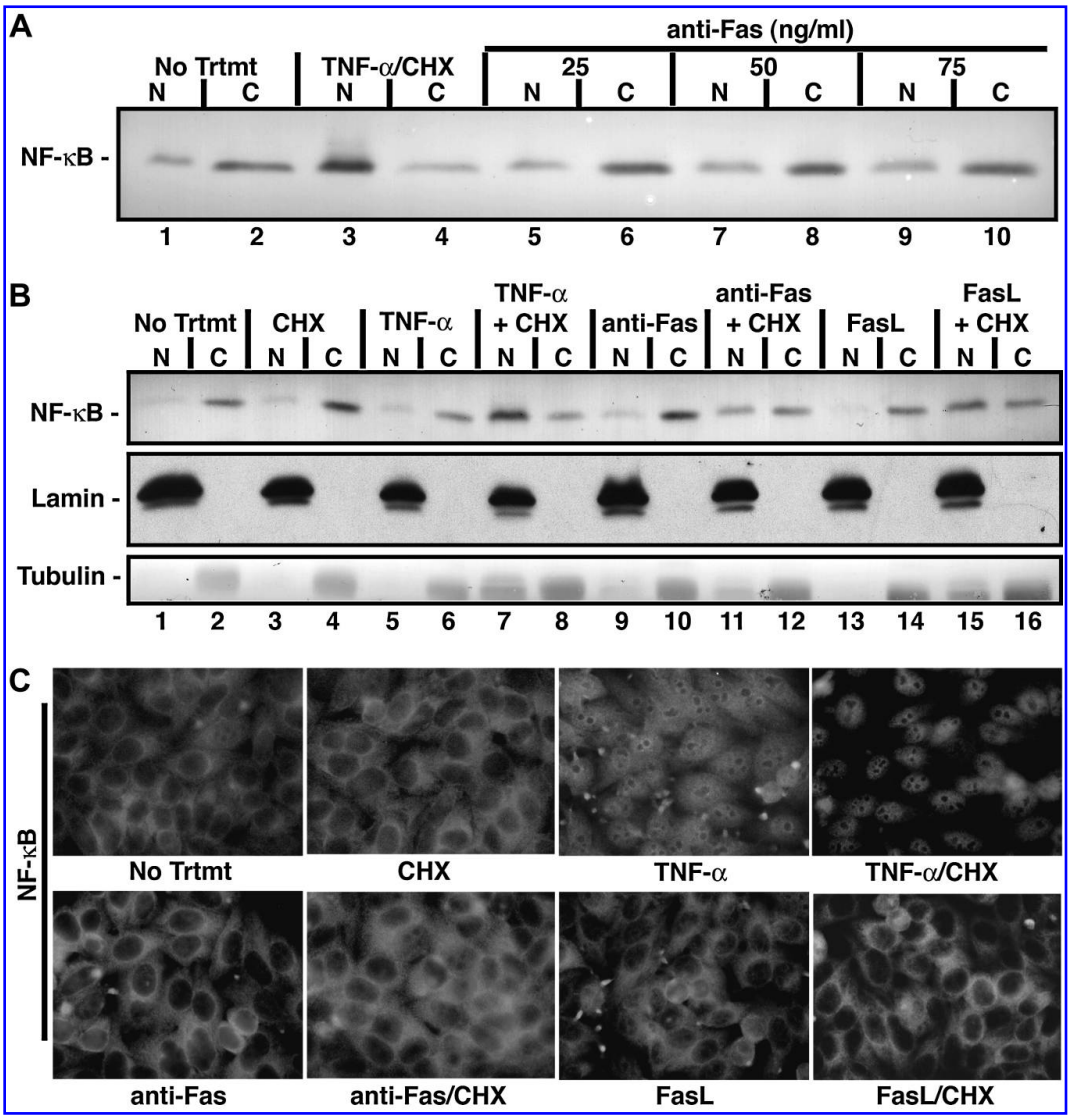

FIG. 1. Treatment with anti-Fas or FasL does not activate NF-κB of HEp-2 cells. (**A**) HEp-2 cells were left untreated (No Trtmt), were treated with TNF-α plus CHX, or treated with increasing amounts of Fas antibody. At 24 h, nuclear (N) and cytoplasmic (C) extracts were prepared, separated in a 15% polyacrylamide gel, transferred to nitrocellulose, and probed with an antibody specific for the p65 subunit of NF-κB. (**B, C**) HEp-2 cells were either left untreated or treated with TNF-α, anti-Fas (75 ng/mL), or FasL (2 ng/mL). All treatments were given alone and in the presence of CHX. (**B**) Nuclear and cytoplasmic extracts were prepared at 20 h posttreatment, and localization of NF-κB was assessed by immunoblotting. Specific antibodies were used as controls for nuclear (lamin) and cytoplasmic (tubulin) fractions. (**C**) Cells were treated as above but at 2 h posttreatment, they were fixed and stained with anti-NF-κB (the p65 subunit) antibody, followed by FITC-conjugated goat antimouse IgG. Cells were then visualized by fluorescence microscopy. $\times$100.

treatment at the concentrations used in this study leads to the nuclear translocation of NF-κB in HEp-2 cells. For all subsequent experiments, either 50 or 75 ng/mL of anti-Fas and either 2 or 3 ng/mL of FasL were used.

Upon close inspection of the results in Figure 1B, we detected a slight amount of nuclear tubulin in the anti-Fas-treated cells but not in the FasL-treated cells (compare lanes 9 and 13). In contrast, when CHX was added, nuclear NF-κB could be seen with both anti-Fas and FasL (Fig. 1B, compare lanes 11, 12 with lanes 15, 16). The presence of tubulin in the nuclear fractions of these samples implied that this result was due to a loss of cellular integrity. However, NF-κB was predominantly cytoplasmic at 2 h after anti-Fas or FasL plus CHX treatments, as measured by indirect immunofluorescence (Fig. 1C), suggesting that the addition of CHX may have made the cells sensitive to the manipulations required for the biochemical subcellular fractionations.

Apoptosis in HEp-2 cells following treatment with anti-Fas or FasL in presence of CHX

The results of the previous experiments demonstrated the inability of FasL or anti-Fas to stimulate the nuclear translocation of NF-κB in HEp-2 cells. However, the observation that

slight amounts of NF-κB and tubulin were observed in the nuclear fractions of cells exposed to these treatments in the presence of CHX raised the possibility that a loss in cellular integrity might have occurred. Previous studies demonstrated that treatment of HEp-2 cells with TNF-α in conjunction with CHX results in apoptosis,[16] and we now also observed tubulin in the nuclear fraction of similarly treated cells (Fig. 1B, lane 7). Accordingly, the purpose of this experiment was to examine the consequences of protein synthesis inhibition during anti-Fas or FasL treatment of HEp-2 cells.

HEp-2 cells were treated with TNF-α, anti-Fas, or FasL in either the presence or absence of CHX. Controls included untreated cells and cells treated with CHX alone. At 19 h posttreatment, cells were stained with the DNA binding dye, Hoechst, and visualized by phase-contrast and fluorescence microscopy. At 20 h posttreatment, nuclear and cytoplasmic extracts were prepared, and immune reactivities with anti-PARP antibody were examined as described in Materials and Methods. We used the cleavage of PARP to assess levels of apoptosis, where intact PARP is represented as the 116,000 molecular weight protein and the cleavage product is an 85,000 molecular weight protein. The ratio of cleaved/uncleaved PARP directly correlates with the percent of cells undergoing apoptosis.[26]

As expected, there was no PARP cleavage in the untreated, CHX-treated, or TNF-α only-treated cells (Fig. 2A, lanes 1–6). There was total PARP cleavage in TNF-α plus CHX-treated cells, indicating that nearly all cells were apoptotic (Fig. 2A, lanes 7, 8). In HEp-2 cells treated with anti-Fas or FasL, slight but detectable levels of cleaved PARP were observed (Fig. 2A, lanes 9, 10 and 13, 14). However, the cells cotreated with CHX and either anti-Fas or FasL were dramatically different, inasmuch as they had essentially full PARP processing (Fig. 2A, lanes 11, 12 and 15, 16). Thus, the response of HEp-2 cells to anti-Fas or FasL plus CHX appeared identical to their response to TNF-α plus CHX.

Visualization of the morphologies of these treated HEp-2 cells 1 h prior to the preparation of nuclear and cytoplasmic extracts confirmed the results. Similar morphologies were observed with untreated, TNF-α, anti-Fas, or FasL-treated cells, whereas those cotreated with CHX exhibited condensed chromatin (80%, 96%, and 100%, respectively) and membrane blebbing, indicative of cells undergoing apoptosis (Fig. 2B). In addition, a significant percentage of anti-Fas-treated or FasL-treated cells in the presence of CHX had detached and were floating in the medium (data not shown). Based on these findings, we conclude that the Fas receptor density on HEp-2 cells is sufficient to activate the proapoptotic cascade, but optimal cell death requires CHX.

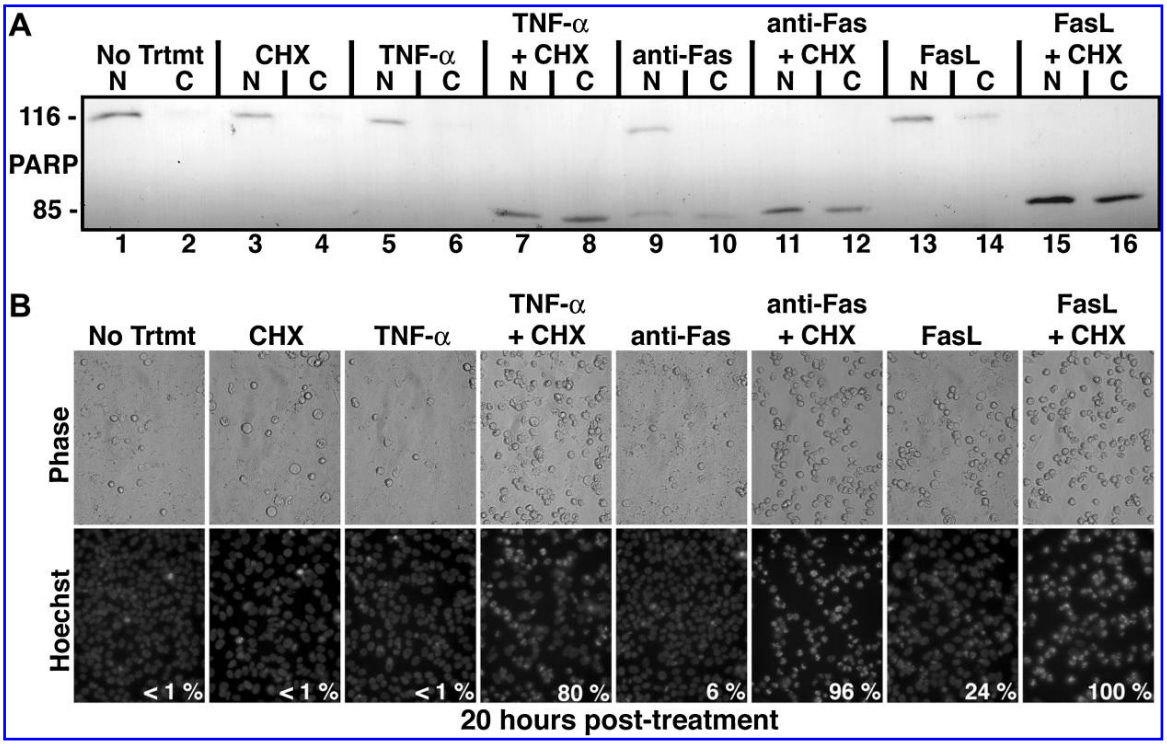

FIG. 2. Anti-Fas- and FasL-induced apoptosis in HEp-2 cells is dependent on inhibition of protein synthesis by CHX. (**A, B**) Cells were treated with TNF-α, anti-Fas (75 ng/mL), or FasL (2 ng/mL), either alone or in the presence of CHX. (**A**) At 20 h posttreatment, nuclear (N) and cytoplasmic (C) extracts were prepared, and cellular proteins were separated in a 15% denaturing gel, transferred to nitrocellulose and probed with anti-PARP antibody. Full-length and cleaved PARP (116,000 [116] and 85,000 [85] molecular weight, respectively) are indicated. (**B**) Visualization of treated cells by phase-contrast and fluorescence microscopy showing cellular morphologies and chromatin condensation. Prior to preparation of the cellular extracts in **A**, live cells were stained with the fluorescent DNA-binding dye, Hoechst, at 17 h posttreatment and photographed at 20 h. The percentages of cells demonstrating apoptotic, condensed chromatin are shown in the bottom right-hand corner of each panel. $\times$40.

Initially, these results seemed to suggest certain similarities between the Fas and TNFR signaling pathways in these cells. None of the three treatments alone, anti-Fas, FasL, or TNF-α, was sufficient to induce significant apoptosis (Fig. 2A, lanes 3, 5, 7), whereas CHX cotreatment led to optimal apoptosis. In the TNF-α system, however, cell survival is contingent on translation of NF-κ-dependent proteins. In contrast, anti-Fas or FasL treatment did not appear to activate NF-κB in these cells (Fig. 1C). During the anti-Fas/FasL treatments plus CHX, NF-κB and tubulin were seen in nuclear cell extracts only when the cells are highly apoptotic (compare Fig. 1B, lanes 11, 12 and 15, 16 with Fig. 2A, lanes 6 and 8). Thus, the observed nuclear NF-κB in cells treated with anti-Fas or FasL in the presence of CHX was likely due to the loss of nuclear membrane integrity rather than the result of the transcription factor's actually being activated. Given the evidence that NF-κB was not active in these cells, it is conceivable that there is some NF-κB-independent survival factor(s) synthesized in HEp-2 cells on treatment with anti-Fas or FasL that prevents them from undergoing extensive apoptosis.

Proteins synthesized prior to 6 hpi in HSV-1-infected HEp-2 cells block anti-Fas and TNF-α-induced apoptosis

Our results represent the initial characterization of the response of HEp-2 cells to signaling through Fas. Treatment with anti-Fas and FasL neither activated NF-κB nor induced apoptosis. However, inhibition of protein synthesis during these treatments caused massive apoptotic cell death. This cellular response is similar to that observed when CHX is added during treatment with TNF-α which, by itself, does not lead to cell death because NF-κB-dependent antiapoptotic proteins are made that block the process. The next question we had to address was what happens to Fas signaling during infection by HSV-1.

Early studies showed that the addition of CHX to HEp-2 cells during HSV-1 infection leads to biochemical and morphologic changes characteristic of apoptosis because of the absence of infected cell proteins.[28,31] The proteins required to block HSV-1-induced apoptosis are synthesized between 3 and 6 hpi,[22,47] and NF-κB has been implicated as participating in this prevention.[16] We set out to determine whether HSV-1 infection could block anti-Fas plus CHX-induced apoptosis and whether this might correlate with the virus-induced nuclear translocation of NF-κB.

In duplicate, simultaneous experiments, monolayers of HEp-2 cells were either mock-infected, infected with HSV-1(F) (moi = 10), or treated with TNF-α, or anti-Fas antibody with or without CHX. Thus, one set of infected cells was treated with TNF-α plus CHX, and the other was treated with anti-Fas antibody plus CHX. In each experiment, infected cells were either untreated or treated at 1, 3, or 6 hpi. At 23 hpi, the DNA binding dye, Hoechst, was added to the medium of cells, and cellular morphologies and chromatin condensation were observed using phase-contrast and fluorescence microscopy. At 24 hpi, nuclear and cytoplasmic extracts were prepared, and immunoblotting with antibodies specific for PARP and NF-κB was used to assess levels of apoptosis and the localization of NF-κB, respectively. Antitubulin antibody was used as a cytoplasmic control to monitor cellular integrity. The positive controls for apoptosis were mock-infected cell cotreatment with CHX and TNF-α for one set and anti-Fas plus CHX for the other. The results (Fig. 3) were as follows.

Neither the mock-infected nor HSV-1(F)-infected cells demonstrated any PARP cleavage (Fig. 3A, lanes 1, 2 and 7, 8). The positive controls for apoptosis, TNF-α plus CHX and anti-Fas plus CHX-treated cells, showed significant levels of PARP cleavage (Fig. 3A, lanes 3 and 9), confirming that in the absence of infection, inhibition of protein synthesis during TNF-α or anti-Fas treatment causes massive apoptosis. Infected cells that received treatments (TNF-α plus CHX or anti-Fas plus CHX) at 1 and 3 hpi were highly apoptotic, with levels of PARP cleavage comparable to those seen in the positive controls (Fig. 3A, lanes 4, 5 and 10, 11). Those cells that received treatment after 6 h of infection (Fig. 3A, lanes 6, 12) were protected from programmed cell death. Cellular and nuclear morphologies of treated/infected cells substantiated these results (Fig. 3B). Nearly all infected cells treated at 1 or 3 hpi demonstrated cell shrinkage, membrane blebbing, and condensed chromatin, whereas the number of those treated at 6 hpi showing these features was quite reduced (from 80% and 83% to 18% and 17%, respectively). These results suggest that proteins synthesized between 3 and 6 hpi during HSV-1(F) infection are capable of blocking both TNF-α plus CHX-induced and anti-Fas plus CHX-induced apoptosis.

As expected,[16,18] HSV-1(F) infection of untreated cells led to the accumulation of NF-κB in the nuclear fractions of these cells, and there was clear segregation between nuclear and cytoplasmic fractions (Fig. 3C, lanes 3, 4 and 15, 16). In contrast, the highly apoptotic cells had significant nuclear contamination of tubulin (lanes 5–8 and 17–20). The observation of NF-κB in the nuclear fractions of the TNF-α plus CHX-treated cells was expected,[16] because of the inability of the TNF-α-stimulated NF-κB to recycle.[48] NF-κB predominated in the cytoplasmic fraction of the 3 hpi, anti-Fas/CHX-treated cells (Fig. 3C, lanes 21, 22), confirming our earlier finding that anti-Fas does not activate NF-κB in HEp-2 cells. Interestingly, NF-κB accumulated in the nuclear extracts of the nonapoptotic (6 hpi), anti-Fas/CHX-treated cells (Fig. 3C, lanes 23, 24). The implication here is that the nuclear translocation of NF-κB was an effect of HSV-1(F) infection and not anti-Fas treatment. This finding suggests that NF-κB may play a role in viral blocking Fas-mediated cell death.

NF-κB is not activated by HSV-1(F) infection, anti-Fas, or TNF-α treatment in IκBαDN cells

These results showed that uninfected HEp-2 cells underwent apoptosis during anti-Fas and FasL treatment only when protein synthesis was inhibited (Figs. 1 and 2). Additionally, proteins synthesized during HSV-1(F) infection between 3 and 6 hpi prevented this killing (Fig. 3). Interestingly, nuclear NF-κB was observed in the nonapoptotic, infected cells treated with anti-Fas plus CHX at 6 hpi (Fig. 3). Because of the breakdown of compartmentalization in apoptotic cells, however, the definitive role of NF-κB in this blocking process remained unclear.

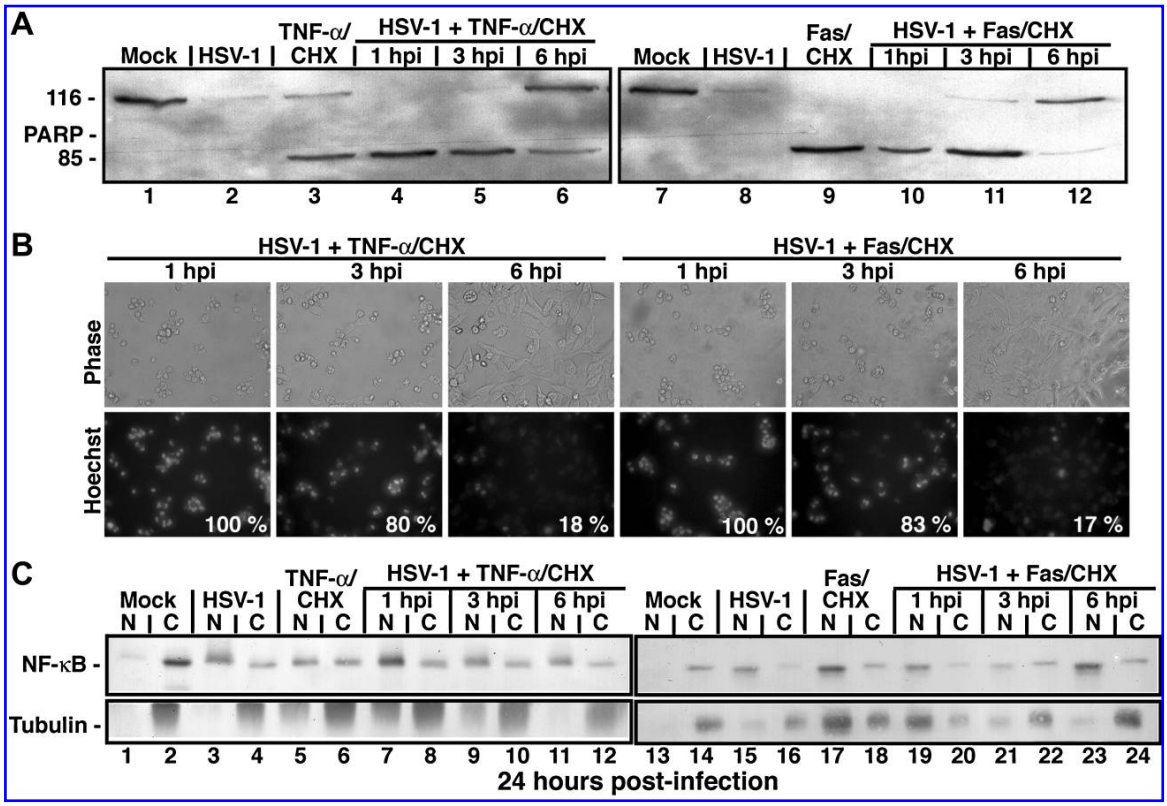

FIG. 3. HSV-1(F) infection of HEp-2 cells prevents both anti-Fas plus CHX-induced and TNF-α plus CHX-induced apoptosis at 6 hpi. Detection of (**A**) cellular death factor PARP processing, (**B**) cellular and nuclear morphologies, and (**C**) subcellular localization of NF-κB. Mock and HSV-1(F)-infected (moi = 10) HEp-2 cells were left untreated or treated with either anti-Fas antibody (Fas) (50 ng/mL) plus CHX or TNF-α plus CHX at either 1, 3, or 6 hpi. (**A, C**) At 24 hpi, nuclear (N), cytoplasmic (C), and whole cell extracts were prepared. Cellular proteins were separated in denaturing gels, transferred to nitrocellulose, and probed with antitubulin, anti-NF-κB (the p65 subunit), and anti-PARP antibodies. Full-length and cleaved PARP (116,000 [116] and 85,000 [85] molecular weight, respectively) are indicated. (**B**) At 23 hpi, live cells were stained with DNA-binding dye, Hoechst, and photographed using phase-contrast and fluorescence microscopy. The percentages of cells demonstrating apoptotic, condensed chromatin are shown in the bottom right-hand corner of each panel. $\times$40.

Previous studies showed that the time frame during productive HSV-1 infection in which necessary prosurvival, antiapoptotic factors are synthesized correlates with the kinetics of NF-κB activation.[16] In its inactive form, NF-κB exists in the cytoplasm bound to its inhibitor IκBα, and upon activation by IκBα kinase (IKK), IκBα is phosphorylated at serine residues 32 and 36, targeting it for ubiquitination and subsequent degradation by the 26S proteosome.[49,50] Upon release from IκBα, NF-κB translocates to the nucleus where it binds DNA, initiating transcripton of target genes; some of which include IκBα, interferons (IFNs), and inhibitors of apoptosis (IAPs).[46,51–53]

To explore NF-κB's specific role in apoptosis prevention on cotreatment with anti-Fas or FasL plus CHX during HSV-1(F) infection, we made use of a clonal HEp-2-derviative cell line, IκBαDN, which stably expresses a dominant-negative form of IκBα.[16] The form of IκBα in this cell line contains two stabilizing serine-to-alanine mutations at key serines 32 and 36, which prevent the protein from being phosphorylated by IKK and subsequently ubiquitinated, targeting it for degradation.[54,55] IκBαDN and control HEp-2 cells were either left untreated for 2 h, infected with HSV-1(F) (moi = 10) for 12 h, or treated with anti-Fas, TNF-α, TNF-α plus CHX, or anti-Fas plus CHX for 2 h. At 2 h post treatment or 12 hpi, cells were fixed, permeablized, and incubated within antibodies specific for the p65 subunit of NF-κB, as described in Materials and Methods. Subcellular localization of NF-κB was then observed at two magnifications by fluorescence microscopy.

After 12 h of HSV-1(F) infection, NF-κB was predominantly nuclear in the HEp-2 cells but not the IκBαDN cells (Fig. 4), indicating that the virus did not activate NF-κB in the cells expressing this mutant form of IκBα.[16] NF-κB was cytoplasmic at 2 h in all of the IκBαDN cells, regardless of the treatment. This was in contrast to the concentrated nuclear staining observed in the TNF-α and TNF-α plus CHX-treated control HEp-2 cells. Consistent with the previous experiments, anti-Fas did not activate NF-κB in either cell line. That NF-κB remained cytoplasmic during cotreatment of anti-Fas eliminates the possibility that NF-κB shuttling may have occurred in these cells. Based on these results, we conclude that the IκBαDN cells have lost their ability to activate NF-κB.

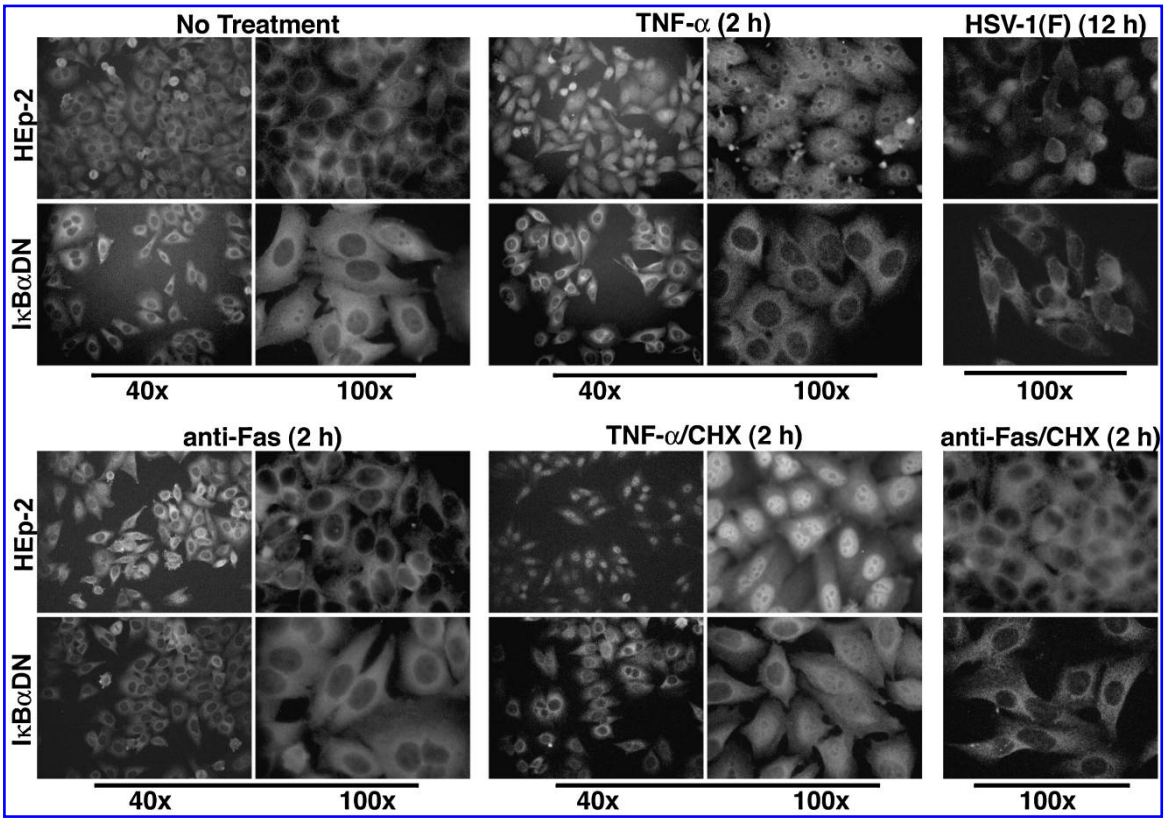

FIG. 4. Absence of nuclear NF-κB in IκBαDN cells. IκBαDN cells and control HEp-2 cells were left untreated, treated with TNF-α, TNF-α plus CHX, anti-Fas (75 ng/mL), or anti-Fas plus CHX or infected with HSV-1(F) (moi = 10). At either 2 h post-treatment or 12 hpi, cells were fixed, permeablized, and stained with an antibody specific for the p65 subunit of NF-κB, as described in Materials and Methods. Magnifications are as indicated.

Proteins synthesized prior to 6 hpi in HSV-1(F)-infected IκBαDN cells block FasL plus CHX-induced apoptosis

To determine if synthesis of NF-κB-dependent proteins was required for the survival of FasL plus CHX-treated HSV-1(F)-infected cells, we made use of the IκBαDN cell line characterized in Figure 4. In this experiment, IκBαDN and control HEp-2 cells were either mock-infected, infected with HSV-1(F) (moi = 10), or treated with CHX, FasL, or FasL plus CHX. One group of infected cells was left alone, while the other received FasL plus CHX treatment at 6 hpi. At 17 h, the DNA binding dye, Hoechst, was added to the medium of treated/infected cells, and cellular and nuclear morphologies were observed by fluorescence microscopy. At 18 h post infection or treatment, nuclear and cytoplasmic extracts were prepared, and proteins were separated in a denaturing gel and transferred to nitrocellulose. Immune reactivities using antibodies specific for ICP4, PARP, NF-κB, lamin, and tubulin were examined. ICP4 is an HSV-1 immediate-early protein and was used as a marker for viral infection at early times.[28] As previously, PARP cleavage from the full-length form to the cleaved form was used to assess levels of apoptosis. Lamin and tubulin antibodies were used as markers for nuclear and cytoplasmic fractions, respectively.

IκBαDN and HEp-2 cells responded similarly to treatments with CHX, FasL, or FasL plus CHX (Fig. 5A). Neither FasL alone nor CHX alone had any visible affect on nuclear morphologies of these cells. In contrast, both types of cells treated with FasL plus CHX were highly apoptotic, exhibiting cell shrinkage (data not shown) and condensed chromatin (100%); many of these cells had lost adherence to the plates. It is important to note that the IκBαDN cells were quite fragile, did not grow to confluency, and exhibited morphologic characteristics that differed from those of HEp-2 cells under mock conditions. However, the apoptotic features of these cells were easily distinguishable. In HEp-2 cells, allowing the HSV-1(F) infection to proceed to 6 hpi prior to addition of FasL plus CHX precluded these apoptotic features (100% to 8%), and these cells were indistinguishable from cells infected without treatment (Fig. 5A). In general, the IκBαDN cells were more sensitive to HSV-1(F) infection than the HEp-2 cells, as expected.[16] As observed with the HEp-2 cells, the infected IκBαDN cells that were treated at 6 hpi resembled those that were only infected but not treated (100% to 4%). These findings suggest that HSV-1(F) may be capable of blocking FasL plus CHX-induced apoptosis in the IκBαDN cells.

Biochemical analyses further substantiated these results, as immune reactivities with extracts from both cell lines looked nearly identical (Fig. 5B,C). As expected, there was little to no PARP cleavage in any mock, CHX, or FasL-treated cells (lanes 1–6). Our positive control for apoptosis induction in uninfected cells, FasL plus CHX treatment, led to total PARP cleavage in

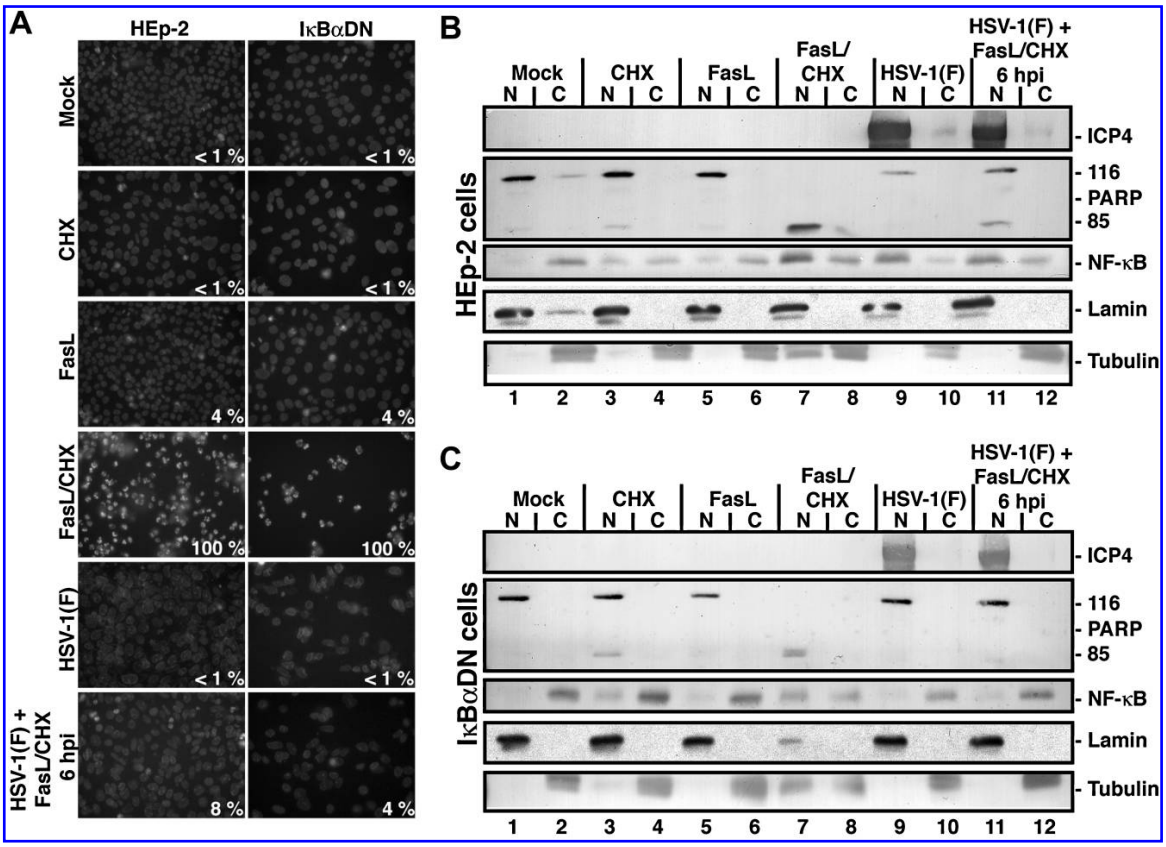

FIG. 5. Prevention of FasL plus CHX-induced apoptosis by HSV-1(F) does not require activation of NF-κB. IκBαDN or control HEp-2 cells were mock-infected or infected with HSV-1(F) (moi = 10) either with or without FasL (2 ng/mL) plus CHX addition at 6 hpi. As controls, cells were either left alone or treated with FasL, CHX, or FasL plus CHX. (**A**) At 17 h postinfection/treatment, cells were stained with the DNA binding dye, Hoechst, and visualized by fluorescence microscopy. The percentages of cells demonstrating apoptotic, condensed chromatin are shown in the bottom right-hand corner of each panel ×40. Nuclear (N) and cytoplasmic (C) extracts of both (**B**) HEp-2 and (**C**) IκBαDN were prepared at 18 h postinfection/treatment. (**B, C**) Proteins were separated on a 15% denaturing gel, transferred to nitrocellulose, and probed with antibodies specific for viral ICP4, cellular death factor PARP, the p65 subunit of NF-κB, lamin, and tubulin as described in Materials and Methods. Full-length and cleaved PARP (116,000 [116] and 85,000 [85] molecular weight, respectively) are indicated. HSV-1(F) infection of IκBαDN leads to a slight increase in PARP cleavage compared to that in HEp-2 cells,[16] which is below the level of detection in this experiment.

both cell types (Lanes 7, 8). That the IκBαDN cell line (Fig. 5C) responded to FasL treatment like the control HEp-2 cell line (Fig. 5B) supports a theory that NF-κB does not play a role in the prevention of FasL-induced apoptosis in uninfected cells. Allowing HSV-1(F) infection to precede to 6 hpi prior to FasL plus CHX addition significantly reduced the level of PARP cleavage in both cells (compare lanes 11, 12 with lanes 7, 8). Importantly, NF-κB was present in nuclei of the HSV-1(F)-infected HEp-2 cells but not the IκBαDN cells (compare lanes 9, 10 between Fig. 5B and 5C). Detection of NF-κB in the nuclear fractions of FasL plus CHX-treated HEp-2 and IκBαDN cells (lanes 7, 8) was presumably due to cytoplasmic contamination of these highly apoptotic cells, as the strictly cytoplasmic tubulin was also seen in the nuclear fractions of these cells.

Taken together, these results indicate that translocation of NF-κB to the nucleus is not required to block apoptosis induced by FasL plus CHX in HEp-2 cells. Thus, some other prosurvival factor(s) was synthesized during HSV-1 infection that is capable of preventing cell death induced by FasL plus CHX treatment. Based on our findings, we conclude that these factors are synthesized prior to 6 h post HSV-1(F) infection.

DISCUSSION

Our goal in this study was to investigate the ability of HSV-1 to block apoptosis initiated through Fas. Because HEp-2 cells are the prototypical cell line for defining HSV-1-dependent apoptosis, we first had to document their response to signaling through Fas. In addition, we considered whether NF-κB played a role in the apoptosis prevention process. The significant findings of our study may be summarized as follows.

FasL or anti-Fas treatments alone were insufficient to induce apoptosis in HEp-2 cells

There have been some discrepancies as to the effects of anti-Fas and FasL on cells expressing the Fas receptor.[42,56–59] For

example, it has been suggested that in Jurkat cells, CHX treatment alone induces apoptosis in a FADD-dependent mechanism.[60] In addition, neutralizing anti-Fas antibodies does not prevent this CHX-mediated apoptosis, implying that FADD can function independently of the Fas receptor. In our hands, neither anti-Fas nor FasL treatment could efficiently induce apoptosis in the absence of CHX in HEp-2 cells. Because apoptosis does not occur without inhibition of protein synthesis, these extrinsic treatments may also initiate the synthesis of yet undefined survival factor(s) that act to block the process of programmed cell death. Perhaps cotreatment of CHX with either anti-Fas or FasL may activate the FADD-dependent death pathway, whereas any of these treatments alone cannot. Further analyses of the response of HEp-2 cells to such extrinsic effectors are required to resolve these important issues.

NF-κB was not activated through Fas in HEp-2 cells

We demonstrated that both FasL and anti-Fas function similarly to trigger apoptosis without activation of NF-κB. Although we observed NF-κB in the nuclear fractions of anti-Fas plus CHX or FasL plus CHX-treated cells, we also observed the presence of tubulin, a strictly cytoplasmic protein, in the nuclei of these cells. As we found that NF-κB was not activated during anti-Fas or FasL treatment using indirect immunofluorescence, it appears that the leakage of cytoplasmic proteins into treated, apoptotic cell nuclei likely occurred as a result of nuclear membrane disruption.

Because anti-Fas treatment alone did not activate NF-κB in our system and cotreatment with CHX caused apoptosis, it is likely that the NF-κB-dependent survival factors that prevent TNF-α plus CHX-induced apoptosis are not responsible for preventing anti-Fas plus CHX-induced apoptosis. Thus, the host cell survival factors involved in modulating Fas-dependent apoptosis may also participate in apoptosis prevention during viral infection.

HSV-1 blocks apoptosis triggered through Fas receptor in HEp-2 cells

The time frame during HSV-1 infection, in which the pro-survival factors necessary to block virus-triggered apoptosis are synthesized, is between 3 and 6 hpi.[22] It has been shown previously that these survival factors are also capable of preventing apoptosis induced by treatment with TNF-α plus CHX, and NF-κB is activated within this time frame.[16] Based on our new results presented here, we conclude that HSV-1 also blocks FasL-induced apoptosis in HEp-2 cells, and this effect is dependent on the synthesis of proteins during the first 6 h of infection. We recognize that the required survival factor(s) could be of either viral or cellular origin. This is in contrast to monocytoid cells, in which it was reported that virus binding to its surface receptor is sufficient to prevent Fas-mediated apoptosis.[20] Although there are likely cell type differences involved, it could also be that the same survival proteins that are synthesized in uninfected cells are made stable in some way by viral infection in HEp-2 cells. To test whether the factors involved in apoptosis prevention in uninfected HEp-2 cells were constitutively active upon treatment with anti-Fas, we performed a time course study (data not shown) in which CHX was added at various times postanti-Fas treatment. We observed that inhibition of protein synthesis at any time resulted in extremely high levels of apoptosis (nearly 100%). Clearly, these findings together imply that infected cell protein synthesis is required to circumvent death by apoptosis in HEp-2 cells.

Viral prevention of apoptosis induced through Fas occurs in the absence of NF-κB activation

This conclusion is based on our demonstration that HSV-1(F) infection in a cell line that stably expresses a dominant-negative form of IκBα (the inhibitor of NF-κB) can still block FasL plus CHX-induced apoptosis. Our results also imply that HSV-1(F) is not capable of activating NF-κB by any alternative method other than through phosphorylation of IκBα. It is clear that the virus has the ability to regulate the activity of NF-κB, and when it is under viral control, NF-κB demonstrates an anti-apoptotic function against virus-induced cell death. The significance of our findings is that HSV-1 does not block all forms of apoptosis by activating NF-κB. NF-κB may be simply another accessory participant[14] in the apoptosis modulation process.[47] Thus, although there is sufficient evidence to conclude that NF-κB is a very important player in productive HSV infection,[16–21,61] it seems that the virus has alternative mechanisms for evading the host cell's apoptotic response to infection that are independent of NF-κB. That NF-κB is not solely responsible for blocking death signaling through Fas in infected cells is, perhaps, not surprising, as HSV-1 itself encodes several proteins that function in apoptosis prevention.[62]

It is intriguing that HSV-1 infection of HEp-2 cells induces both apoptosis and synthesis of proteins that block the process.[22] In this respect, HSV-1 parallels the dual effects seen during treatment with TNF-α. Both TNF-α and HSV-1(F) infection are potent activators of NF-κB, whose downstream effectors participate in the apoptosis prevention process. In certain cell lines, FasL and anti-Fas have also been shown to have similar effects as TNF-α, in terms of apoptosis induction. We now show that HSV-1(F) infection is also capable of blocking anti-Fas or FasL plus CHX-induced apoptosis. Our results indicate that HSV-1(F) infection can block the process of Fas-mediated apoptosis by a viral or cellular mechanism that is independent of NF-κB. These findings have important implications in defining the molecular mechanisms involved in viral evasion of the cytokine-driven, innate immune response.

ACKNOWLEDGMENTS

We thank Margot Goodkin for helpful discussions during the course of these experiments. These studies were supported in part by grants from the United States Public Health Service (AI 48582 and AI 38873 to J.A.B).

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Received 18 September 2006/Accepted 14 November 2006

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