Efficient Replication by Herpes Simplex Virus Type 1 Involves Activation of the I κ B Kinase-I κ B-p65 Pathway

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Infection by herpes simplex virus type 1 (HSV-1) induces a persistent nuclear translocation of NF κ B. To identify upstream effectors of NF κ B and their effect on virus replication, we employed mouse embryo fibroblast (MEF)-derived cell lines with deletions of either IKK1 or IKK2, the catalytic subunits of the I κ B kinase (IKK) complex. Infected MEFs were assayed for virus yield, loss of I κ B α , nuclear translocation of p65, and NF κ B DNA-binding activity. Absence of either IKK1 or IKK2 resulted in an 86 to 94% loss of virus yield compared to that of normal MEFs, little or no loss of I κ B α , and greatly reduced NF κ B nuclear translocation. Consistent with reduced virus yield, accumulation of the late proteins VP16 and gC was severely depressed. Infection of normal MEFs, Hep2, or A549 cells with an adenovirus vector expressing a dominant-negative (DN) I κ B α , followed by superinfection with HSV, resulted in a 98% drop in virus yield. These results indicate that the IKK-I κ B-p65 pathway activates NF κ B after virus infection. Analysis of NF κ B activation and virus replication in control and double-stranded RNA-activated protein kinase-null MEFs indicated that this kinase plays no role in the NF κ B activation pathway. Finally, in cells where NF κ B was blocked because of DN I κ B expression, HSV failed to suppress two markers of apoptosis, cell surface Annexin V staining and PARP cleavage. These results support a model in which activation of NF κ B promotes efficient replication by HSV, at least in part by suppressing a host innate response to virus infection.

Cells activate the transcription factor NF κ B in a wide variety of situations, including responses to stress-inducing insults such as UV irradiation and virus infection or in response to cytokines such as tumor necrosis factor alpha (TNF- α). NF κ B has an important role in suppression of apoptosis and regulates the expression of many important antiapoptotic functions (5, 31, 48, 50). NF κ B, as a p65/p50 heterodimer, is normally sequestered in the cytoplasm in a complex with inhibitor of κ B (I κ B). I κ B α is targeted for phosphorylation at serine residues 32 and 36, and I κ B β is targeted for phosphorylation at serine residues 19 and 23 (42, 46, 51), by the multisubunit I κ B kinase (IKK) (11, 23, 36, 52, 58). This phosphorylation triggers its polyubiquitylation and destruction by the 26S proteasome (6, 7, 12), and, as a result, NF κ B is translocated to the nucleus (14, 57). Key roles for IKK and I κ B in NF κ B signaling were demonstrated in studies in which overexpression of a kinase-dead, *trans*-dominant form of IKK prevents I κ B phosphorylation and inhibits NF κ B activation (36, 52). IKK is activated by phosphorylation mediated by mitogen-activated protein (MAP) kinase kinases (MAP3Ks) MEKK1, -2, or -3 or NF κ B-inducing kinase (NIK) (23, 30, 37, 59). Under stress conditions, the double-stranded RNA-activated protein kinase (PKR) has been shown to activate NF κ B through a pathway dependent on NIK and IKK (57). Distinct roles for the catalytic components of the IKK have been recognized. IKK α appears to play a major role in transducing signals for NF κ B activation during embryonic development (18, 45), while IKK β is essential for cytokine and other stress-induced signaling pathways (10, 27,

29). Besides cytoplasmic roles in the activation of NF κ B, recent studies have identified IKK α and the IKK scaffold protein IKK γ /NEMO in direct regulation of NF κ B-dependent transcription in the nucleus (2, 49, 53). NF κ B activation is also dependent on distinct signaling pathways which target p65 for phosphorylation (32).

The ability of herpes simplex virus type 1 (HSV-1) to activate NF κ B has been well documented (1, 15, 40). Beginning at 3 to 5 h postinfection (hpi), HSV-1 induced a strong and persistent nuclear translocation, increased p50/p65-dependent DNA binding activity as measured by electrophoretic mobility shift assay (EMSA), and induced activation of a 3XNF κ B-luciferase reporter. Persistent NF κ B activation required virus binding and entry as well as *de novo* infected cell protein synthesis, including expression of functional viral immediate-early (IE) protein ICP27. Activation was also accompanied by increased IKK activity and loss of both I κ B α and I κ B β . Interference with NF κ B activation occurred following expression of a dominant-negative I κ B α (DN I κ B) containing alanine substitutions for serine residues 32 and 36 normally targeted by IKK. The resulting substantial reduction in NF κ B EMSA activity correlated with a reduction in virus yield. The latter may be related to the reported role of NF κ B in preventing HSV-1-induced apoptosis (15). The foregoing results argue that the observed persistent NF κ B activation, rather than being a host response to virus infection, plays a positive role in the promoting efficient virus replication.

Toll-like receptors (TLRs) and associated downstream signaling components can also mediate activation of NF κ B (35, 44). While HSV-1 infection stimulated interferon production in mice by a mechanism dependent on TLR9/MyD88 (20) and TLR2 mediated an inflammatory response that contributes to

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lethal encephalitis in a murine model of HSV-1 pathogenesis (22), as yet no direct evidence links TLRs to NF κ B activation during HSV-1 infection.

The experiments presented here were designed to more fully characterize the activation pathway for NF κ B in HSV-infected cells and to assess how components of the classical NF κ B activation pathway (IKK β -I κ B α -p65/p50) contribute to virus replication. Our approach was to assess virus replication and markers of NF κ B activation in mouse embryo fibroblasts (MEFs) with targeted deletions in key components of the signaling pathway (IKK α or IKK β) or through overexpression of a DN I κ B α and to assess HSV replication and the ability of HSV to suppress markers of apoptosis.

MATERIALS AND METHODS

Viruses and cells. All experiments were performed with the KOS1.1 strain of HSV-1. Adenovirus vectors expressing a dominant-negative I κ B α (DN I κ B α) or green fluorescent protein (GFP) were obtained from the Virus Vector Core Facility at University of North Carolina (UNC)-Chapel Hill. Spontaneously immortalized mouse embryo fibroblasts derived from normal mice or mice with targeted deletions in IKK α or IKK β (26, 27) were obtained from Tal Kafri (UNC-Chapel Hill). MEFs from normal mice or mice with a targeted deletion of the double-stranded RNA-activated protein kinase PKR (54) were obtained from Bryan Williams (Cleveland Clinic). These cell lines as well as A549 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine calf serum, 100 U of penicillin/ml, 1% streptomycin, and 1% L-glutamine (all from Gibco). Hep2 cells were maintained in minimal essential medium supplemented as described for DMEM.

Plaque assay. Monolayers of cells in 60-mm-diameter dishes were infected with HSV at a multiplicity of infection (MOI) of 5, and cells and medium were harvested at various times postinfection, followed by three cycles of freezing and thawing. Serial 10-fold dilutions of the lysates were assayed in triplicate on monolayers of Vero cells in 12-well dishes. After 1 h, monolayers were covered with DMEM-H containing 2% calf serum and 0.3% methylcellulose. After 3 days of incubation at 37°C, medium was aspirated from the wells and plaques were stained with 0.8% crystal violet in 50% ethanol (EtOH).

Preparation of cell extracts and Western blotting. For preparation of whole-cell extracts, medium was removed and monolayers were rinsed with ice-cold Dulbecco's phosphate-buffered saline (PBS). Cells were scraped directly into 1× sodium dodecyl sulfate (SDS) sample buffer (3.85 mM Tris base [pH 6.8], 9.1% β -mercaptoethanol, 1.82% SDS, 4.6% glycerol, and 0.023% bromophenol blue [in 100% EtOH]) and denatured by boiling. Cell-equivalent amounts of lysate were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Fractionated cytoplasmic and nuclear extracts were prepared as described previously (38). Briefly, cells were collected by trypsinization, spun through a cushion of bovine calf serum, washed twice in PBS, resuspended in three packed cell volumes (PCV) of CE buffer (10 mM HEPES, [pH 7.8], 1 mM EDTA, 60 mM KCl, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1% NP-40, 25% glycerol, 0.4 mM NaF, 0.4 mM Na₃VO₄, 10 μ M pepstatin, and Complete Protease Inhibitor Cocktail [Roche]), and incubated on ice for 10 min. Following a 10-s spin in a benchtop microcentrifuge, the supernatant was removed and the nuclear pellet was resuspended in CW buffer (CE buffer without NP-40 or glycerol) and repelleted. Nuclei were resuspended in 2 PCV of NE buffer (20 mM Tris-HCl [pH 8.0], 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM PMSF, 25% glycerol, and phosphatase and protease inhibitors as described above). After incubation on ice for 10 min, nuclei were pelleted at 60,000 rpm at 4°C for 20 min in a Beckman TL100.3 rotor. The nuclear extract supernatant was carefully removed, and aliquots were stored at -80°C.

Equal amounts of protein were transferred to PolyScreen polyvinylidene difluoride (PVDF) membranes (Perkin-Elmer Life Sciences) followed by blocking in TBST (150 mM NaCl, 20 mM Tris [pH 7.6], 0.05% Tween 20) with 5% milk. All probing and washing of membranes was done in TBST. Rabbit polyclonal antibodies for IKK β (H-470, sc-7607), IKK α (H-744, sc-7218), and I κ B α (C-21, sc-371) from Santa Cruz Biotechnology were used at a 1:1,000 dilution overnight at 4°C per the manufacturer's instructions. Rabbit polyclonal antibody for p65 (100-4165) was from Rockland and was used at a 1:2,000 dilution for 1 h at room temperature. Monoclonal antibodies against HSV IE ICP4 (1101) and ICP27 (1119) were purchased from the Rumbaugh-Goodwin Institute for Cancer Research (Plantation, Fla.) and were used at a 1:800 dilution. Polyclonal antibody

for VP16 (Clontech) was used at a 1:5,000 dilution. Polyclonal antibody against ICP8 (3-83) was a generous gift from David Knipe (Harvard University) and was used at a 1:1,000 dilution. Polyclonal antibody against gC (R47), used at 1:5,000, was a generous gift of Gary Cohen (University of Pennsylvania). Rabbit polyclonal antibody to PARP (H-250, sc-7150) was used at a 1:1,000 dilution. Goat anti-rabbit and anti-mouse secondary antibodies were purchased from Amersham Biosciences. The secondary antibody was detected with SuperSignal West Pico Chemiluminescent substrate agent (Pierce). Autoradiographic films were scanned, and images were stored as 8-bit grayscale JPEG files. The density of each band was determined by using Image J (National Institutes of Health). Relative density values were corrected for average background by subtracting the density of a blank portion of the film. The corrected values were then used to calculate fold change relative to the control sample (e.g., the ratio of the mock-infected cell value to the infected cell value).

EMSA. Preparation of nuclear extracts and mobility shift assays for NF κ B were performed as described previously (17, 38, 40). Nuclear extract was incubated with a radiolabeled probe containing a κ B-binding site, TGGGGATTC CCCA, in buffer containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 0.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 2 μ g of poly(dI-dC) · poly(dI-dC). Following 20 min of incubation at room temperature, aliquots were fractionated at 4°C on nondenaturing 4% polyacrylamide gels prepared in 0.25× TBE (1× TBE is 10 mM Tris base [pH 8.3], 9 mM boric acid, and 2 mM EDTA). Gels were then placed on 3MM paper, dried under vacuum and heat, and exposed to Kodak BMR film at -70°C.

Annexin V staining. Infected Hep2 cells were stained with Annexin V by using the Vybrant Apoptosis Assay kit #2 (Molecular Probes) per the manufacturer's instructions. Briefly, cells were harvest by trypsin treatment and were resuspended in DMEM supplemented with 10% fetal bovine calf serum, 100 U of penicillin/ml, 1% streptomycin, and 1% L-glutamine. Following pelleting, cells were resuspended at 10⁶/ml in 1× binding buffer followed by addition of 50 μ l of Annexin stain/ml and 10 μ l of propidium iodide (100 μ g/ml in 1× binding buffer)/ml. Cells were incubated at room temperature for 15 min, and then 400 μ l of binding buffer was added for a total volume of 500 μ l. Stained cells were kept on ice until Annexin staining was quantified by fluorescence-activated cell sorter (FACS) analysis.

RESULTS

Both IKK α and IKK β contribute to virus yield and NF κ B activation in HSV-infected cells. The multisubunit IKK complex phosphorylates I κ B, triggering the latter's destruction and subsequent nuclear localization of NF κ B (14, 57). IKK activity was reported to increase following HSV infection (1). To determine the importance of IKK catalytic subunits IKK α and IKK β on the replication efficiency of HSV-1, we prepared lysates at 8 hpi from cell lines derived from normal MEFs or MEFs from embryos with targeted deletions of either IKK α or IKK β and assessed the effect of loss of either catalytic component of IKK on levels of I κ B α and nuclear translocation of p65, two markers of NF κ B activation. The results of Western blots are presented in Fig. 1A. First, cytoplasmic extracts were probed for IKK subunits to confirm their presence or absence in IKK α ^{-/-} and IKK β ^{-/-} MEF lines (compare IKK α and IKK β in lanes 1 to 3 with 4 to 6 and lanes 7 to 9 with 10 to 12). IKK α and IKK β levels were used as loading controls for cytoplasmic samples, and PARP was used as a loading control for nuclear samples. As an assessment of our fractionation procedure, we did not detect IKK α or IKK β in nuclear lysates or PARP in the cytoplasm (data not shown). Following infection or TNF- α treatment of wild-type (WT), IKK α ^{-/-}, and IKK β ^{-/-} cells, we observed nuclear translocation of p65 (compare lanes 1 to 6 and 7 to 12). Compared to their corresponding mock-infected nuclear samples, increases in nuclear p65 following HSV infection or TNF- α treatment were approximately twofold greater in WT cells than in either of the IKK knockout cell types. Importantly, infection of WT and

FIG. 1. Both IKK α and IKK β contribute to the activation of NF κ B in HSV-infected cells. Western blot analysis for proteins involved in NF κ B activation (A) and for viral proteins (B and C) in murine cell lines. Nuclear and cytoplasmic extracts were prepared from normal (WT), IKK α ^{-/-}, IKK β ^{-/-}, p65^{-/-}, and p65^{+/-} cells at 8 hpi, and they were then probed for the indicated cellular and viral proteins as described in Materials and Methods. M, mock infected; H, HSV infected; T, TNF- α treated. (B) Nuclear extracts were probed for ICP27 and ICP8, while cytoplasmic extracts were probed for gC and VP16.

IKK α ^{-/-} cells also resulted in two- to threefold reductions in cytoplasmic p65 compared to that of mock-infected cells, while no reduction in cytoplasmic p65 was detected in IKK β ^{-/-} cells following infection or TNF- α treatment. We observed an apparent increase in the total amount of p65 after TNF- α treatment of both WT and IKK α ^{-/-} cells (lanes 3 and 6). Whether this reflects the ability of this cytokine to activate Sp1 and transcriptionally activate the p65 promoter (47) is presently unknown. Quantification by Image J, as described in Materials and Methods, revealed 29- and 20-fold reductions, respectively, in IκB α following HSV infection or TNF- α treatment of WT cells. Reductions of IκB α in IKK α ^{-/-} cells were 4.4-fold following HSV infection and 8.7-fold after TNF- α treatment. Consistent with the reduced nuclear translocation of p65 observed in IKK β ^{-/-} cells, IκB α was reduced in these cells only 1.3- and 1.1-fold following infection or TNF- α treatment.

Filters were also probed for several viral proteins representative of different kinetic classes. While IE ICP27 levels were comparable between WT and IKK β ^{-/-}, we observed decreased ICP27 accumulation (1.7-fold relative to that of the WT) in IKK α ^{-/-} cells. Delayed early (DE) ICP8 accumulation

TABLE 1. Virus yield assays from normal, IKK α ^{-/-}, and IKK β ^{-/-} MEFs^a

Cell type	Results at 16 hpi		Results at 24 hpi	
	Yield (PFU/culture)	Fold decrease	Yield (PFU/culture)	Fold decrease
Normal	(3.86 $\pm$ 1.5) $\times$ 10 ⁷		(3.74 $\pm$ 0.36) $\times$ 10 ⁷	
IKK α ^{-/-}	(7.09 $\pm$ 3.4) $\times$ 10 ⁶	5.4	(6.0 $\pm$.37) $\times$ 10 ⁶	6.2
IKK β ^{-/-}	(2.4 $\pm$ 0.16) $\times$ 10 ⁶	16.3	(2.62 $\pm$ 0.33) $\times$ 10 ⁶	28.5

^a Cells and culture media from three independent experiments were collected at the indicated times postinfection, and yields were determined as described in Materials and Methods. Relative fold decrease is the ratio of yield on control cells to knockout cells.

was comparable in the three cell types (Fig. 1B, lanes 2, 5, and 8). In contrast, accumulation of the L proteins VP16 and gC was considerably reduced in both knockout cell lines (Fig. 1B, compare lanes 2 and 5 and lanes 8 and 11). Here we observed decreases in VP16 of 5- to 6-fold and decreases in gC of 6- to 10-fold, respectively, in IKK α ^{-/-} and IKK β ^{-/-} lysates compared to those of WT.

Next, we determined virus yields in cell lines derived from normal MEFs compared to those of IKK α ^{-/-} and IKK β ^{-/-} MEFs. Cultures were harvested at 16 and 24 hpi, and infectious virus was titered on Vero cell monolayers. Results, presented in Table 1, indicated that both IKK α ^{-/-} and IKK β ^{-/-} deficient cells were impaired in supporting virus replication, because virus yield at 24 h was reduced 6- to 28-fold compared to that of normal cells. Taken together, the results of yield assays in Table 1 are consistent with results of Western blots indicating that both IKK α ^{-/-} and IKK β ^{-/-} cells were impaired in nuclear translocation of p65 and in aspects of late viral protein accumulation.

We also prepared nuclear extracts from mock-infected, TNF- α -treated, and HSV-infected cells and performed EMSA to determine the effect of loss of IKK α or IKK β on NF κ B DNA-binding activity. In these cell lines we could detect an abundant NF κ B complex consisting of p50/p65 under conditions of TNF- α treatment or HSV-1 infection (Fig. 2B). Thus, incubation of lysates with a p65 antibody supershifted a relatively low-mobility complex, while an incubation with a p50 antibody supershifted both the relatively low-mobility complex and a higher mobility complex. Incubation with an irrelevant antibody to E2F served as a control. While the ability of p65 and p50 antibodies to shift complexes from TNF- α -treated cells appeared quantitative, a small amount of unshifted p50/p65 and p50/p50 complexes persisted following incubation of lysates from infected cells with p65 and p50 antibodies. The precise nature of these complexes is presently unknown. The results presented in Fig. 2A indicate that the absence of either of the IKK catalytic subunits resulted in decreased levels of nuclear NF κ B (p50/p65), consistent with their role in nuclear translocation of p50/p65. Interestingly, the amounts of p50 homodimer activity increased in all three cell types following HSV infection or TNF- α treatment (see Fig. 2B for a better example of p50 homodimer activity in WT cells). This latter result suggests that the SCF(β)-TrCP ubiquitin ligase required to initiate limited proteolysis of p105 to yield p50 is activated following both TNF- α treatment and HSV-1 infection (39).

FIG. 2. IKK α and IKK β contribute to NF κ B activation and efficient replication in HSV-infected cells. (A) EMSA for NF κ B DNA-binding activity in WT, IKK $\alpha^{-/-}$, and IKK $\beta^{-/-}$ MEFs was performed with extracts prepared from mock-infected cells (M), cells infected for 8 h with HSV (I), or cells treated for 15 min with TNF- α (T). (B) Extracts prepared from WT MEFs treated for 15 min with TNF- α or infected for 8 h with HSV were incubated with antibody to NF κ B subunits p50 or p65 or an irrelevant antibody against E2F4 prior to incubation with the NF κ B-specific oligonucleotide. Filled circles to the right of each lane identify supershifted complexes in response to incubation with anti-p50 or anti-p65. For both panels, only the portion of the autoradiograph containing specific complexes is shown.

I κ B α contributes to efficient replication of HSV. NF κ B is sequestered in the cytoplasm by I κ B. Following phosphorylation of I κ B α at serine residues 32 and 36, which triggers its polyubiquitylation and destruction by the 26S proteasome, NF κ B translocates into the nucleus. DNI κ B, in which these serines are mutated to alanines, is resistant to cytokine-induced turnover, and p50/p65 is retained in the cytoplasm (42). It was previously reported that infection of pooled clones of C33-A cells stably expressing DNI κ B resulted in reduced nuclear translocation of NF κ B and reduced virus yield compared to that of control cells (40). This experimental approach could not control for variable expression of DNI κ B among individual cells and could potentially accommodate some NF κ B activation and virus replication. We therefore employed infection with an adenovirus vector to transiently express DNI κ B in all cells in the culture. A549 cells were infected with an adenovirus vector expressing GFP (Ad-eGFP) or with adenovirus expressing DNI κ B (Ad-DNI κ B), and after 16 h the cells were superinfected with HSV for 8 h or were treated with TNF- α for 15 min prior to harvest. Figure 3A represents Western blots for I κ B α in cytoplasmic extracts. Infection with HSV or treatment with TNF- α of cells infected with Ad-eGFP resulted in the almost complete loss of endogenous I κ B α (Fig. 3A, lanes 2 and 3). A 1.45-fold decrease in I κ B α was detected in cells infected with Ad-DNI κ B and superinfected with HSV, consistent with loss of only the endogenous form of the protein. The total level

FIG. 3. Characterization of cells expressing DNI κ B. (A) Western blot for I κ B α and viral proteins. Replicate cultures of A549 cells were infected with a 100-fu/cell concentration of adenovirus vector expressing enhanced GFP (Ad-eGFP) or DNI κ B (Ad-DNI κ B). After 16 h, monolayers were mock infected (lanes 1 and 4), infected with HSV-1 at an MOI of 5 for 8 h (lanes 2 and 5), or treated with 50 ng of human recombinant TNF- α /ml (lanes 3 and 6) for 15 min prior to harvest. Cytoplasmic lysates were prepared and analyzed for I κ B α , gC, or α -tubulin by Western blot. Nuclear lysates were analyzed for ICP8. (B) EMSA for NF κ B binding activity. Nuclear lysates were prepared from A549 cells initially infected with either Ad-eGFP or Ad-DNI κ B, and then they were either mock infected, infected with HSV-1, or treated with TNF- α as described for cultures analyzed in the experiment presented in panel A. NS, nonspecific binding activity.

of I κ B α present following TNF- α treatment was unchanged compared to that of mock treatment (Fig. 3A, compare lanes 4 and 6). Whether this reflected minimal loss of endogenous I κ B α following treatment is unclear, but analysis of nuclear extracts by EMSA (Fig. 3B) indicated efficient suppression of NF κ B DNA-binding activity in cells infected with Ad-DNI κ B (compare lanes 1 to 3 with lanes 4 to 6). The effect of DNI κ B on viral gene expression was also analyzed. Lysates from control and DNI κ B lysates were probed for the DE protein ICP8 and the L protein gC and were quantified by Image J. While there was a twofold decrease in the amount of ICP8, the level of gC declined 6.5-fold in DNI κ B-expressing cells compared to that in the control lysate. These results were reminiscent of those presented in Fig. 1 for the effect on viral protein accumulation in cells lacking IKK α or IKK β .

We then determined the effect of DNI κ B expression on virus replication following a similar infection protocol. We infected murine 3T3 cells and the human tumor cell lines A549 and Hep2 with Ad-DNI κ B, followed by HSV superinfection. Production of progeny virus was determined by a plaque assay on Vero cells, and the results are presented in Tables 2 and 3. Two separate experiments involving murine fibroblasts were performed. Infectious virus was assayed at 16 and 24 hpi following low-MOI (0.2) infection or at 20 hpi following infection at an MOI of 0.2 or 1. Virus yields were reduced ~15- to 25-fold in DNI κ B-expressing cells compared to that of Ad-eGFP control cells (Table 2). Following low-MOI (0.2) infection of A549 or Hep2 cells, virus yield was reduced to 1,300-fold (Table 3). When the input MOI of A549 cells was increased to 1, the fold decrease in virus yield was reduced accordingly (42- to 62-fold) and still represented a significant decrease of 18-fold even at an MOI of 5. Replication of HSV in Hep2 cells was extremely sensitive to expression of DNI κ B. Even at an MOI of 5, virus yield was reduced almost 800-fold (Table 3).

TABLE 2. Virus yield in WT MEFs^a

Expression vector and expt no.	Results at 16 hpi		Results at 20 hpi		Results at 24 hpi	
	Yield (PFU/culture)	Fold decrease	Yield (PFU/culture)	Fold decrease	Yield (PFU/culture)	Fold decrease
Expt 1						
Ad-eGFP (HSV MOI = 0.2)	1.4×10^7				2.5×10^7	
Ad-DN κ B (HSV MOI = 0.2)	2.3×10^6	6			1.7×10^6	14.7
Expt 2						
Ad-eGFP (HSV MOI = 0.2)			1.95×10^7			
Ad-DN κ B (HSV MOI = 0.2)			7.4×10^5	26.4		
Ad-eGFP (HSV MOI = 0.1)			3.7×10^7			
Ad-DN κ B (HSV MOI = 0.1)			2.23×10^6	16.6		

^a Cells and culture media were collected at the indicated time points, and infectious titers were determined as described in Materials and Methods. Relative fold increase in virus yield is the ratio of yield from control cultures to yield from DN κ B-expressing cultures.

PKR is not necessary for NF κ B activation in HSV-infected cells. Because HSV induces IKK activity (1) and because IKK α and IKK β both contribute to NF κ B activation by HSV-1 (Fig. 1 and 2), we next asked whether PKR, a stress- and virus-induced kinase, is necessary for NF κ B activation and efficient virus replication. The double-stranded RNA-activated protein kinase, PKR, activates NF κ B by stimulating IKK activity and inducing turnover of I κ B α and I κ B β (57), though it is not known whether the critical serine residues on I κ B α and I κ B β are phosphorylated (21, 33). In fibroblasts from PKR-deficient (PKR^{0/0}) mouse embryos, double-stranded RNA, alpha interferon, and gamma interferon failed to activate NF κ B, while TNF- α still functioned as an inducer (57). PKR is known to be activated with late kinetics in cells infected with WT HSV-1 and γ 1 34.5 mutants of HSV-1, but it is not known whether this is via autophosphorylation or by a virus-encoded or -induced kinase (8). PKR-dependent phosphorylation of eIF2 α is reversed in virus-infected cells through the action of the γ 1 34.5 protein (16). Recently, NF κ B activation in HSV-infected cells was reported to be dependent on PKR, based on infection of PKR^{+/+} and PKR^{0/0} MEFs (43). MEFs from normal mice (PKR^{+/+}) or mice with a targeted deletion of PKR (PKR^{0/0}) (54) were infected with HSV, and virus yields at 16 and 24 hpi

were determined by plaque assay (Table 4). Virus yields from PKR^{0/0} cells were similar at 16 h and were approximately fourfold increased at 24 h relative to yields from PKR^{+/+} cells. EMSA for NF κ B DNA-binding activity from nuclear extracts of PKR^{+/+} and PKR^{0/0} cells are presented in Fig. 4A. Little NF κ B activity was detectable in the extract from mock-infected PKR^{+/+} cells, while both TNF- α treatment and HSV infection induced NF κ B activities consisting of p50/p65 or operationally defined as p50 homodimer, based on effects of p65 and p50 antibodies on the mobility of the complexes (data not shown). Mock-infected PKR^{0/0} cells constitutively expressed the p50 activity, which increased substantially following HSV infection or TNF- α treatment. HSV infection also induced p50/p65 activity comparable to that seen in the PKR^{+/+} cells, while TNF- α induction was impaired. Finally, we asked whether the absence of PKR affected the turnover of I κ B α normally observed after virus infection. Equivalent amounts of cell lysate were separated by SDS-PAGE and probed by Western blot (Fig. 4B). Amounts of I κ B α were considerably reduced after HSV infection of both PKR^{+/+} and PKR^{0/0} cells, while the amount of ICP4 detected was greater in the PKR^{0/0} lysate. Together, these results indicate that PKR is not essential for HSV-induced NF κ B activation, but it does contribute to NF κ B

TABLE 3. Virus yield in A549 and Hep2 cells expressing DN κ B^a

Cell type, expression vector, and expt no.	Results at HSV MOI of 0.2		Results at HSV MOI of 1.0		Results at HSV MOI of 5.0	
	Yield (PFU/culture)	Fold decrease	Yield (PFU/culture)	Fold decrease	Yield (PFU/culture)	Fold decrease
A549						
Expt 1						
Ad-eGFP	4.1×10^8		4.1×10^8		4.5×10^8	
Ad-DN-I κ B	4.1×10^6	100	9.8×10^6	41.8	2.4×10^7	18.4
Expt 2						
Ad-eGFP	5.7×10^7		8.7×10^7		1.05×10^8	
Ad-DN-I κ B	2.13×10^5	267	1.41×10^6	61.7	5.6×10^6	18.7
Hep2						
Expt 1						
Ad-eGFP	1.3×10^7		7.8×10^7		1.5×10^8	
Ad-DN-I κ B	1×10^4	1,300	6.7×10^4	1,164	1.9×10^5	789

^a Cells and culture medium were collected, and infectious titers were determined as described in Materials and Methods. Cultures were harvested at 24 hpi. Relative fold decrease in virus yield is the ratio of yield from control cultures to yield from DN-I κ B expressing cultures. The analysis of virus yield in Hep2 cells was performed once.

TABLE 4. Effect of loss of PKR on virus yield^a

Cell type	Results at 16 hpi		Results at 24 hpi	
	Yield (PFU/culture)	Fold decrease	Yield (PFU/culture)	Fold increase
PKR ^{+/+}	1.65 × 10 ⁷		2.2 × 10 ⁷	
PKR ^{0/0}	1.25 × 10 ⁷	1.3	9.0 × 10 ⁷	4

^a Monolayers of PKR^{+/+} and PKR^{0/0} cells were infected with HSV at an MOI of 5. Cells and culture media were collected at the indicated times postinfection, and infectious titers were determined as described in Materials and Methods. Virus yield on these cell types was analyzed once.

activation by TNF-α. It remains possible that PKR can have offsetting effects on virus replication. For example, PKR may contribute to NFκB activation when present, but in its absence the pathway leading to eIF2α phosphorylation is disrupted. At this time we cannot rule out the possibility that the greatly increased amount of p50 homodimer also contributes to efficient virus replication (see Discussion). The basis for the differences between our results and those previously published (43) is unclear, though they could reflect differences in HSV strain or the time of harvest postinfection.

NFκB promotes an antiapoptotic response in HSV-infected cells. HSV efficiently suppresses apoptosis (3, 4, 19, 24, 25, 62), and one recent report pointed to activation of NFκB as an important mediator of this effect (15). NFκB has a well-documented role in suppressing apoptosis under a variety of conditions (5, 31, 48, 50). One way in which the NFκB activation pathway might be linked to enhanced virus yields would be through the mechanism of apoptosis suppression. To test this model we asked whether expression of DN-IκB eliminated apoptosis suppression by HSV. We assayed for cell surface phosphoserine by staining with fluorochrome-conjugated Annexin V and for cleavage of PARP (Fig. 5). Parallel cultures of Hep2 cells were infected with Ad-DN-IκB, followed at 16 h by super-

FIG. 4. PKR is not necessary for NFκB activation in HSV-infected cells. (A) EMSA for NFκB was performed as described in Materials and Methods with nuclear extracts prepared from mock-infected, TNF-α-treated, or HSV-infected PKR^{+/+} and PKR^{0/0} MEFs. (B) Western blot of IκBα and IE ICP4 was performed on whole-cell extracts prepared from mock-infected (M) or HSV-infected (I) PKR^{+/+} and PKR^{0/0} MEFs.

FIG. 5. NFκB promotes an antiapoptotic response in HSV-infected cells. (A) Replicate monolayers of Hep2 cells were infected with an adenovirus vector expressing DN-IκB from a cytomegalovirus promoter at the indicated MOI. After 24 h, monolayers were either mock infected or infected with HSV at an MOI of 5. At 24 hpi cells were harvested, stained for Annexin V, and processed for FACS analysis as described in Materials and Methods. (B) Parallel cultures of Hep2 cells were infected with adenovirus vector expressing DN-IκB at the indicated MOIs. After 24 h, monolayers were mock infected or infected with WT HSV-1 or the ICP27 null mutant d27-1 at an MOI of 5. Whole-cell extracts were prepared at 24 hpi for Western blot analysis of PARP.

infection with HSV. Uninfected cultures or cultures receiving Ad-DN-IκB but not superinfected with HSV served as controls, and all cultures with HSV were harvested at 24 hpi. The fraction of HSV-infected cells stained with Annexin V increased from ~20 to ~43% by 24 hpi as the MOI of Ad-DN-IκB increased from 0 to 200 focus-forming units (ffu)/cell (Fig. 5A). By contrast, only 7% of cells infected with 300 ffu of Ad-DN-IκB but not superinfected with HSV displayed cell surface Annexin V staining. In a second experiment, we infected Hep2 cells with different amounts of Ad-DN-IκB, followed by mock infection, infection with WT HSV-1, or infection with the ICP27 mutant d27-1. The latter virus has been reported to efficiently induce apoptosis in human cells (3, 4). Lysates were prepared and assayed for PARP by Western blot, and the results are shown in Fig. 5B. Whereas unprocessed (~116 kDa) PARP was present in all lysates, increased amounts of processed PARP (85-kDa) were present in lysates of WT HSV-infected cells only when DN-IκB was expressed (lanes 3 to 4) or in lysates from all cultures infected with d27-1 (lanes 6 to 9).

DISCUSSION

Previous studies have described NFκB activation after HSV infection (1, 15, 40). Results of studies reported here extend this observation, documenting the roles of upstream effectors of NFκB in this process as well as in efficient virus replication. Specifically, IKKα and IKKβ as well as IκBα are critical for

efficient activation, as measured by nuclear localization of p65 and increases in nuclear NF κ B-dependent EMSA activity. Though activated after HSV infection, no role for PKR in NF κ B activation was observed. As measured by either accumulation of specific viral proteins or production of infectious virus, the intact IKK-I κ B α -NF κ B pathway contributed to efficient virus replication. This efficiency of virus replication may in part reflect the suppression of apoptosis by a mechanism requiring activation of the NF κ B. It was previously reported (40) that nuclear translocation of NF κ B after HSV infection was not accompanied by NF κ B-dependent promoter activation. Amici and coworkers (1) reported functional activation of NF κ B after infection, and the work of Goodkin et al. (15) and work presented here imply that NF κ B is functional and participates in the program of apoptosis suppression following virus infection. We attribute the previous inability to detect NF κ B activity largely to the cell type (C33-A) and the chloramphenicol acetyltransferase reporter used in previous studies. We can readily detect functional NF κ B in HeLa and HEK293T cells following infection by using a 3XNF κ B luciferase-based reporter (D. Hargett, J. Prince, and S. Bachenheimer, unpublished observations).

The requirement for IKK in NF κ B activation during HSV infection of murine fibroblasts largely mirrors its requirement for activation by TNF- α . Thus, loss of cytoplasmic I κ B α and nuclear accumulation of p65 were either modestly impaired (IKK α ^{-/-}) or largely impaired (IKK β ^{-/-}) relative to normal cells, and NF κ B-dependent EMSA activity was greatly reduced. It has been reported that IKK β plays a critical role (27, 29), while IKK α plays a redundant (18, 26, 45) or distinct (28, 41) role, in cytokine-mediated NF κ B activation in murine cells. By using an RNA interference approach, both IKK catalytic subunits were shown to play critical roles in cytokine-induced NF κ B in human cells (44). Future experiments will be directed at understanding whether a requirement for both IKK α and IKK β also holds for NF κ B activation by HSV in human cells. Both IKK α ^{-/-} and IKK β ^{-/-} cells supported comparable amounts of IE ICP27 and DE ICP8 accumulation relative to normal cells, but they had greatly reduced expression of L proteins VP16 and gC. These results are consistent with the reduced yield of infectious virus from these cell lines and with previous reports on virus yield (1, 40).

Infection of murine fibroblasts deleted for PKR resulted in NF κ B-dependent EMSA activity and yield of infectious virus comparable to or greater than that seen following infection of normal cells. We also noted constitutive expression of a p50 homodimer form of NF κ B and its substantial increase following either TNF- α treatment or HSV infection. While p50 homodimers would not be expected to have transcriptional transactivation potential, Bcl-3, an I κ B α family member, complexes with and relocates p50 homodimers to the nucleus, where it can also serve as a transcriptional coactivator of p50 homodimers (9, 13). Thus, it remains possible that increased p50 homodimer formation in part mediates efficient HSV replication in PKR^{0/0} cells and that preventing p50/p65 activation by, for example, ectopic expression of DN I κ B may not reduce HSV replication.

HSV infection induces and then efficiently suppresses apoptosis. Consistent with the role of NF κ B as a cell survival factor, there is a strong correlation between the ability of HSV

to express functional ICP27 and the suppression of apoptosis (3) and between expression of NF κ B and suppression of apoptosis (15). Coupled with the requirement for functional ICP27 to activate NF κ B (D. Hargett and S. Bachenheimer, submitted for publication), we propose that a delayed-type activation of NF κ B, dependent on viral gene expression, plays an important role in prolonging functional cell survival and, thus, efficient virus replication. This delayed-type activation has also been observed following infection of cells with human cytomegalovirus (HCMV) (55) and was termed second-tier activation. Another mechanism of virus-induced NF κ B activation is inferred from experiments which characterized the ability of HSV-1 to both trigger and inhibit apoptosis (4, 25). A key finding in the recognition of a rapid-type activation of NF κ B stemmed from experiments which demonstrated that ectopic expression of virion glycoproteins gD or gJ could rescue cells from apoptosis following infection with virus deleted for the gD gene (61, 62). Interestingly, the domains of gD involved in blocking apoptosis for the most part do not overlap those domains involved in binding HveC, the ubiquitously expressed gD receptor (60). More recently, either infection with UV-inactivated HSV or exposure of cells to gD was shown to protect cells from Fas-mediated apoptosis by a mechanism that was dependent on NF κ B activation (34). One inference that could be drawn from both these experiments was that the protective effect of gD occurred at the time of virus binding and entry and, thus, did not have a requirement for de novo viral gene expression. This situation is similar to that of the first-tier activation of NF κ B observed during infection with HCMV (56) and suggests that two mechanistically distinct mechanisms of NF κ B activation occur during HSV infection.

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