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Herpes Simplex Virus Disrupts NF-κB Regulation by Blocking Its Recruitment on the IκBα Promoter and Directing the Factor on Viral Genes*

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Herpes simplex viruses (HSVs) are able to hijack the host-cell IκB kinase (IKK)/NF-κB pathway, which regulates critical cell functions from apoptosis to inflammatory responses; however, the molecular mechanisms involved and the outcome of the signaling dysregulation on the host-virus interaction are mostly unknown. Here we show that in human keratinocytes HSV-1 attains a sophisticated control of the IKK/NF-κB pathway, inducing two distinct temporally controlled waves of IKK activity and disrupting the NF-κB autoregulatory mechanism. Using chromatin immunoprecipitation we demonstrate that dysregulation of the NF-κB-response is mediated by a virus-induced block of NF-κB recruitment to the promoter of the IκBα gene, encoding the main NF-κB-inhibitor. We also show that HSV-1 redirects NF-κB recruitment to the promoter of ICP0, an immediate-early viral gene with a key role in promoting virus replication. The results reveal a new level of control of cellular functions by invading viruses and suggest that persistent NF-κB activation in HSV-1-infected cells, rather than being a host response to the virus, may play a positive role in promoting efficient viral replication.

Herpes simplex virus type 1 (HSV-1)[4] represents a prototype for understanding the fundamental replication mechanisms of herpesviruses, a large family of medically important double-stranded DNA viruses. As other members of the family, HSV-1 can establish productive and latent infections (1). During productive infection HSV-1 efficiently redirects the host transcriptional machinery to express its own genes in a tightly regulated temporal cascade, consisting of the sequential expression of three gene classes: the immediate-early (IE), delayed-early (DE) and late (L) genes. The five IE genes are expressed shortly after entry into the host cell, and the resulting IE proteins (infected cell proteins ICP-0, -4, -22, -27, and -47) are essential for the subsequent temporally controlled expression of DE genes, the majority of which encode proteins involved in viral DNA replication, as well as of later L genes, which encode predominantly structural proteins. In particular, the multifunctional phosphoprotein ICP0 acts as a strong activator of all classes of HSV-1 genes, as well as of other eukaryotic genes (1). The molecular mechanism responsible for ICP0 transactivating activity is not yet understood. No specific DNA-binding sequence for ICP0 could be identified, and the transactivating activity seems to be dependent on one or more of the different functions of the ICP0 protein (2). The facts that ICP0-negative mutants grow poorly in most tissue systems and are reactivation-impaired indicate that adequate ICP0 activity confers a growth advantage and is essential to promote initiation of the lytic-phase transcriptional events (1).

Several distinct cis-acting elements are important for ICP0 expression during productive infection (3). In addition to the transactivating activity of the virion VP16 protein-induced complex, ICP0 expression can be modulated by a variety of host-transactivating factors, including the nuclear factor-κB (NF-κB).

NF-κB is a collective term referring to a class of dimeric transcription factors consisting of homo- and heterodimers of five structurally related Rel/NF-κB proteins (4). In most cells NF-κB exists as an inactive cytoplasmic complex, whose predominant form is a heterodimer composed of p50 and p65/RelA subunits, bound to inhibitory proteins of the IκB family, including IκBα, IκBβ, and IκBε (5). IκB proteins consist of an N-terminal regulatory domain followed by a series of ankyrin repeats important in the binding to the NF-κB heterodimer. The interaction with IκB masks the nuclear localization sequence in the NF-κB complex, sequestering the factor in the cytoplasmic compartment. Different stimuli for NF-κB activation initiate different signal transduction pathways most of which converge on the IκB kinase (IKK) signalosome that plays a major role in NF-κB activation (6). IKK is a multisubunit complex, containing two catalytic subunits (IKK-α and IKK-β), which are able to form homo- or heterodimers, and the IKK-γ or NEMO regulatory subunit, which is not a kinase *per se*, but acts as a docking protein for IKK kinases or other signaling proteins (7). Following stimulation, the NF-κB/IκB complex is activated via the phosphorylation of the inhibitory protein. In the case of IκBα, IKK-mediated phosphorylation occurs at serine residues 32 and 36 in the N-terminal portion of the molecule (6). Phosphorylation targets IκBα for ubiquitination by the β-TrCP (transducin repeat-containing protein)-containing SCF (Skp1-Cul1-F-box-protein) ubiquitin ligase complex at lysines 21 and 22, which leads to degradation of the inhibitory subunit by the 26 S proteasome, allowing the release of NF-κB. Following the degradation of the inhibitory protein and exposure of the nuclear localization sequence motif, freed NF-κB dimers translocate to the nucleus and bind to DNA

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[4] The abbreviations used are: HSV-1, herpes simplex virus type 1; ChIP, chromatin immunoprecipitation; $CPE_{50\%}$, 50% cytopathic effect; ICP0, infected cell protein 0; IκBα, κB inhibitory protein α; IKK, IκB kinase; NF-κB, nuclear factor-κB; PGA_1, prostaglandin A_1; p.i., post infection; IE, immediate-early gene; DE, delayed-early gene; L, late gene; EMSA, electrophoretic mobility shift assay.

consensus sequences (κB elements), activating the transcription of several target genes, including the NF-κB-inhibitory protein IκBα, which provides a negative feedback mechanism to limit NF-κB activity (8). IκBα displays nucleocytoplasmic shuttling properties and, after NF-κB-dependent resynthesis, it enters the nucleus and promotes NF-κB removal from DNA, restoring the inducible pool of the transcription factor into the cytoplasm (9).

NF-κB was found to be activated early during HSV-1 infection (10, 11), and was shown to be involved in up-regulation of several host genes (12), as well as in promoting the progression of the virus replication cycle (11, 13). However, the mechanisms governing NF-κB activity in the nucleus of HSV-1-infected cells have still not been defined.

In the present report we show that, in its target cell, the human keratinocyte, HSV-1 infection induces two separate, temporally controlled waves of IKK activity with distinct characteristics. For the first time we demonstrate that the virus recruits NF-κB to the ICP0 promoter, enhancing ICP0 gene transcription. We also demonstrate that, during the second wave of IKK activity, the virus disrupts the NF-κB autoregulatory loop, by interfering with the recruitment of the factor to the IκBα promoter. The results give new insights on how viruses have evolved sophisticated control mechanisms to redirect the cellular signaling machinery to their own advantage.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Virus Infection—Human HaCaT keratinocytes were grown at 37 °C in a 5% CO_2 atmosphere in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics. Transfections were carried out using Lipofectamine Plus (Invitrogen) according to the manufacturer's protocols. HaCaT cell monolayers were infected for 1 h at 37 °C with HSV-1 strain F1 at a multiplicity of infection of 10 plaque forming units/cell, unless stated otherwise. Virus titers were determined by plaque assay or by cytopathic effect 50% ($CPE_{50\%}$) assay on confluent VERO cell monolayers (11). Inactivated HSV-1 virus was prepared by exposure to UV light (254-nm wavelength) on ice for 15 min. UV exposure reduced HSV-1 infectivity by $>10^6$-fold, as verified by plaque assay. Prostaglandin A_1 (PGA_1, Cayman Chemicals) was added after the 1-h adsorption period and maintained in the medium for the duration of the experiment. Statistical analysis was performed using Student's t test for unpaired data. Data were expressed as the mean ± S.E., and p values of <0.05 were considered significant.

Plasmid Construction and Generation of a Keratinocyte Cell Line with Stably Integrated ICP0-promoter (HaCaT-ICP0-Luc)—A fragment of the ICP0 promoter spanning from −809 to +150 derived from the pIE1-CAT vector (kind gift of Dr. R. D. Everett) was subcloned into the PGL3 basic vector (Promega). To obtain HaCaT cells, which presented the HSV-1 ICP0-promoter integrated in their chromatin (HaCaT-ICP0-Luc), the PGL3-ICP0 promoter vector was cotransfected with pBABE-puro plasmid, and stable integrants were selected by using puromycin (1 μg/ml) for 12 days. Selected pools of HaCaT cells were tested for luciferase induction after HSV-1 infection. For the construction of the pcDNA3-ICP0-vector used in the run-on experiments, the cDNA corresponding to sequence 1494–2152 of the first ICP0-exon was obtained by amplifying the viral HSV-1 DNA using synthesized primers 5′-GGATGTCTGGGTGTTTCCCTGC-3′ (1494–1515, sense) and 5′-CGTCGTCCAGGTCGTCGTCATCC-3′ (2130–2152, antisense). The fragment was subcloned into the pcDNA3 vector EcoRV site, and the construct was confirmed by DNA sequencing.

Electrophoretic Mobility Shift Assay—Whole cell extracts (15 μg) prepared after lysis in a high salt extraction buffer (14) were incubated with ^{32}P-labeled κB DNA probe (15) followed by analysis of DNA-binding activity by EMSA. Binding reactions were performed as described previously (15). Complexes were analyzed by nondenaturing 4% polyacrylamide gel electrophoresis. Specificity of protein-DNA complexes was verified by immunoreactivity with polyclonal antibodies specific for p65 (Rel A). Quantitative evaluation of NF-κB-DNA complex formation was determined by Typhoon 8600 imager (Molecular Dynamics PhosphorImager, MDP) with the use of ImageQuaNT (MDP analysis).

Kinase Assay and Western Blot Analysis—Cell lysates were incubated with anti-IKKα antibodies in the presence of 15 μl of protein-A-Sepharose at 4 °C for 12 h. After washing, endogenous IKK activity was determined using GST-IκBα-(1–54) as substrate (11). For immunoblot analysis, equal amounts of protein (40 μg/sample) from HaCaT whole cell extracts were separated by SDS-PAGE, blotted to nitrocellulose, and filters were incubated with polyclonal anti-IκBα/MAD3 (Santa Cruz Biotechnology), anti-IKKα, or anti-p65 antibodies followed by decoration with peroxidase-labeled anti-mouse or anti-rabbit IgG (ECL, Amersham Biosciences) (11). Filters were analyzed by Versadoc-1000 system (Bio-Rad) for protein quantitative determination.

Transcriptional Run-on Assay and Protein Synthesis—In vitro run-on transcription reactions were performed in isolated HaCaT nuclei as previously described (16). RNA, ^{32}P-labeled, was hybridized with nitrocellulose filters containing plasmids for HSV-1 ICP0 (ICP0-pcDNA3), human IκBα (IκBα-pcDNA3), or glyceraldehyde-3-phosphate dehydrogenase gene, as a control. Following hybridization, filters were visualized by autoradiography, and the radioactivity was quantified by MDP analysis. For determination of protein synthesis, cells were pulse-labeled with [^{35}S]methionine (10 μCi/10^5 cells, 45-min pulse) and lysed in L buffer (20 mM Tris-Cl, pH 7.4, 0.1 M NaCl, 5 mM $MgCl_2$, 1% Nonidet P-40, 0.5% SDS). Samples containing the same amount of radioactivity were separated by SDS-PAGE (3% stacking gel, 10% resolving gel) and processed for autoradiography (11).

Chromatin Immunoprecipitation Assay—HaCaT cells were fixed by adding formaldehyde (Sigma) to the medium to a final concentration of 1%. After 15 min, cells were washed with ice-cold phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride and scraped. After centrifugation, cells were lysed in L1 buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, and protease inhibitors) and centrifuged for 5 min at 3,000 rpm at 4 °C. After removal of supernatants, nuclei were resuspended in L2 buffer (50 mM Tris, pH 8.0, 1% SDS, 5 mM EDTA), and chromatin was sheared by sonication. After removal of nuclear debris by centrifugation at 13,000 rpm for 5 min at 8 °C, lysates were diluted 10-fold with DB buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 200 mM NaCl, 0.5% Nonidet P-40) and then precleared for 3 h using 80 μl of 50% salmon sperm-DNA-saturated protein A-agarose beads.

Immunoprecipitation was carried out at 4 °C overnight, and immune complexes were collected with salmon sperm-DNA-saturated protein A-agarose beads. Antibodies utilized included anti-p65 (Santa Cruz Biotechnologies) or pre-immune rabbit serum as control for nonspecific interaction. After washing three times with high salt WB buffer (20 mM Tris, pH 8.0, 0.1% SDS, 1% Nonidet P-40, 2 mM EDTA, 0.5 M NaCl) and twice with low salt TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), immunocomplexes were eluted with TE containing 1% SDS. Protein-DNA cross-links were reversed by incubating at 65 °C overnight. After proteinase K digestion, DNA was extracted with phenol-chloroform and precipitated with ethanol using 15 μg of tRNA as carrier. PCR was performed (30 cycles, denaturing at 94 °C for 45 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s) using primers specific for the human IκBα promoter IκBα-proS (5′-ACTTGCAGAGGGACAGGA-

TTACAG-3′) and IκBα-proAS (5′-AGGCTCGGGGAATTTCCAAG-3′); for the ICP0 viral promoter ICP0-proS (5′-TAATGGGGTTCTT-TGGGGGACACC-3′) and ICP0-proAS (5′-TGCAAATGCGACCA-GACTGTC-3′); for the ICP8 viral promoter ICP8-proS (5′-AGCACC-TGACCGTAAGCATCTG-3′) and ICP8-proAS (5′-CTTTGTCTCC-ATGTCCTCCTGG-3′). To discriminate the integrated form of the ICP0 promoter from the non-integrated viral promoter in the ChIP analysis, we have utilized the same upstream primer ICP0-proS but different downstream primers: the internal ICP0 gene primer ICP0-AS1 (5′-CATGGCGGCCGGTTCCAGTGTAAGG-3′) for the viral form and the Luc-AS primer (5′-TCCATCTTCCAGCGGATAGAATGG-3′) for the integrated ICP0-Luc fragment, respectively.

RESULTS

HSV-1 Induces a Biphasic Wave of IKK-dependent NF-κB Activation in Human Keratinocytes—To investigate how herpes simplex viruses modulate the IKK/NF-κB pathway in human keratinocytes, confluent HaCaT cell monolayers were infected with HSV-1 for 60 min at 37 °C. After this time, the virus inoculum was removed and cells were incubated at 37 °C in Dulbecco's minimum essential medium supplemented with 2% fetal calf serum. Control cells were treated identically. In a parallel experiment, cell monolayers were exposed to infectious or UV-inactivated HSV-1, to determine whether virus replication was necessary for NF-κB activation. Immediately after the adsorption period (time 0) and at different times post infection (p.i.), whole cell extracts were prepared and analyzed for IKK activity by kinase assay, IκBα degradation by immunoblot analysis, and NF-κB activation by EMSA. In human keratinocytes, HSV-1 infection was found to activate IKK in a biphasic way (Fig. 1*A*, *upper panels*). During virus entry, a first wave of IKK activity was observed. IKK activation at this time was rapid, transient, and independent of virus replication, because it occurred also after exposure of cells to UV-inactivated virions (Fig. 2). Induction of IKK function was rapidly followed by IκBα degradation (Fig. 1*A*, *middle panels*) and triggering of NF-κB DNA-binding activity, which lasted for ~2 h (Fig. 1*A*, *lower panels*). As expected, transcriptionally active NF-κB switched on IκBα resynthesis, rapidly restoring the intracellular pool of the inhibitory protein and, consequently, activating the NF-κB autoregulatory turn-off signal (Fig. 1*A*, *middle panels*). At later times (3 h) p.i., HSV-1 infection induced a second wave of IKK activity (Fig. 1*A*, *upper panels*), which, differently from the early transient phase of induction, persisted at elevated levels for several hours p.i. This second wave of IKK activity was dependent on active virus replication, because UV-inactivated virus particles were unable to turning it on (Fig. 2). The second wave of IKK activity also led to complete IκBα degradation, and it induced massive and persistent NF-κB activation (Fig. 1*A*, *middle* and *lower panels*); however, no detectable IκBα resynthesis was observed up to 24 h p.i. (Fig. 1*B*), suggesting that HSV-1 could interfere with the NF-κB autoregulatory loop at this stage of infection. In the absence of the inhibitory protein, NF-κB remained in the activated DNA-binding state for at least 24 h in HSV-1-infected keratinocytes (Fig. 1*B*).

Recruitment of NF-κB Dimers to the IκBα Promoter Is Impaired during Lytic HSV-1 Infection—To investigate whether the lack of IκBα resynthesis following the second wave of HSV-1-induced IKK activity was merely the consequence of a general shut-off of cell protein synthesis after viral infection, HaCaT cells were infected with HSV-1, and, at different time intervals, cellular and viral protein synthesis was determined by SDS-PAGE and autoradiography, after [^{35}S]methionine labeling. In parallel samples, whole cell extracts were analyzed by EMSA for NF-κB activity and by Western blot for detection of IκBα. As expected,

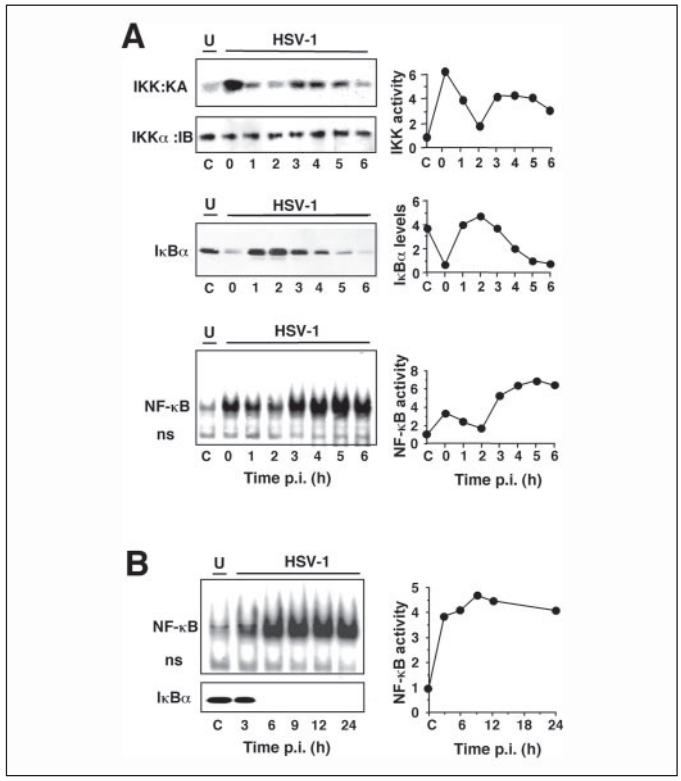

FIGURE 1. **HSV-1 induces a biphasic wave of IKK-dependent NF-κB activation in human keratinocytes.** Confluent monolayers of HaCaT cells were mock infected (*U*) or infected with HSV-1 (10 plaque forming units/cell) (HSV-1) for 1 h at 37 °C. *A*, at the end of the adsorption period (time 0) and at different times post infection (*p.i.*), whole cell extracts were analyzed for IKK activity and recovery by kinase assay (*KA*) and immunoblotting (*IB*), respectively (*upper panels*), for IκBα degradation by Western blot analysis (*middle panel*), and for NF-κB activation by EMSA (*lower panel*). Sections of fluorograms from native gels are shown (*right panels*). Positions of NF-κB-DNA (*NF-κB*) and nonspecific protein-DNA (*ns*) complexes are indicated. Levels of IKK and NF-κB activity were quantified by MDP analysis and expressed as fold induction of the levels detected in uninfected control cells (*C*, *left panels*). IκBα protein levels were quantified by densitometric analysis and expressed as arbitrary units. *B*, in a parallel experiment, levels of IκBα (*lower panel*) and NF-κB activity (*upper panel*) were determined up to 24 h after HSV-1 infection (*right panels*). Levels of NF-κB DNA-binding activity were quantified by MDP analysis and expressed as -fold induction of uninfected control (*C*, *left panel*).

two peaks of NF-κB activity were detected immediately after the adsorption period and at 3 h p.i., respectively (Fig. 3*A*, *filled circles*).

As determined by [^{35}S]methionine incorporation into trichloroacetic acid-insoluble material, HSV-1 was found not to significantly alter protein synthesis in the host cell up to 6 h after infection (Fig. 3*A*, *open circles*). In addition, analysis of autoradiographic patterns after SDS-PAGE separation of labeled proteins did not reveal major differences in cellular protein synthesis in infected cells up to 6 h p.i. (data not shown), excluding the possibility that the lack of IκBα could be the consequence of a general protein synthesis shut-off at this time.

The kinetics of the IκBα gene transcription was then analyzed by *in vitro* run-on assay on nuclei isolated from duplicate samples. As shown in Fig. 3*B* (*upper panel*), IκBα transcription was rapidly induced upon virus entry. The transcription rate attained a 5-fold induction at the end of the virus adsorption period, leading to IκBα resynthesis at this time (Fig. 3*B*, *lower panels*) and declined thereafter to reach basal levels at 2 h p.i. However, IκBα gene transcription was not observed at later times p.i., even when NF-κB DNA-binding activity had reached maximal levels (Fig. 3, compare *A* and *B*), indicating that HSV-1-induced NF-κB complexes are unable to transactivate this cellular target gene at this stage of infection. Supershift assay using antibodies against various members of the Rel/NF-κB family identified p65 as a component of the

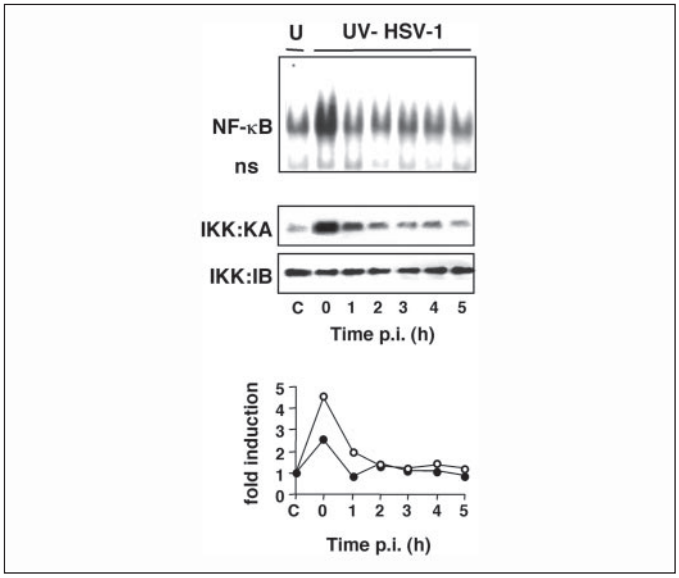

FIGURE 2. **Induction of IKK and NF-κB activity by UV-inactivated HSV-1 in HaCaT cells.** HaCaT cells were exposed to UV-inactivated HSV-1 for 1 h at 37 °C. At the end of the adsorption period (time 0) and at different times p.i., whole cell extracts from uninfected (*U*), or UV-inactivated-HSV-1-infected (*UV-HSV-1*) cells were assayed for NF-κB activation by EMSA (*upper panel*), and for IKK activity and recovery by kinase assay (*KA*) and immunoblotting (*IB*), respectively (*middle panels*). Sections of fluorograms from native gels are shown. Levels of IKK (○) and NF-κB (●) activity were quantified by MDP analysis and expressed as -fold induction of uninfected control (*C*, *lower panel*).

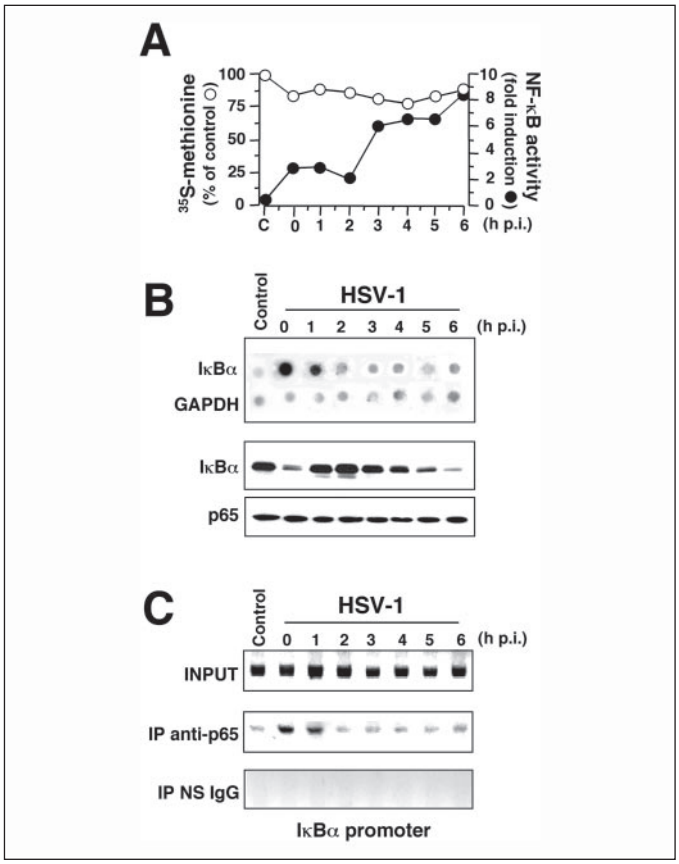

FIGURE 3. **IκBα gene transcriptional regulation in HSV-1-infected keratinocytes.** *A*, mock infected or HSV-1-infected HaCaT cells were labeled with [^{35}S]methionine at different time intervals. Protein synthesis was determined by [^{35}S]methionine incorporation into trichloroacetic acid-insoluble material. Data, expressed as percent mock infected control (*C*), represent the mean of duplicate samples (○). Whole cell extracts from parallel samples were assayed for NF-κB activity by EMSA. NF-κB DNA-binding activity levels were quantified by MDP analysis and expressed as -fold induction of mock infected control (●). *B*, in parallel samples, IκBα mRNA transcription rates were measured by *in vitro* run-on assay performed on isolated nuclei from HSV-1-infected (*HSV-1*) or mock infected (*Control*) cells. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA transcription was determined as control (*upper panel*). IκBα levels (*IκBα*) were analyzed by immunoblotting. Levels of p65/RelA protein (*p65*) were determined as loading control (*lower panels*). *C*, recruitment of p65/RelA to the IκBα promoter in HSV-1-infected cells was analyzed at different times p.i. by ChIP assay using anti-p65 rabbit polyclonal antibodies. p65/RelA-coprecipitating DNA was analyzed by semiquantitative PCR with promoter-specific primers amplifying the IκBα promoter (*IP anti-p65*, *middle panel*). An unrelated rabbit polyclonal antiserum was used as control (*IP NS IgG*, *lower panel*). Genomic DNA obtained from mock infected and infected cells was employed to normalize the DNA subjected to immunoprecipitation (*INPUT*, *upper panel*).

DNA-binding complex (data not shown), excluding the possibility that the defect in IκBα gene transactivation could be due to the formation of transcriptionally inactive NF-κB dimers. On the other hand, control glyceraldehyde-3-phosphate dehydrogenase gene transcription levels were not affected by the virus up to 6 h p.i.

NF-κB recruitment to the IκBα promoter was then analyzed *in vivo* by ChIP assay in HSV-1-infected and mock infected keratinocytes at different times p.i. Formaldehyde cross-linked, sonicated chromatin fragments from HaCaT cells were immunoprecipitated with an affinity purified antibody against p65. DNA released from immunocomplexes was analyzed by semiquantitative PCR to detect an enrichment of the IκBα promoter in the immunoprecipitates. The rate of amplification was verified at all time points using cross-linked, not immunoprecipitated chromatin (Fig. 3*C*, *upper panel*, *INPUT*). The specificity of chromatin immunoprecipitation was determined by using a control unrelated antibody (Fig. 3*C*, *lower panel*, *NS IgG*). The virus entry process was found to induce a rapid recruitment of p65 to the IκBα gene promoter (Fig. 3*C*, *middle panel*), which, driving gene transcription, led to restoration of IκBα levels by 1 h p.i., as shown in Fig. 3*B*. *De novo* synthesized IκBα is known to induce the removal of p65 from its promoter switching off gene transcription. As expected, recruitment of p65 to the IκBα promoter ceased when IκBα levels went back to normal between 1 and 2 h p.i. (Fig. 3, *B* and *C*). Interestingly, p65 recruitment could not be detected on IκBα κB-elements at later times p.i., indicating that the defect in IκBα gene transcription is due to an impairment of NF-κB recruitment to the promoter of this target gene.

NF-κB Is Recruited to the Viral ICP0 Promoter during HSV-1 Lytic Infection—Several NF-κB binding elements have been described in the promoter region, as well as in the first intron sequence, of the HSV-1 ICP0 gene (17). However, little is known about the requirement of NF-κB for induction of ICP0 gene transcription.

We then investigated whether NF-κB is actually recruited to the viral ICP0 gene promoter. HaCaT cells were infected with HSV-1 and analyzed by ChIP assay using anti-p65 polyclonal antibodies. p65/RelA-coprecipitating DNA was analyzed by semiquantitative PCR with promoter-specific primers amplifying the ICP0 viral promoter (Fig. 4*A*, *IP anti-p65*). An unrelated rabbit polyclonal antiserum was used as control. The viral ICP8 promoter was also analyzed with specific primers in the p65-coprecipitating DNA. In a parallel experiment, viral ICP0 and cellular IκBα mRNA transcription rates were measured by *in vitro* run-on assay performed on isolated nuclei.

Similarly to the IκBα gene, p65 was found to be recruited to the ICP0 promoter rapidly after virus entry into the host cell (Fig. 4*A*, *ICP0*, *middle panel*). p65/RelA recruitment to the ICP0 promoter corresponded with a remarkable burst in ICP0 transcription (Fig. 4*B*), indicating that NF-κB bound to the viral enhancer may contribute significantly to ICP0 transcriptional activation. Interestingly, p65 recruitment to the ICP0 promoter was also detected at 2 h p.i., when IκBα resynthesis had been completed and NF-κB DNA-binding activity had partially declined. p65/RelA recruitment to the viral promoter persisted for several hours, accompanied by a further increase in ICP0 gene transcription, which

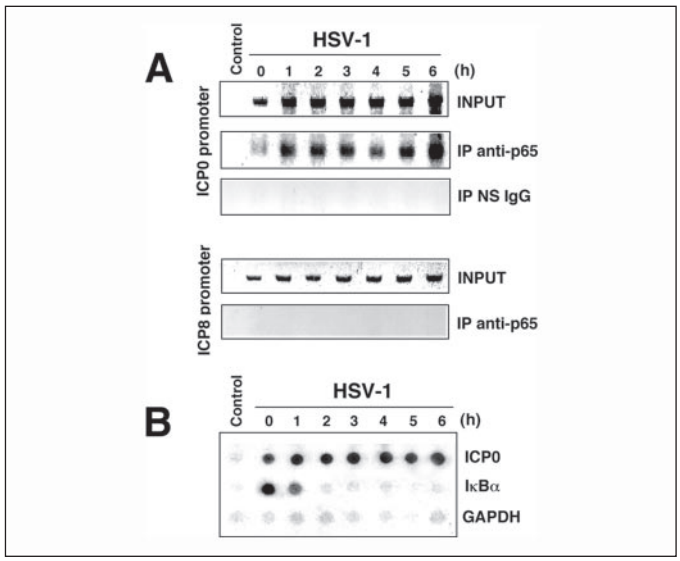

FIGURE 4. **Recruitment of HSV-1-induced NF-κB to the viral ICP0 promoter is associated with ICP0 mRNA transcription.** *A*, mock infected (*Control*) or HSV-1-infected (*HSV-1*) HaCaT cells were analyzed at different times p.i. by ChIP assay using anti-p65 polyclonal antibodies. p65/RelA-coprecipitating DNA was analyzed by semiquantitative PCR with promoter-specific primers amplifying the ICP0 viral promoter (*IP anti-p65*, *middle panel*). An unrelated rabbit polyclonal antiserum was used as control (*IP NS IgG*, *lower panel*). The viral ICP8 promoter (lacking NF-κB binding sites) was analyzed with specific primers in the p65-coprecipitating DNA (*IP anti-p65*, *lower panel*). Genomic DNA obtained from mock infected and infected cells was employed to normalize the DNA subjected to immunoprecipitation (*INPUT*, *upper panels* for ICP0 and ICP8 promoters). *B*, in a parallel experiment, viral ICP0 and cellular IκBα mRNA transcription rates were measured by *in vitro* run-on assay performed on isolated nuclei. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA transcription was determined as control.

reached maximal levels at 6 h p.i. No amplification of chromatin immunoprecipitated with the anti-p65 antibody was detected using primers to the ICP8 promoter, which lacks NF-κB consensus sequences, demonstrating the specificity of p65 occupancy on the ICP0 promoter (Fig. 4*A*, *lower panels*).

Viral ICP0 Promoter Shows Remarkable Avidity for NF-κB—To investigate whether the differences observed in NF-κB recruitment could be a consequence of the different status of viral and cellular DNA organization (episomal *versus* chromosomal), we have generated HaCaT cells (HaCaT-ICP0-Luc cells) in which the ICP0 promoter controlling the expression of the luciferase reporter gene is stably integrated into the chromatin structure. HaCaT-ICP0-Luc cells were infected with HSV-1, and, at different times post-infection, were processed for luciferase or ChIP analysis. As shown in Fig. 5*B*, HSV-1 infection induces luciferase activity in these cells, indicating that the ICP0-Luc promoter is transcriptionally activated by the virus. In the same experiment, NF-κB recruitment to the integrated and to the free viral ICP0 promoters was analyzed at the end of the virus adsorption period and at 5 h post-infection, which correspond to the first and second waves of NF-κB activation, respectively. To discriminate the integrated form of the ICP0 promoter from the non-integrated viral promoter in the ChIP analysis, we have utilized the same upstream primer but different downstream primers (Fig. 5*A*). As shown in Fig. 5*C*, at the end of the 1-h adsorption period, NF-κB is recruited to the IκBα promoter and to both forms (viral and integrated form) of the ICP0 promoter, indicating that during the first wave of NF-κB activation all the promoters analyzed behave similarly in respect to NF-κB recruitment. As expected, at 5 h p.i. NF-κB was not recruited to the IκBα promoter. Interestingly, at this time, NF-κB was recruited selectively to the viral form of the ICP0 promoter. When integrated into the host chromatin structure, the ICP0 promoter looses its ability to recruit the nuclear factor, behaving like IκBα (Fig. 5*C*). These results suggest that the differences in NF-κB recruitment observed between ICP0 and IκBα promoters could be due to differences in the status of DNA and/or to general chromatin modifications induced by HSV-1 during infection.

The IKK Inhibitor PGA_1 Prevents p65 Recruitment to the ICP0 Promoter and Inhibits Virus Replication—We have shown that cyclopentenone prostaglandins are potent inhibitors of NF-κB activation by direct inhibition and modification of the IKK β-subunit (14). We then investigated the effect of the cyclopentenone PGA_1 on HSV-1-induced NF-κB activation and recruitment to the ICP0 promoter in human keratinocytes. HaCaT cells were infected with HSV-1 and treated with PGA_1 (30 μM) or control diluent soon after the 1-h adsorption period. Mock infected cells were treated identically. At 6 h p.i., cell extracts were analyzed for NF-κB activity by EMSA and IκBα degradation by Western blot analysis. As shown in Fig. 6*A*, treatment with PGA_1 completely prevented IκBα degradation and NF-κB activation by HSV-1 in this cell system. Inhibition of HSV-1-induced NF-κB activity resulted in the block of p65 recruitment to the ICP0 promoter as determined by ChIP assay in infected cells (Fig. 6*B*). To determine the effect of PGA_1 treatment on ICP0 transcription, HaCaT cells were transiently transfected with the PGL3-ICP0-LUC vector. After 16 h, transfected cells were mock infected or infected with HSV-1 and then treated with PGA_1 soon after the 1-h adsorption period. As shown in Fig. 6*C*, PGA_1 treatment resulted in inhibition of viral RNA expression, as measured by luciferase activity. As previously shown in other types of cells, PGA_1 treatment was effective in reducing virus yield in HSV-1-infected keratinocytes, as determined by $CPE_{50\%}$ assay at 24 h p.i. (Fig. 6*D*). Because cyclopentenone prostaglandins are also known to interfere with the activity of the heat shock transcription factor 1 (15, 16), it cannot be excluded that different mechanisms could contribute to the potent antiviral activity of PGA_1 in this model.

DISCUSSION

The nuclear factor NF-κB is a key regulator of cellular events. NF-κB-binding sites have in fact been identified in the promoter region of more than 300 cellular genes whose expression is dependent on a sophisticated multilevel control of the factor activity. Proteins encoded by NF-κB target genes include the NF-κB-inhibitory proteins A20 and IκBα, which provide a negative feedback mechanism to limit NF-κB activity, and several proteins that participate in the control of cell proliferation and survival, as well as in the activation of the host immune and inflammatory responses (8, 17). Finally, functionally important NF-κB-binding sites have been located also in the genome of several viruses, including different members of the herpesvirus family (18, 19).

In view of the central role of NF-κB in regulating cellular metabolic events and of the fact that activation of the NF-κB pathway does not require protein synthesis, NF-κB is an attractive tool for the invading virus to control cellular functions. Many human viral pathogens have evolved different strategies to modulate the NF-κB pathway (4). Among herpesviruses, it has been shown that the β-herpesvirus cytomegalovirus and the γ-herpesvirus Epstein-Barr virus are potent inducers of NF-κB activation representing an example of biphasic kinetics of NF-κB induction (20–23).

Herein we show that also α-herpesviruses are able to activate NF-κB in a biphasic way. In human keratinocytes HSV-1 induces a first wave of NF-κB activation dependent on a rapid and dramatic induction of IKK activity, which reaches a 6-fold increase above the control level at the end of the 1-h virus adsorption period. IKK activity at this time is independent of virus replication, because it occurs also after exposure of cells to UV-inactivated virions, indicating that activation could be trig-

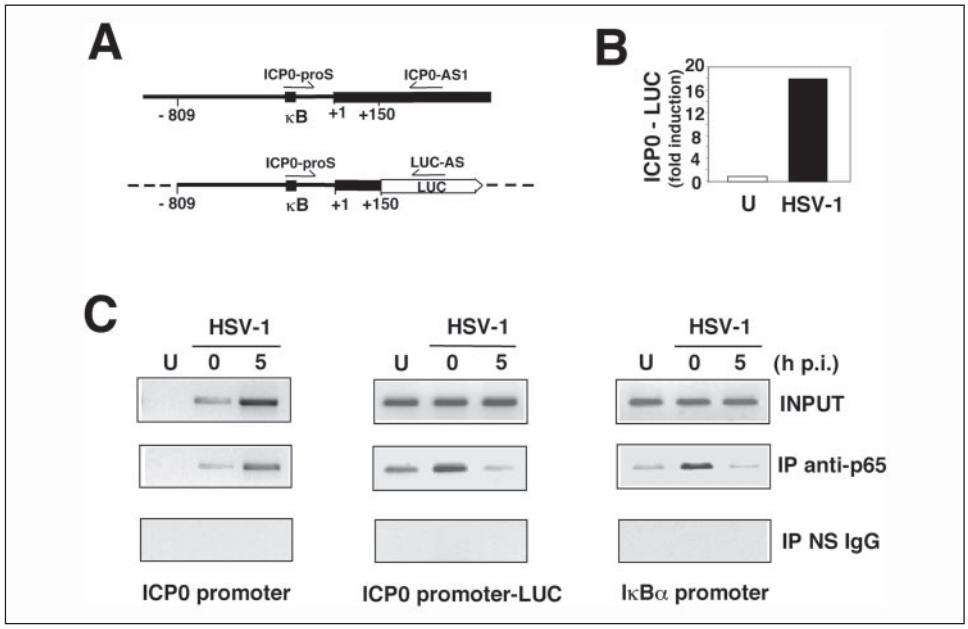

FIGURE 5. **Recruitment of p65/RelA to chromatin-integrated ICP0 promoter during HSV-1 infection of human keratinocytes.** *A*, positions of primers (indicated by *arrows*) utilized to discriminate the ICP0 viral promoter (*upper*) from the integrated ICP0 promoter (*lower*). *B*, HaCaT cells stably transfected with the luciferase gene under the control of the viral ICP0 promoter (HaCaT-ICP0-Luc) were mock infected (*U*) or infected with HSV-1 for 1 h at 37 °C. At 5 h p.i., the transcriptional activity of the integrated ICP0 promoter was determined by luciferase activity. Data are expressed as -fold induction of uninfected control. *C*, recruitment of p65/RelA to the chromatin-integrated and to the viral genome ICP0 promoters in HSV-1 infected HaCaT-ICP0-Luc cells. Soon after the adsorption period (time 0) and at 5 h p.i. p65/RelA recruitment to the ICP0-promoters was analyzed by ChIP assay with specific primers able to discriminate the viral ICP0 promoter (*ICP0 promoter*, *right panels*) from the chromatin-integrated ICP0 promoter (*ICP0 promoter-LUC*, *central panels*) (see "Experimental Procedures" for details). For the IκBα promoter (*left panels*) the same primers as in Fig. 3 were used.

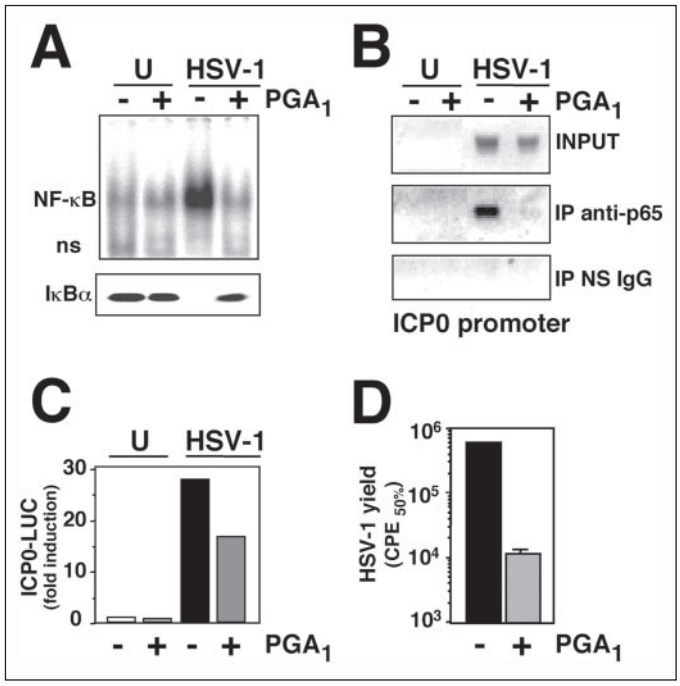

FIGURE 6. **Prostaglandin A_1 blocks p65/RelA recruitment to the ICP0 promoter and inhibits ICP0-driven transcription and HSV-1 replication.** *A*, mock infected (*U*) or HSV-1-infected (*HSV-1*) HaCaT cells treated with PGA_1 (30 μM) (+) or control diluent (−) were analyzed for NF-κB activity by EMSA (*upper panel*), and IκBα degradation was analyzed by immunoblot analysis (*lower panel*) at 5 h p.i. *B*, parallel samples were analyzed by ChIP assay for p65/RelA recruitment to the ICP0 viral promoter. *C*, HaCaT cells were transiently transfected with the PGL3-ICP0-LUC vector and, after 16 h, were infected with HSV-1 (*HSV-1*) or mock infected (*U*) and treated with PGA_1 (+) or control diluent (−). Luciferase activity was determined at 6 h p.i. in triplicate samples. Data are expressed as -fold induction of uninfected controls. *D*, virus yield in untreated (−) or PGA_1-treated (+) infected cells was determined by $CPE_{50\%}$ assay at 24 h p.i. Data represent the mean ± S.D. of duplicate samples and are representative of three separate experiments with similar results.

gered by the binding of the gD envelope glycoprotein component to cellular receptors/coreceptors such as the herpesvirus entry mediator A, a member of the TNF receptor superfamily (24). Induction of IKK function at this time is transient and is rapidly followed by IκBα degradation and triggering of NF-κB DNA-binding activity. Active NF-κB switches on IκBα resynthesis, rapidly restoring the intracellular pool of the inhibitory protein and consequently activating the autoregulatory turn-off signal. At later times after infection, a second wave of IKK activity is stimulated by HSV-1, which requires active virus replication and viral protein synthesis (11). This second wave of IKK activity also causes complete IκBα degradation, inducing massive NF-κB activation. However, no detectable IκBα resynthesis occurs at this stage of infection, and, in the absence of the inhibitory protein, NF-κB remains in the activated DNA-binding state for at least 24 h in HSV-1-infected keratinocytes.

Besides keratinocytes, persistent activation of NF-κB has been detected in different types of epithelial, neuronal and lymphocytic cells, appearing to be a general response of human cells to HSV-1 infection, and has been thought to play an important role in viral pathogenesis (4, 11, 25). However, the mechanism of NF-κB persistence was not known. We have now shown that the lack of IκBα resynthesis following the second wave of HSV-1-induced IKK activity is not merely the consequence of a general shut-off of cell protein synthesis after viral infection and cannot be attributed to enhanced degradation of IκBα mRNA, as previously suggested in SK-N-SH cells infected with HSV-1 (26), but is due to a selective block of IκBα gene transcription, as shown by *in vitro* run-on assay. Furthermore, we demonstrate that the defect in IκBα gene transcription after the second wave of NF-κB activation is due to an impairment of NF-κB recruitment to the promoter of this target gene. ChIP analysis studies of HSV-1-infected keratinocytes show that, whereas NF-κB activated during the virus-entry process is rapidly recruited to the IκBα gene promoter driving gene transcription, and leading to restoration of IκBα levels at 1 h p.i., the factor could not be detected on IκBα κB-elements at later times p.i. At this time, we could not detect recruitment of NF-κB also on promoters of other cellular target genes, such as tumor necrosis factor-α and interleukin-6 (data not shown).

Interestingly, we now show for the first time that NF-κB is recruited to the viral ICP0 promoter. Differently from the IκBα promoter, p65 binding was detected on ICP0 following both waves of virus-induced NF-κB activity, contributing to sustained ICP0 mRNA transcription at both stages of infection. The picture emerging from the results described indicates that NF-κB activated at impressively

high levels in human keratinocytes during HSV-1 infection is preferentially recruited to viral gene promoters, whereas it appears to have no access to the promoter of its own inhibitor IκBα starting few hours after infection.

The mechanism responsible for the differential recruitment of the nuclear factor is unknown at the moment. The lack of NF-κB recruitment to the IκBα promoter could be simply due to the sequestering of the factor by the rapidly increasing levels of viral DNA in infected nuclei. However, the fact that p65 is not recruited to the IκBα promoter already at relatively early stages of infection (2–3 h p.i.) suggests that additional mechanisms, besides competition for κB binding sites, could be contributing to this effect. Different scenarios could be hypothesized. In eukaryotes, transcriptional gene regulation often involves epigenetic factors such as DNA methylation or modification of histone tails. During latent infection the HSV-1 genome is predominantly organized as nucleosomes and histone modification may play a regulatory role during HSV-1 latency (27, 28). At the moment it is still debated whether and at which stage HSV-1 DNA is bound to histones during lytic infection. In contrast to previous evidence of non-nucleosomal HSV-1 DNA (29, 30), viral DNA has been recently found to associate with histones also during lytic infection (31). However, despite its presence on IE gene coding regions and DE and L viral gene promoters, histone-H3 was not detected on IE promoters, which in this respect would then behave as non-nucleosomal DNA in early stages of infection (32). In this context, it appears reasonable that the differences in NF-κB recruitment to the ICP0 and IκBα promoters observed during the second wave of NF-κB activation could reflect differences in the status of viral and cellular DNA structure, and it could be hypothesized that general chromatin modifications induced by HSV-1 infection could impair the recruitment of NF-κB to its consensus sequences on the promoters of cellular genes. If this were the case, it could be predicted that, once integrated into the chromatin structure, the ICP0 promoter would behave like a cellular promoter in respect to NF-κB recruitment. To investigate this possibility we generated HaCaT cells in which the ICP0 promoter controlling the expression of the luciferase reporter gene is stably integrated into the cellular chromatin structure. The results indicate that, during the second wave of activation, NF-κB is not recruited to the ICP0 promoter when integrated into the host genome, supporting the hypothesis that major alterations in chromatin structure and remodeling are involved in the hijacking of the factor on the viral DNA.

An alternative explanation for the differential recruitment of NF-κB to the viral promoter could be the ability of the virus to interfere with the phosphorylation or acetylation of the factor itself. It has in fact been recently reported that NF-κB binding to DNA is controlled by both phosphorylation (or acetylation) of the p65/RelA subunit triggered by different stimuli (33). In view of the ability of HSV-1 to alter the function of several cellular kinases (1), the possibility of virus-induced modifications of the transcription factor represents an attractive hypothesis.

An important question to be answered is the function of the persistently activated NF-κB in HSV-1-infected cells. So far, two roles have been ascribed to NF-κB activation during HSV-1 infection: to enhance viral replication and to block apoptosis. NF-κB activation seems to be indispensable to block apoptosis induced by exogenous pro-apoptotic agents or virus replication itself (34). Accordingly, virus mutants unable to induce NF-κB activation were found to render cells more susceptible to apoptosis (34). However, HSV-1 does not induce apoptosis in cells in which NF-κB was not activated or absent, and apoptosis is not observed in NF-κB−/− cells infected with the d120 mutant (26, 35). Several lines of evidence instead show that NF-κB induction is utilized by HSV-1 to enhance its replication by increasing the synthesis of NF-κB-dependent cellular proteins (12) or by transactivation of relevant κB-containing viral promoters (11, 13). Functional relevance of NF-κB in controlling the progression of the virus replication cycle was previously shown by the fact that inhibition of NF-κB activity by expressing the IκBα-AA super-repressor (a mutated form of IκB in which $Ser^{32/36}$ residues critical for phosphorylation by IKK are replaced by alanine) inhibits transcription of viral genes and reduces virus yield in human cells (10, 11). In addition, deletion of either IKKα or IKKβ has been shown to result in 86–94% loss of viral yield in HSV-1-infected mouse fibroblasts (13). We now demonstrate that NF-κB is in fact recruited for several hours to the viral ICP0 promoter, contributing to sustained ICP0 mRNA transcription. Moreover, hindering virus-induced NF-κB activation by the IKK inhibitor PGA_1 causes a block in viral RNA transcription, resulting in inhibition of HSV-1 replication and in a dramatic reduction in viral progeny production by infected keratinocytes. These results further suggest that persistent NF-κB activation in HSV-1-infected cells, rather than being a host response to the virus, may play a positive role in promoting efficient viral replication.

In conclusion, the results shown herein reveal a new sophisticated level of control of cellular functions by invading viruses. We have shown that HSV-1 not only hijacks the cellular transcription factor NF-κB to utilize it for its replication but is also able to disrupt the autoregulatory mechanism of NF-κB, keeping it in a persistently active state. Because the intricate network of events involved in the modification of both NF-κB and chromatin necessary for recruitment of the factor is still far from being elucidated, the understanding of how human pathogens like HSV-1 may interfere with these cellular programs is a challenging task. However, the results indicate that research in this direction may lead to the discovery of innovative strategies for antiviral therapy.

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