Influenza Virus-induced NF-κB-dependent Gene Expression Is Mediated by Overexpression of Viral Proteins and Involves Oxidative Radicals and Activation of IκB Kinase*

(Received for publication, November 1, 1999, and in revised form, December 21, 1999)

Egbert Flory‡§, Manfred Kunz‡¥, Carsten Scheller‖, Christian Jassoy‖, Roland Stauber**, Ulf R. Rapp‡, and Stephan Ludwig‡‡ ‡‡ To whom correspondence should be addressed: Institut für Medizinische Strahlenkunde und Zellforschung (MSZ), Universität Würzburg, Versbacherstr. 5, D-97078 Würzburg, Germany, the †Klinik für Dermatologie und Venerologie, Universität Rostock, Augustenstr. 20, D-18055 Rostock, Germany, the [Institut für Virologie und Immunobiologie, Universität Würzburg, Versbacherstr. 7, D-97078 Würzburg, Germany, and the **Institut für Klinische und Molekulare Virologie, Universität Erlangen, Schloßgarten 4, D-91054 Erlangen, Germany

Influenza A viruses are capable of inducing the expression of a variety of cytokine and proapoptotic genes in infected cells. The promoter regions of most of these genes harbor binding sites for the transcription factor NF-κB which is an important mediator of immune and inflammatory responses. Our present study is based on an observation that influenza A virus infection of cells stimulates transcriptional activation of the HIV-1 long terminal repeat (LTR) which harbors two regulatory NF-κB elements, and is aimed at identifying the molecular mechanisms involved in this process. We found that the expression of influenza virus hemagglutinin (HA), matrix protein (M), and nucleoprotein (NP), as single factors is sufficient to transcriptionally activate the HIV-1 LTR. This process is mediated by oxidative radicals because treatment of cells with pyrrolidine dithiocarbamate, a scavenger of such radicals, abolished the transactivating ability. Expression of different influenza proteins induces activation of NF-κB-dependent gene expression but not transcriptional activation of an AP-1/Ets-dependent promoter, indicating a selectivity for NF-κB transactivation. Furthermore, influenza protein expression induces activation of IκB kinase (IKK). Accordingly coexpression of a catalytically inactive mutant of IKK abolishes influenza protein induced activation of NF-κB as well as HIV-1 LTR-dependent reporter gene expression, suggesting that IKK is an important intermediate within this signaling process. Taken together, our results show that various influenza virus proteins act as viral transactivators to modulate transcriptional activity of κB-element harboring promoters such as the HIV-LTR.

Influenza A virus is a negative strand RNA virus with a segmented genome coding for 10 different structural and non-structural proteins (reviewed in Refs. 1 and 2). The components of the viral RNA-dependent RNA polymerase complex are coded by RNA segments 1–3 (3). Segments 4 and 6 code for the two integral membrane glycoproteins of the virus, hemagglutinin (HA), and neuraminidase, respectively. The most abundant structural proteins of the virus particle are the products of RNA segment 5 and 7, the nucleoprotein (NP) and the matrix protein (M), respectively, both of which were found to be post-translationally phosphorylated at least in some virus strains. Segment 7 further codes for a third integral membrane protein M2 (4) which acts as an ion channel. Segment 8 also codes for two proteins: nonstructural proteins 1 and 2 (5) the second of which has been shown to be present in the virus particle in very low amounts (6). The limited genetic information of the virus genome requires multifunctionality of most viral proteins and these additional functions may include yet undetected activities toward cellular factors.

Although only little is known about the intracellular signaling pathways which are activated after influenza virus infection, there are numerous reports on downstream target genes which are deregulated upon infection, both in cells which are permissive or non-permissive for viral replication. This includes genes encoding interleukins (IL), tumor necrosis factor α (TNFα), and interferons, but also chemokine genes, proapoptotic genes, and adhesion molecule genes (reviewed in Ref. 7). Remarkably, most of the influenza virus responsive cytokine, chemokine, or adhesion molecule genes that are up-regulated in response to influenza virus infection harbor binding sites for the transcription factor NF-κB in their promoter regions.

The transcription factor NF-κB is a rapidly induced host cell factor and a pleiotropic mediator of immune and inflammatory responses (reviewed in Refs. 8 and 9). In most cell types, NF-κB is sequestered in an inactive, cytoplasmic complex by binding to IκB, an inhibitory subunit (reviewed in Ref. 10). Exposure of cells to a wide variety of pathological stimuli such as viral or bacterial infections, inflammatory cytokines, or UV irradiation leads to activation of NF-κB through the phosphorylation of IκB. This phosphorylation event is at least in the case of TNFα, IL-1, and HTLV-1 Tax protein-induced NF-κB activation mediated by the recently identified IκB kinases (IKK) (reviewed in Ref. 11). Following translocation to the nucleus, NF-κB acti-

---

* This work was supported by Deutsche Forschungsgemeinschaft Grant Lu477/4-1 and the Fonds der Chemischen Industrie. This paper was presented in part at the International Congress of Virology (ICV), August 1999, Sydney, Australia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Paul Ehrlich Institut, Abt. Med. Biotechnologie, Paul Ehrlich Str. 51–59, D-63225 Langen, Germany.

§ Present address: Paul Ehrlich Institut, Abt. Med. Biotechnologie, Paul Ehrlich Str. 51–59, D-63225 Langen, Germany.

‖ Present address: Paul Ehrlich Institut, Abt. Med. Biotechnologie, Paul Ehrlich Str. 51–59, D-63225 Langen, Germany.

© The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.
vates transcription of a large variety of genes including those of cytokines, hematopoietic growth factors, cell-adhesion molecules, and promoter regulatory regions of human pathogenic viruses (12). NF-κB activation is induced by viruses such as human immunodeficiency virus-1 (HIV-1) and HIV-2, human T cell leukemia virus type-1 (HTLV-1), hepatitis B virus, herpes simplex virus type-1, and influenza virus (8, 13, 14). However, the detailed molecular mechanism and viral components underlying this induction remains unclear. In several cases the expression of a single viral protein is sufficient to activate NF-κB, as seen with Tax from HTLV-1 (15), E3/19k from adenovirus (16), and HBx from hepatitis B virus (17, 18). In addition, it was reported that the HA from a highly pathogenic avian influenza virus transactivates NF-κB via an ER-overload mechanism (19, 20).

Transcriptional control of HIV-1 gene expression involves a complex interaction between host cellular as well as viral regulatory proteins and their target sequences within the long terminal repeat (LTR) (21–25). The HIV-1 LTR harbors two consensus sites and a persistent activation of NF-κB when acting as single factors, is sufficient to lead to antioxidation-sensitive stimulation of HIV-1 gene expression via an antioxi-
dation, it was reported that the HA from a highly pathogenic avian influenza virus transactivates NF-κB via an ER-overload mechanism (19, 20).

Our study is based on the observation that influenza A virus infection of cells leads to transactivation of the HIV-LTR. Our objective was to study the molecular mechanism by analyzing the transactivating effects of influenza viral components using a HIV-1 LTR-reporter gene construct as a molecular read out for NF-κB dependent T cell-specific gene expression. We show that expression of different structural influenza viral proteins, when acting as single factors, is sufficient to lead to antioxidation-sensitive stimulation of HIV-1 gene expression via an NF-κB inducing mechanism involving the recently described IKK. This mechanism is likely to account for influenza virus-induced gene expression in cells which are not permissive for viral replication, such as T lymphocytes.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs, Cloning, and Immunoblotting**—The cDNAs for the matrix protein and nucleoprotein gene of influenza A/WSN/33 (H1N1) were kindly supplied by Dr. E. Neumeier and Dr. G. Hobom, Institut für Molekularbiologie und Mikrobiologie, Giessen, Germany. The matrix protein and nucleoprotein genes as well as the hemagglutinin genes of A/sw/Germany/181 (H1N1) (27) and A/Mongolia/231/85 (H1N1) (28) were cloned into the multiple cloning site of pSRSPA (29) for eukaryotic expression under the control of a Rous sarcoma virus promoter. The experiments were performed with both HA proteins with essentially the same results. The data obtained with the HA from A/sw/Germany/181 are shown in the figures. An expression vector for Sendai virus NN protein was kindly provided by Dr. I. Albrecht and Dr. W. Neubert, Max-Planck Institut für Biochemie, München, Germany. The cDNA for the green fluorescence protein was subcloned from pGreenLantern (Life Technologies, Inc.) into the pSRSPA background. An expression vector for an active form of mitogen-activated protein kinase θ (MKθ/EE3) was kindly provided by Dr. R. Davis, University of Massachusetts Medical School, Worcester, MA (30). A plasmid expressing a membrane targeted active mutant of c-Raf-1 was described previously (25). To exclude that lipopolysaccharide potentially contaminating the membrane preparation confounds the results, DNAs were purified using Endo-free DNA purification kit (Qiagen). Expression of proteins was confirmed by Western blotting using a polyclonal rabbit antisera to the M protein (kindly provided by Dr. T. Wolff, Institut für Virologie, Marburg, Germany), a polyclonal goat antiserum to the NP (kindly provided by Dr. R. G. Webster, St. Jude Childrens Research Hospital, Memphis, TN), and mouse monoclonal antibodies against H1 hemagglutinins (27, 31) to detect the M, NP, and HA proteins, respectively. For SDS-polyacrylamide gel electrophoresis, 10% gels were electrophobated onto nitrocellulose BAS-55 membrane (Schleicher & Schuell) and analyzed by Western blot analysis. For Western blot analysis, the membranes were incubated in blocking buffer (5% non-fat dry milk in Tris-buffered saline Tween 20 (TBST)) and washed in TBST as described by Flory et al. (24). As a secondary antibody, protein A-peroxidase (Amersham Pharmacia Biotech) was used, followed by standard enhanced chemiluminescence detection. The 3xκB-tk luciferase model was obtained through A. Rethwilm, Institut für Virologie, University of Würzburg, from the NIH AIDS Research and Reference Reagent Program. The molecular HIV-1 luciferase reporter gene vector, the pNL4-3-HIV-LTR luciferase plasmid, and the AP-1/ets-dependent pB4x luciferase plasmid were described previously (25, 32, 33).

**Viral Stock and Infection**—A virus stock of A/Bratislava 79 (H7N7) with 1 × 10⁶ plaque forming units/ml was kindly provided by Dr. S. Pleschka, Institut für Molekularbiologie und Mikrobiologie, Giessen, Germany. Cells were washed twice with phosphate-buffered saline and infected with a multiplicity of infection of 2 or 10 plaque forming units/cell. Cells were incubated with a virus dilution in phosphate-buffered saline, 0.2% bovine albumin for 30 min at 37°C. Virus dilutions were removed and cells were incubated for 4 h in Dulbecco’s modified Eagle’s medium, 0.2% bovine albumin.

**HIV p24 Enzyme-linked Immunosorbent Assay**—For enzyme-linked immunosorbent assay, culture supernatants were collected 24–120 h post-transfection and stored at -70°C. HIV-1 p24 antigen in culture supernatant was detected using the Abbott Laboratories HIVAG-1 enzyme immunoassay according to the manufacturer’s protocol. The p24 concentration (pg/ml) was calculated according to the Abbott Laboratories Quantification enzyme-linked immunosorbent assay. The cutoff value was A₄₀₀nm = 0.063, which represents 240 pg/ml p24.

**Cell Culture, DNA Transfection, and Reporter Gene Assay**—Human A3.01 T cells were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, streptomycin, and penicillin. The human embryonic kidney cell line HEK293 was cultured in Dulbecco’s modiﬁed Eagle’s medium supplemented with 10% fetal calf serum. For transfection of HEK293 cells 5 × 10⁵ cells were seeded in a 10-cm diameter dish and grown 24 h in Dulbecco’s modiﬁed Eagle’s medium, 10% fetal calf serum prior to transfection. Transfections were performed by a calcium phosphate co-precipitation method using 5–10 μg of DNA unless otherwise indicated. Cells were rinsed twice with phosphate-buffered saline and the DNA constructs were co-precipitated with 6 μL of DMRIE (0.5 ng/μL DNA) and 10 μL of CaCl₂ (250 mM) in Dulbecco’s modiﬁed Eagle’s medium. Transfections were removed and cells were incubated for 5 h in an incubator at 37°C, 7.5% CO₂ in the presence of the DMRIE-nucleic acid complexes. Then 1.5 mL growth medium was added. For luciferase assays, total cell extracts were prepared 24–42 h later. Briefly, cells of each well were harvested in 100 μL of lysis buffer (50 mM sodium-MES, pH 7.8, 50 mM Tris-HCl, pH 7.8, 10 mM dithiothreitol, 2% Triton X-100). The crude cell lysates were cleared by centrifugation and 50 μl of precleared cell extracts were added to 50 μl of luciferase assay buffer (125 mM sodium-MES, pH 7.8, 25 mM magnesium acetate, 2 mg/ml ATP) and activity was measured after injection of 50 μl of 1× β-luciferin (AppliChem) in a Berthold Lumat luminometer. Total protein concentration was measured by the Bradford technique (Bio-Rad). Results are presented as luciferase units normalized to protein concentration and mock transfection with corresponding empty expression vectors. Mean and standard deviations of at least three independent experiments each done in duplicate or triplicate are shown in the figures.

**IKK Immune Complex Kinase Assays**—A3.01 T cells were transiently co-transfected with a DNA construct expressing a SV40-taged form of IKKα (26) to test expression of IFN-κBα and different endogenous viral proteins. Cells were lysed in TBL buffer (20 mM Tris, pH 7.4, 50 mM sodium β-glycerophosphate, 20 mM sodium pyrophosphate, 137 mM NaCl, 1% (v/v) glycerol, 1% (v/v) Triton X-100, 2 mM EDTA, 1 mM Pefabloc, 1 mM sodium orthovanadate, 5 mM benazmide, 5 μM aprotinin, 5 μM leupeptin) on ice for 30 min. Cell debris was removed by centrifugation at 15,000 rpm for 10 min. Supernatants were then
incubated with 1 μg/ml of an anti-VSV mab (Roche Molecular Biochemicals) for 2 h at 4 °C. The immune complexes were precipitated with protein G-agarose, washed once with high-salt TLB buffer (20 mM Tris, pH 7.4, 50 mM sodium β-glycerophosphate, 20 mM sodium pyrophosphate, 500 mM NaCl, 1% (v/v) glycerol, 1% (v/v) Triton X-100, 2 mM EDTA, 1 mM Pefabloc, 1 mM sodium orthovanadate, 5 mM benzamide, 5 μg/ml aprotinin, 5 μg/ml leupeptin) and twice with kinase buffer (10 mM MgCl2, 25 mM β-glycerophosphate, 25 mM HEPES, pH 7.5, 5 mM benzamide, 0.5 mM dithiothreitol, and 1 mM sodium vanadate) supplemented with 3 μg/ml aprotinin. After two final washing steps in kinase buffer immunoprecipitates were assayed in the same buffer supplemented with 5 μCi of [γ-32P]ATP, 0.1 mM ATP, and 1 μg of recombinant GST-IxBSa as a substrate protein at 30 °C for 30 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted onto polyvinylidene difluoride membranes (Millipore), and detected by x-ray film exposure or by a Bio Imaging Analyzer BAS 2000 (Fuji). Equal loading of VSV-HKkb was controlled by Western blotting using the anti-VSV monoclonal antibody.

RESULTS

Influenza A Virus Infection Stimulates HIV-1 LTR-dependent Transcription—To assess whether infection with influenza A virus may transactivate the HIV-1 LTR, transient transfection experiments using an HIV-1 LTR-driven luciferase gene were performed (Fig. 1A). Human HEK293 cells were transfected with this reporter constructs and 24 h after transfection cells were washed twice and subsequently infected with influenza A virus at a multiplicity of infection of 2 and 10 as described under “Experimental Procedures.” Human HEK293 cells, stimulated with TPA (10 ng/ml) for 4 h were used as a positive control. After 4 h luciferase assays were performed. Relative luciferase activities are based on vector control (see “Experimental Procedures” for details). The values represent the mean (± S.D.) of at least three independent experiments.

Fig. 1. A, schematic representation of the HIV-1 LTR spanning the region from nucleotide −150 to +70 of the HIV-1 promoter. NF-κB, SP1-binding sites, and the Tat responsive region (TAR) is indicated. B, influenza A virus infection stimulates HIV LTR-dependent transcription. Human HEK293 cells were transiently transfected with 400 ng of HIV LTR-driven luciferase reporter using DMRIE-C liposome transfection reagent. 24 h post-transfection cells were washed twice and subsequently infected with influenza A virus at a multiplicity of infection of 2 and 10 as described under “Experimental Procedures.” Human HEK293 cells, stimulated with TPA (10 ng/ml) for 4 h were used as a positive control. After 4 h luciferase assays were performed. Relative luciferase activities are based on vector control (see “Experimental Procedures” for details). The values represent the mean (± S.D.) of at least three independent experiments.
strate that expression of various influenza proteins transactivates the HIV-LTR, and that the mechanism of this activation involves the generation of oxidative radicals.

Expression of Influenza NP, M, and HA Proteins Stimulate Antioxidant-sensitive NF-κB Activation, But Not AP-1/Ets-Driven Gene Transcription—The radical scavenger PDTC is a known inhibitor of NF-κB (15), a transcription factor which is essential for HIV-1 gene expression. We performed transient transfection experiments using expression vectors for influenza proteins and a κB-dependent luciferase reporter gene to investigate whether influenza protein expression stimulates NF-κB activation. TPA treatment of cells induced a κB-dependent luciferase activity about 12-fold (Fig. 3, black bars). NF-κB activation is not induced in mock transfected cells or in cells transfected with structural surface HN protein from Sendai virus. Consistent with previous published data (19, 35), expression of influenza-HA stimulates κB-dependent transcription about 10-fold. As observed with the HIV-LTR, other structural viral proteins such as NP and the M protein also induce transcriptional activation of the κB-dependent reporter gene 3–18-fold over mock transfected cells, suggesting that these proteins mediate NF-κB activation. To rule out that the observed effect is simply a result of protein overexpression that for some reason does not occur with Sendai virus HN we expressed other proteins such as the green fluorescence protein or an active mutant of the mitogen-activated protein kinase kinase MKK6 (MKK6EE). Green fluorescence protein was chosen as an irrelevant protein with no signaling capacity, whereas MKK6EE has a known transactivating feature toward various promoters (30, 36) including the AP-1 and Ets-dependent promoter used below. However, we did not observe a transcriptional activation of the κB-dependent promoter in the presence of green fluorescence protein or MKK6(EE), indicating that the influenza virus protein-induced transactivation is not specifically caused by protein accumulation (data not shown). Similar to our observation with the HIV-LTR, incubation of cells with PDTC substantially inhibited NF-κB activation induced by TPA as well as HA and M protein expression (Fig. 3, white bars). Interestingly, PDTC was not as effective on NP induced activity. These findings demonstrate that expression of influenza proteins leads to activation of κB-dependent transcription and that the mechanism is likely to involve the generation of oxidative radicals as critical signaling intermediates.

We next determined whether influenza proteins stimulate signaling pathways leading to activation of other promoters such as AP-1/Ets responsive elements of immediate-early genes. In transient transfection experiments using an AP-1/Ets-dependent luciferase reporter, gene expression could be...
induced about 17-fold by treatment of cells with TPA and by about 34-fold by overexpression of active Raf-kinase (Fig. 4), which is in accordance with previously published data (32). In contrast, no significant transcriptional activation of this reporter was observed after expression of different influenza proteins. These data suggest that there is a certain transcriptional selectivity of these proteins toward the NF-κB transcription factor.

Expression of Influenza NP, M, and HA Proteins Induces Activation of IkB Kinase β (IKKb)—NF-κB activation is controlled by inhibitory proteins of the IkB family (8, 13). These proteins are phosphorylated by IKK and it has been shown that these kinases are critical mediators in IL-1, TNF, and HTLV-1 Tax protein-induced activation of NF-κB (11). Thus, we analyzed whether influenza proteins are also capable to induce IKK activity. A3.01 T cells were co-transfected with empty vector or DNA constructs expressing influenza virus NP, M, HA, or Sendai virus HN proteins together with a plasmid expressing a VSV-tagged form of IKKb. Cell lysates were subjected to immunoprecipitation with an anti-VSV antibody and immune complexes were subsequently assayed in an in vitro kinase assay using recombinant GST-IκBa as a substrate. Consistent with the NF-κB transactivation data (Fig. 3) coexpression of empty vector or Sendai virus NH protein has no effect on VSV-IKKb activity, however, in the presence of influenza virus NP, M, and HA proteins VSV-IKKb activity is significantly induced as measured by an increased phosphorylation of GST-IκBa (Fig. 5).

Influenza Virus Protein Expression Stimulates NF-κB Activity Dependent on IKKb Activity—To assess whether IKK activation plays a functional role in influenza protein-induced NF-κB dependent gene expression we coexpressed kinase inactive versions of both IKKa and -β. These mutants act as dominant negative forms over their cellular counterparts. Fig. 6A shows that TPA induced NF-κB activation was significantly inhibited by co-expression of a kinase-dead mutant of IKKβ (IKKβ-KD), but, surprisingly, not by a dominant negative mutant of IKKa (IKKa-KD). High amounts of IKKβ-KD but not of IKKa-KD act very efficiently and even inhibit basal NF-κB activity. To analyze the induced promoter activity a semi-inhibiting concentration of kinase-dead IKKβ resulting in a 50–60% inhibition of TPA-induced NF-κB activation was chosen for the following experiments. As seen after stimulation of cells with TPA, NF-κB activation induced by expression of HA, M, and NP was inhibited by co-expression of IKKβ-KD but not by IKKa-KD (Fig. 6B). In addition, coexpression of wild type VSV-IKKb cooperates with the influenza proteins in NF-κB activation (data not shown). These data clearly indicate that cellular IKKb is an important intermediate in influenza protein-induced NF-κB activation.

Expression of an HIV-1 Molecular Clone Induced by Different Influenza Viral Proteins Is Abolished by a Kinase-dead Mutant of IKKb—To determine whether single influenza virus protein expression is sufficient to induce transcription, subsequent expression, and processing of HIV-1 genes from a molecular vector, we developed a reporter-gene based assay using an HIV-1 molecular vector carrying a luciferase gene (33). Transient transfection of cells with increasing amounts of this construct leads to an enhancement in luciferase activity indicating that the HIV-1 genome is functionally expressed (data not shown). TPA treatment of cells was used as a positive control and shows an 4.5-fold stimulation of HIV-1 gene expression (Fig. 7A). Expression of influenza viral proteins HA, M, and NP induced HIV-1 gene expression up to 15-fold. HIV-1 molecular vector gene expression was not influenced in mock transfected cells, or in cells expressing Sendai HN-protein. Using an infectious wild type HIV-1 molecular clone, we confirmed our results and observed that co-transfection of HIV-1NL4-3 DNA with influenza HA and NP enhanced cell-free p24gag synthesis within 24 h, similar to TPA treatment of cells (data not shown). Taken together, these data clearly show that expression from HIV-1 molecular clone is induced by expression of single influenza
Influenza Proteins Transactivate NF-κB-dependent Promoters

Figure 6. A, expression of kinase-dead IKKβ (IKKβ-KD) inhibits TPA-induced NF-κB activation. Human T cells were co-transfected with 400 ng of 3xκB-tk luciferase construct together with either 500 ng of IKKα-KD or IKKβ-KD or empty expression vector (mock). Cells were stimulated with TPA (10 ng/ml) for 16 h or left untreated. 42 h post-transfection cells were harvested and luciferase assays were performed as described. The values represent the mean (±S.D.) of three independent experiments. B, influenza protein-induced NF-κB activation is inhibited by expression of kinase-dead IKKβ. As described above, cells were co-transfected with 400 ng of 3xκB-tk luciferase construct together with either 500 ng of IKKα-KD or IKKβ-KD or empty expression vector (mock) and HN, HA, M, and NP, respectively. 42 h post-transfection cells were harvested and luciferase assays were performed as described above. The values represent the mean (±S.D.) of three independent experiments.

Discussion

The mechanism by which influenza virus infection induces NF-κB-dependent gene expression is unknown. Here, we have shown that different influenza virus proteins have a transactivating capacity to stimulate HIV-1 gene expression via activation of NF-κB. The molecular mechanism of this transactivation by influenza NP, M, and HA proteins involves the generation of oxidative radicals, and integrates activation of IKKβ as a signal transduction intermediate, a kinase which phosphorylates IkB thereby regulating NF-κB activity.

The target genes of NF-κB are numerous and most share the common feature of being quickly induced in response to a variety of extracellular stimuli (8–10, 12). This characteristic rapidity of NF-κB induction can be utilized by viruses as a strategic tool to initiate self-replication, or place its life cycle under the control of the host cell. In this respect, Knobih et al. (14) reported that influenza virus infection induces nuclear translocation and DNA binding of NF-κB factors as determined by electrophoretic mobility shift assay, and increases the production of intracellular inactive oxygen intermediates (14). NF-κB activation is judged to be a survival signal (37) and thus may be recruited by the virus to suppress programmed cell death in infected cells.

Our observation that both, virus protein-induced and TPA-induced NF-κB dependent reporter gene activity is blocked by dominant negative IKKβ but not by dominant negative IKKα is consistent with recent data obtained from IKK-deficient mice. These data show that only cells from mice deficient in IKKβ but not in IKKα exhibit a defect in NF-κB activation (37–39). This indicates that the β isoform of IKK represents the major enzyme responsible for IkBα phosphorylation which may be blocked by the corresponding dominant negative mutant.

Expression of single viral proteins are known to have a transactivating or a signal-transducing capacity, indicating that infection with an intact virus is not always required for NF-κB activation. One example of this is HTLV-1-Tax, which activates NF-κB via IKK (40). It was previously reported that a subtype H7 HA from a highly pathogenic avian influenza virus also activates NF-κB when expressed as a single factor (19). We now demonstrate that hemagglutinins of the subtype H1 derived from a human and a swine isolate, respectively (27, 28), also activate NF-κB and that IKKβ and inactive oxygen intermediates are critical intermediates in this process. The molecular mechanism may involve the accumulation of this protein in the endoplasmic reticulum due to the synthesis of large amounts of HA following viral infection (20). This intracellular accumulation of structural viral proteins has been proposed to activate NF-κB via an oxidant sensitive pathway (19). However, this might not be a general mechanism for ER-processed viral proteins, since we did not observe NF-κB activation by expression of a Sendai virus glycoprotein, HN. In addition, the ER-overload mechanism does not apply for the observed activity of influenza NP and M proteins, since these proteins are not processed via the ER. Thus, influenza M and NP represent a
novel class of viral NF-κB inducers, stimulating an oxidant-sensitive pathway involving the IκB activating IKK. Both the M and HA proteins are synthesized late during influenza virus replication, but NP represents an early viral protein. NP may initiate NF-κB activation and gene expression before the virus-induced shut off of protein synthesis takes place. This may explain the efficient release of cytokines from infected cells although there is no cellular protein synthesis going on in later stages of productive infection.

Interestingly, influenza NP is only a weak inducer of the HIV-LTR and NF-κB driven reporter gene, but a strong activator of gene expression from the luciferase HIV-1 molecular vector. The HIV-LTR used in this study is shorter than the LTR from the luciferase HIV-1 molecular vector. Additional cis-acting elements in the promoter region of the molecular vector may be responsible for the enhanced activity of NP, suggesting that binding factors of these cis-elements may interact with NF-κB factors to modulate promoter activity. This might also explain the observation that only the NP induced HIV-1 gene expression is dependent on both, IKKα and -β, activity.

It is still an open issue which structural features of the viral proteins are recognized by the cell to activate IKK and NF-κB. Influenza virus NP, M, and HA proteins do not only differ in structure, but also in their time of synthesis post-infection and in their processing by the cellular machinery. However, there are several transactivators from different viruses described so far, all of which have developed individual strategies to accomplish NF-κB activation. Thus it is not unlikely that there exist three distinct modes of IKK and NF-κB activation by three different proteins of the same virus, one mechanism of which might be HA-induced ER overload. Baumann et al. (35) have recently demonstrated that the mechanism of HA-induced NF-κB activation is different from that which is involved when mitogens are used. Stimuli such as TPA, or the expression of viral proteins such as HIV-Tax (40), EBV-LMP-1 (41–44), or HBx protein (17, 18) are pleiotropic activators in the cell and activate a variety of intracellular signaling pathways including mitogen-activated protein kinase signaling cascades and their target AP-1/Ets transcription factors. In contrast, the expression of influenza proteins seems to act more selectively, neither activating AP-1/Ets-dependent transcription (Fig. 4), nor inducing ERK, p38, or JNK kinase activity. Selectivity for NF-xB activation is also reflected by the regulation of genuine influenza A virus-induced cellular genes. The monocyte-chemoattractant protein-1 gene is induced after influenza virus infection in monocytes (45) and its transcription is strictly dependent on two NF-xB-binding sites in the promoter (46).

The IL-8 gene is also activated in response to influenza virus infection in different cell types (47, 48) and requires the cooperation of NF-xB with factors of the AP-1 family (49). Accordingly, the MCP-1 promoter is strongly activated by the influ-

\[ S. \text{ Ludwig, unpublished results.} \]
Influenza Proteins Transactivate NF-κB-dependent Promoters

enza proteins in a monocytic cell line and in A3.01 T cells, whereas the IL-8 promoter which additionally requires AP-1 activation is not induced by expression of influenza HA, M, and NP proteins in these two cell lines. Thus, AP-1 activation requires other virus-induced mechanisms than overexpression of viral proteins.

Most viral proteins with transactivating capacity are found in viruses which require the host cell DNA/RNA synthesis machinery for replication, indicating that these viral activators of cellular gene expression are required to drive resting cells into the cell cycle. Negative strand RNA viruses such as influenza virus do not need this synthesis machinery because the required RNA-dependent RNA polymerases are coded by the viral genome. In this respect, the transactivating feature of influenza HA, M, and NP proteins is surprising. It is yet unclear whether this is a viral function to support viral replication, or a cellular recognition event to initiate a protective response. The latter mechanism might be more plausible since several cytokines which contribute to the antiviral immune response carry NF-κB-binding sites in their promoters.

Acknowledgment—We thank Heide Häfner for excellent technical assistance. For providing reagents we thank I. Albrecht, R. J. Davis, M. Karin, W. Neubert, E. Neumeier, S. Pleschka, T. Wirth, R. G. Webster, and T. Wolff. The following reagents were obtained from the NIH AIDS Research and Reference Reagent Program: A3.01 T cell line, HIV-1 p24 antibody (183-H12-5C), and pNL4-3 HIV expression plasmid. We are greatly indebted to Joseph Slupsy for a piercing critique and for the efficiency bestowed upon the manuscript.

REFERENCES
1. Krug, R. M. (1989) in The Influenza Viruses (Krug, R. M., ed) Plenum Press, New York
2. Lamb, R. A., and Krug, R. M. (1996) in Fields Virology (Fields, B. N. E. A., ed) Third Ed., pp. 1353–1395, Lippincott-Raven Publishers, Philadelphia, PA
3. Deijen, B. M., St. Angelo, C., Katze, M. G., and Krug, R. M. (1987) J. Virol. 61, 16–22
4. Lamb, R. A., Lai, C. J., and Choppin, P. W. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 4170–4174
5. Lamb, R. A., Choppin, P. W., Chanoz, R. M., and Lai, C. J. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 1857–1861
6. Richardson, J. C., and Akkina, R. K. (1991) Arch. Virol. 116, 69–80
7. Ludwig, S., Pleschka, S., and Wolff, T. (1999) Virological 12, 175–196
8. Baeuerle, P. A., and Henkel, T. (1994) Annu. Rev. Immunol. 12, 141–179
9. Baeuerle, P. A., and Baltimore, D. (1996) Cell 87, 13–20
10. Baldwin, A. S., Jr. (1996) Annu. Rev. Immunol. 14, 649–683
11. Zandi, E., and Karin, M. (1999) Mol. Cell. Biol. 19, 4547–4551
12. Siebenlist, U., Franzoso, G., and Brown, K. (1994) Annu. Rev. Cell Biol. 10, 405–455
13. Grilli, M., Choi, J. J.-S., and Lenard, M. J. (1993) Int. Rev. Cytol. 143, 1–61
14. Knobli, K., Choi, A. M., Weigand, G. W., and Jacoby, D. B. (1998) Am. J. Physiol. 274, L134–142
15. Schreck, R., Grassmann, R., Fleckenstein, B., and Baeuerle, P. A. (1992) J. Virol. 66, 6288–6293
16. Pahl, H. L., Sester, M., Bargetz, H. G., and Baeuerle, P. A. (1996) J. Cell Biol. 132, 511–522
17. Su, F., and Schneider, R. J. (1996) J. Virol. 70, 4558–4566
18. Doris, M., Klein, N., Lucito, R., and Schneider, R. J. (1995) EMBO J. 14, 4747–4757
19. Pahl, H. L., and Baeuerle, P. A. (1995) J. Virol. 69, 1480–1484
20. Pahl, H. L., and Baeuerle, P. A. (1997) Trends Biochem. Sci. 22, 63–67
21. Nabel, G., and Baltimore, D. (1987) Nature 326, 711–713
22. Gaynor, R. (1992) AIDS 6, 347–363
23. Faucci, A. S. (1996) Nature 384, 529–534
24. Flory, E., Hoffmeyer, A., Smola, U., Rapp, U. R., and Bruder, J. T. (1996) J. Virol. 70, 2260–2268
25. Flory, E., Weber, C., Chen, P., Hoffmeyer, A., Jassoy, C., and Rapp, U. R. (1998) J. Virol. 72, 2788–2794
26. Jacque, J. M., Fernandez, B., Arenasza-Seidados, P., Thomas, D., Baleux, F., Virelizier, J. L., and Baeuerle, P. A. (1996) J. Virol. 70, 2930–2938
27. Ludwig, S., Stitz, L., Planz, O., Van, H., Fitch, W. M., and Scholtissek, C. (1995) Virology 212, 555–561
28. Anchen, D., Ludwig, S., Nymadawa, P., Mensahkhan, J., and Scholtissek, C. (1996) Arch. Virol. 141, 1553–1569
29. Dorn, P. L., DaSilva, L., Matarano, L., and Derse, D. (1990) J. Virol. 64, 1616–1624
30. Raingeaud, J., Whitmarsh, A. J., Barrett, T., Derijard, B., and Davis, R. J. (1996) Mol. Cell. Biol. 16, 1247–1255
31. Brown, I. H., Ludwig, S., Olsen, C. W., Hannoun, C., Scholtissek, C., Hinshaw, V. S., Harris, P. A., McCauley, J. W., Strung, I., and Alexander, D. J. (1997) J. Gen. Virol. 78, 553–562
32. Bruder, J. T., Heidecker, G., and Rapp, U. R. (1992) Genes Dev. 6, 545–556
33. Staubner, R. H., Rulon, S., Palm, G., and Tarasova, N. I. (1990) Biochem. Biophys. Res. Commun. 228, 695–702
34. Israel, N., and Gougerot-Pocidalo, M. A. (1997) Cell. Mol. Life Sci. 53, 864–870
35. Baumann, B., Kistler, B., Kirilliev, A., Bergman, Y., and Wirth, T. (1998) J. Biol. Chem. 273, 11448–11455
36. Hoffmeyer, A., Grosse-Wilde, A., Flory, E., Neufeld, B., Kunz, M., Rapp, U. R., and Ludwig, S. (1999) J. Biol. Chem. 274, 4519–4537
37. Li, Z. W., Chu, W., Hu, Y., Delhase, M., Deering, T., Ellisman, M., Johnson, R., and Karin, M. (1999) J. Exp. Med. 189, 1839–1845
38. Tanaka, M., Fuentes, M. E., Yamaguchi, K., Durnin, M. H., Dalrymple, S. A., Hardy, K. L., and Goeddel, D. V. (1999) Immunity 10, 421–429
39. Li, Q., Lu, Q., Hwang, J. Y., Buscher, D., Lee, K. F., Izpisua-Belmonte, J. C., and Verma, I. M. (1999) Genes Dev. 13, 1322–1328
40. Geleziunas, R., Ferrell, S., Lin, X., Mu, Y., Cunningham, E. T., Jr., Grant, M., Connelly, M. A., Hambor, J. E., Marca, K. B., and Greene, W. C. (1998) Mol. Cell. Biol. 18, 5157–5165
41. Hammerskold, M. L., and Simurda, M. C. (1992) J. Virol. 66, 6496–6501
42. Kieser, A., Kilger, E., Gires, O., Ueffing, M., Kolch, W., and Hammerschmidt, W. (1997) EMBO J. 16, 6478–6485
43. Gires, O., Kohlhuber, F., Kilger, E., Baumann, M., Kieser, A., Kaiser, C., Zeidler, R., Schefer, B., Ueffing, M., and Hammerschmidt, W. (1999) EMBO J. 18, 3064–3073
44. Kieser, A., Kaiser, C., and Hammerschmidt, W. (1999) EMBO J. 18, 2511–2521
45. Sprenger, H., Meyer, R. G., Kaufmann, A., Bussfeld, D., Rischkowsky, E., and Verma, I. M. (1995) Nature 372, 1839–1845
46. C. Erhardt and S. Ludwig, unpublished results.
Influenza Virus-induced NF-κB-dependent Gene Expression Is Mediated by Overexpression of Viral Proteins and Involves Oxidative Radicals and Activation of IκB Kinase

Egbert Flory, Manfred Kunz, Carsten Scheller, Christian Jassoy, Roland Stauber, Ulf R. Rapp and Stephan Ludwig

*J. Biol. Chem.* 2000, 275:8307-8314.

doi: 10.1074/jbc.275.12.8307

Access the most updated version of this article at [http://www.jbc.org/content/275/12/8307](http://www.jbc.org/content/275/12/8307)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 47 references, 27 of which can be accessed free at [http://www.jbc.org/content/275/12/8307.full.html#ref-list-1](http://www.jbc.org/content/275/12/8307.full.html#ref-list-1)