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NF-κB-Mediated Modulation of Inducible Nitric Oxide Synthase Activity Controls Induction of the Epstein-Barr Virus Productive Cycle by Transforming Growth Factor Beta 1▽†

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Transforming growth factor beta 1 (TGF-β1) signal transduction has been implicated in many second-messenger pathways, including the NF-κB pathway. We provide evidence of a novel TGF-β1-mediated pathway that leads to extracellular signal-regulated kinase (ERK) 1/2 phosphorylation, which in turn induces expression of an Epstein-Barr virus (EBV) protein, ZEBRA, that is responsible for the induction of the viral lytic cycle. This pathway includes two unexpected steps, both of which are required to control ERK 1/2 phosphorylation: first, a quick and transient activation of NF-κB, and second, downregulation of inducible nitric oxide synthase (iNOS) activity that requires the participation of NF-κB activity. Although necessary, NF-κB alone is not sufficient to produce downregulation of iNOS, suggesting that another uncharacterized event(s) is involved in this pathway. Dissection of the steps involved in the switch from the EBV latent cycle to the lytic cycle will be important to understand how virus-host relationships modulate the innate immune system.

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus associated with numerous malignancies of both B-cell and epithelial cell origins (32, 43). *In vitro*, infection of B lymphocytes usually results in viral latency, with expression of six nuclear proteins (EBNA 1, 2, 3A, 3B, 3C, and LP), three latent membrane proteins (LMP1, LMP2A, and LMP2B), two small EBV-encoded RNAs (EBERs) (latency III pattern of expression) (24), and microRNAs (37). Entry into the viral lytic cycle is initiated by expression of two immediate-early (IE) EBV proteins, i.e., Z Epstein-Barr virus replication activator (ZEBRA), encoded by BZLF1(44), and Rta, encoded by BRLF1 (50). The two IE proteins activate the viral early genes, resulting in a cascade of events that leads to progeny virions. EBV reactivation can be modeled by treating EBV-positive B-cell lines with pleiotropic agents, such as phorbol 12-myrisate 13-acetate (PMA) (an activator of protein kinase C), calcium ionophore (inducing calcium entry and upregulation of the calcium-dependent factors calcineurin and calcium/calmodulin-dependent kinase type IV [CAMK-IV]), *n*-butyrate (a histone deacetylase inhibitor), cross-linking of cell surface immunoglobulin G (which activates the B cell receptor) (for a review see reference 23), transforming growth factor beta 1 (TGF-β1) (11, 19, 31, 48), and nitric oxide (NO) inhibitor (26). Type 2 nitric oxide synthase (iNOS or NOS2) generates nitric oxide (NO) from the amino acid L-arginine and thereby contributes to the control of replication or killing of intracellular microbial pathogens.

The transforming growth factor beta family of peptides is involved in the control of several biological processes, including the immune response (inflammation) (13, 39). Binding of TGF-β to the TGF-β type II receptor (TβRII) triggers heterodimerization and phosphorylation of the TGF-β type I receptor (TβRI). The signal is then propagated through phosphorylation of receptor-regulated Smads (R-Smads), Smad2 and Smad3, which oligomerize with the common mediator (Co-Smad) Smad4 and then translocate to the nucleus, where they induce the expression of a large number of target genes (27, 41, 51). However, depending on the cell type, TGF-β1 signal transduction has been implicated in many second-messenger pathways, including the NF-κB pathway (3, 14). NF-κB is a dimeric transcription factor composed of homo- or heterodimers of p50 (NF-κB1), p52 (NF-κB2), p65 (RelA or NF-κB3), RelB, and c-Rel. These are endogenously complexed to inhibitor proteins called IκB that sequester NF-κB in the cytoplasm. In response to various stimuli, inhibitory proteins are phosphorylated at specific serine residues and are rapidly processed by the proteasome after polyubiquitination. This exposes the nuclear localization signal of NF-κB, leading to its nuclear translocation. The phosphorylation step is usually controlled by the IκB kinase (IKK) complex (38). The most frequent NF-κB dimer is p50/p65, which hence is considered to be the prototype (29).

Activation of NF-κB is a feature of many viral infections (30). Viruses may modulate the NF-κB pathway to enhance viral replication or prevent virus-induced apoptosis (18, 40).

Because NF-κB is involved in the maintenance of EBV

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latency and has an effect on lytic cycle progression, we investigated NF-κB activation in TGF-β1-treated positive Burkitt's lymphoma (BL) cell lines which exhibit a restricted, latency I (Lat I) pattern, in which EBNA1 is the only expressed viral protein. We identified a novel pathway that includes two unexpected steps, (i) a quick transient activation of NF-κB and (ii) NF-κB modulation of iNOS activity, both of which are required to control extracellular signal-regulated kinase (ERK) 1/2 phosphorylation; this last event is necessary for expression of the EBV protein ZEBRA, which is responsible for the induction of the viral lytic cycle.

MATERIALS AND METHODS

Cell culture and reagents. The EBV-positive BL cell lines Mutu-I, Kem-I, Sav-I, and B95-8 and the EBV-negative BL cell lines BJAB and DG75 were grown in RPMI 1640 supplemented with 2 mM glutamine (GIBCO BRL Life Technologies, Grand Island, NY), 100 μg/ml primocin (Cayla, Toulouse, France), and 10% heat-inactivated fetal calf serum (FCS) (GIBCO BRL Life Technologies, Grand Island, NY). For induction of the EBV lytic cycle, Mutu-I, Kem-I, and Sav-I cells were stimulated with 2 ng/ml of TGF-β1 (R&D Systems Minneapolis, MN) in reduced 0.5% FCS medium. U0126, phorbol 12-myristate 13-acetate (PMA), BAY1170-82, IKK2 inhibitor V, and MG262 were obtained from Calbiochem (VWR International, France), and SB-431542, N^G-monomethyl-L-arginine acetate salt (L-NMMA) and *S*-nitroso-*N*-acetylpenicillamine (SNAP) were purchased from Sigma (St. Louis, MO).

Antibodies. The anti-ZEBRA monoclonal antibody Z125 was obtained from E. Drouet (Faculté de Pharmacie, Grenoble, France). Antibodies against p44/p42 mitogen-activated protein kinase (MAPK), phospho-p44/p42 MAPK (Thr202/Tyr204), HP1γ, tubulin, p65, and IKBα were purchased from Ozyme (St. Quentin-en-Yvelines, France). Human antiserum to EA (D, R) was kindly provided by Jean H. Joncas (Sainte Justine Hospital, Montreal, Canada). Human anti-VCA antibody was obtained from Ortho Diagnostic Systems, Inc. (Raritan, NJ), and human anti-IgG was obtained from Dako (Denmark). Rabbit peroxidase-conjugated Ig, human peroxidase-conjugated IgG, and mouse peroxidase-conjugated IgG from Ozyme (St. Quentin-en-Yvelines, France) were used as secondary antibodies.

Cell fractionation. Cells were harvested at 4°C and lysed, and cytoplasmic and nuclear extracts were prepared using an NE-PER nuclear and cytoplasmic extraction reagent kit (Pierce Biotechnology) as described by the manufacturer. Protease inhibitors were added (complete mini-protease inhibitor cocktail tablets, obtained from Roche Diagnostic, Meylan, France). Briefly, 10 mg of cells was resuspended in 50 μl cytoplasmic extraction reagent I (CERI), mixed, and incubated on ice for 10 min; 2.75 μl of cytoplasmic extraction reagent II (CERII) was then added, followed by mixing and incubation on ice for 1 min. The intact nuclei were pelleted, and the supernatant cytoplasmic extract was collected. The nuclei were resuspended in 25 μl of ice-cold nuclear extraction reagent (NER), incubated on ice repeatedly, and centrifuged to obtain the supernatant containing nuclear proteins. Protein concentrations were determined using micro-bicinchoninic acid (BCA) protein assay kit from Pierce Biotechnology.

NF-κB activity. The activity of NF-κB was assessed by the levels of p65 in the nuclear fractions using an NF-κB/p65-active enzyme-linked immunosorbent assay (ELISA) kit (Imgenex, San Diego, CA). Nuclear and cytoplasmic extracts were prepared and subjected to an enzyme-linked immunosorbent assay according to the manufacturer's instructions. All experiments were repeated at least twice at different times.

Immunoblot analysis. Cells were harvested, washed briefly with phosphate-buffered saline, resuspended in a buffer composed of 100 mM Tris-Cl (pH 7.6), 50 mM NaCl, 2 mM EDTA, 0.5% NP-40, phenylmethylsulfonyl fluoride (100 μg/ml), and 1 μg each of leupeptin, pepstatin, and aprotinin per ml, and sonicated. The protein concentration was determined using a micro-BCA protein assay kit from Pierce Biotechnology. Equal amounts of protein in loading buffer, heated for 5 min at 100°C and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 to 12% gels, were transferred by electroblotting to a nitrocellulose membrane (Schleicher & Schuell, Ecquevilly, France). Membranes were reacted with the indicated primary antibodies using the appropriate dilution as indicated by the manufacturer, followed by treatment with a horseradish peroxidase-conjugated IgG (anti-rabbit, anti-mouse, or anti-human IgG antibody [1:2,000 to 1:4,000]) from Sigma (St. Louis, MO). Membranes were visualized with enhanced chemiluminescence (West-Pico or -Femto; Pierce Biotechnology, Rockford, IL). Images were captured using a charge-coupled device (CCD) camera (LAS-1000; Fuji system).

Reverse transcription-PCR (RT-PCR). Total RNA from each uninduced and induced EBV-positive cell lines was isolated using TRIzol reagent and RNase inhibitors (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The RNA concentration was determined spectrophotometrically. The template cDNA was synthesized by reverse transcription of total RNA (3 μg) with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) using a dT_{15} oligonucleotide primer (Promega). The primers used for PCR amplifications included 5′-TTACACCTGACCCATACCAG-3′ and 5′-ACATCTGCTTC AACAGGAGG-3′ for ZEBRA. Hypoxanthine phosphoribosyltransferase (HPRT) cDNA was used as internal control as previously described (2, 36).

Determination of cell viability. Cells were treated with different combinations of TGF-β1, BAY11-7082, IKK2 inhibitor V, U0126, SNAP, L-NMMA, for the indicated periods of time and then tested for viability using the LIVE/DEAD reduced-biohazard viability/cytotoxicity test (Molecular Probes, Invitrogen, Cergy Pontoise, France). Staining and analysis were performed as recommended by the manufacturer. The ability of this kit to detect cell death was controlled using a treatment with 0.4 mM $MnCl_2$ (10). Cell number was determined with a Kova counting chamber (Hycor, Edinburgh, United Kingdom). All experiments were repeated at least twice at different times.

Quantification of iNOS protein. The amount of iNOS in cell lysates was quantified with the Quantikine human iNOS immunoassay (R&D) according to the recommendations of the manufacturer. This assay employs a quantitative sandwich enzyme immunoassay technique. Briefly, a monoclonal antibody specific for iNOS is preapplied to a microplate. Standards and samples are pipetted into the wells, and any iNOS present is bound by the immobilized antibody. After any unbound substances are washed away, an enzyme-linked monoclonal antibody that is specific for iNOS is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells, and color develops (450 nm) in proportion to the amount of iNOS bound in the initial step.

Nitrate/nitrite assays. The amount of NO produced by cells was indirectly measured by the formation of nitrate (NO_3^-) and nitrite (NO_2^-) (two stable end products of NO) in culture supernatants. The total amounts of nitrate and nitrite were determined with a nitrate/nitrite assay kit (Cayman Chemical Co., Ann Arbor, MI), using the manufacturer's protocols. Briefly, supernatant (100 μl) was incubated with nitrate reductase (20 μl) at room temperature for 1 h, followed by incubation with 100 μl Griess reagent (1% sulfanilic acid, 0.1% naphthyl ethylenediamine dihydrochloride, 2.5% phosphoric acid) for 10 min. The optical density of each sample was analyzed at 540 nm in a microplate reader with sodium nitrite as a standard.

Expression and reporter plasmids. Plasmids pEGFP-p65 and active mutant NRAS have been described (33, 46). pEGFP, pRL-TK, and pNF-κB-Luc were purchased from Promega (Madison, WI). The plasmid coding for NF-κB-inducing kinase (NIK) was obtained from Open Biosystems (Thermo Scientific Open Biosystems, Huntsville, AL). The plasmid −234Zp-CAT(16) was generously provided by Henri Gruffat and contained bp −234 to +12 (relative to the transcription initiation site) of the BZLF1 promoter cloned upstream of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene.

Transient transfection and CAT assay. Plasmid DNA (5 μg) and pRL-TK vector (0.1 μg) were mixed with DG75 cells in 500 μl of RPMI 1640. The cells were exposed to a single pulse at 230 V and 960 mF (Bio-Rad, Richmond, CA). The transfected cells were resuspended in 5 ml of complete culture medium. Cells were harvested 24 h later and washed with phosphate-buffered saline, and the cell extract was subjected to the CAT ELISA as recommended by the manufacturer (Roche Diagnostics Mannheim, Germany). Each transfection and reporter assay result shown was compiled from two independent experiments. A *Renilla* luciferase assay was performed as recommended by the manufacturer (Promega, Madison, WI).

Luciferase assay. A total of 10^7 DG75 cells were transfected with 5 μg of pNF-κB-luc (Promega, Madison, WI) and 0.1 μg of pRL-TK internal control plasmid (Promega, Madison, WI), and combined with 5 μg pEGFP-p65 or 5 μg NIK (Open Biosystems, Thermo Scientific Open Biosystems, Huntsville, AL)-expressing plasmids in 6-well plates. After 6 h of incubation in complete culture medium, transfected cells were lysed, and the luciferase activity was assayed using a dual-luciferase reporter assay system (Promega, Madison, WI). Both firefly and *Renilla* luciferase activities were monitored with a FLUOstar Optima luminometer (BMG, Labtech, France). Normalized reporter activity was determined by dividing the firefly luciferase value by the *Renilla* luciferase value.

Silencing of p65. DG75 cells were transfected with a 20 nM concentration of either a small interfering oligonucleotide RNA (siRNA) specific for p65 or a

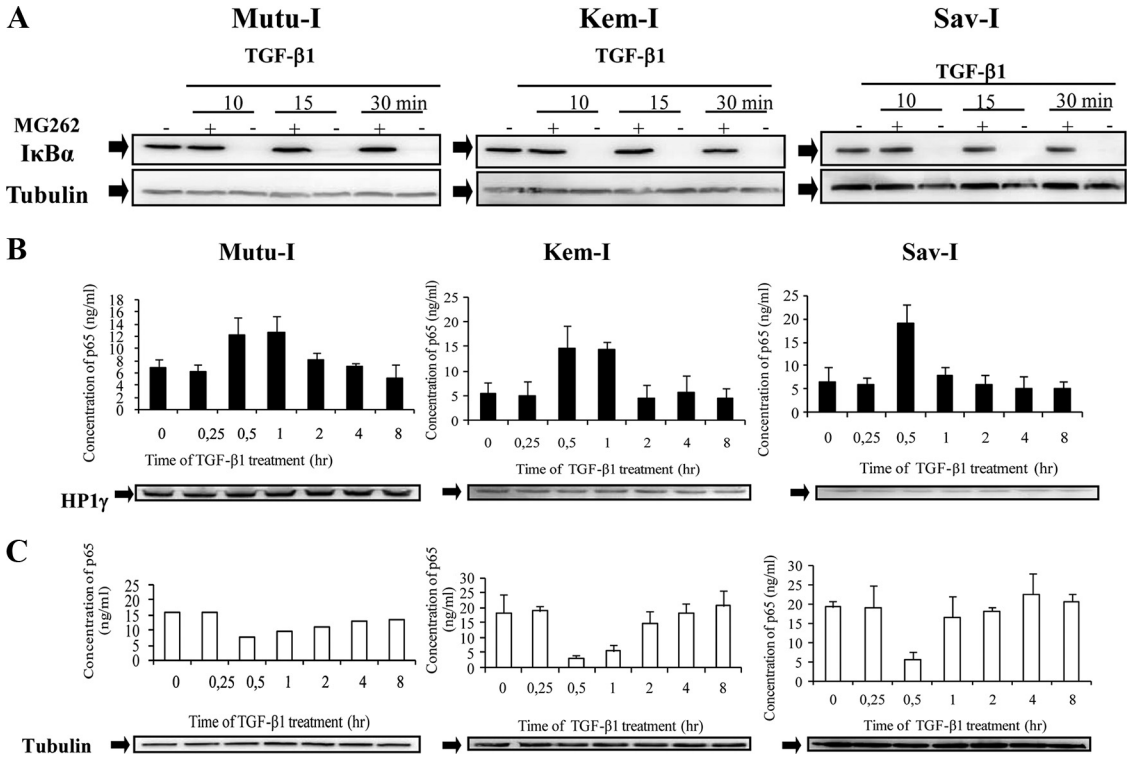

FIG. 1. TGF-β1 induces rapid and transient activation of NF-κB in BL cell lines. (A) Mutu-I, Kem-I, and Sav-I cells were pretreated or not with MG262 prior to stimulation with TGF-β1 (2 ng/ml) for 10, 15, or 30 min. Cells were harvested, washed, and lysed, and then equal amounts of protein were separated by SDS-PAGE and analyzed by Western blotting with antibodies to IκBα and tubulin. (B and C) Mutu-I, Kem-I, and Sav-I cells were treated with TGF-β1 (2 ng/ml) for various periods of time. At the indicated time points, cells were harvested; the nuclear (B) and cytoplasmic (C) extracts were isolated, and the content of NF-κB p65 protein was determined by ELISA (Imgenex). Loading of nuclear and cytosolic fractions was assayed by blotting with antibodies to HP1γ (for the nuclear fraction) and tubulin (for the cytosolic fraction).

scrambled nonsilencing control oligonucleotide (control siRNA) purchased from Ambion (Applied Biosystems, Courtaboeuf, France). Assessment of knockdown efficiency was performed 2 days later by immunoblotting.

RESULTS

TGF-β1 induces a rapid activation of NF-κB in BL cell lines. During latency, NF-κB activation is crucial for cell survival (for a review, see reference 42). Thus, we examined the effect of TGF-β1 on NF-κB, i.e., degradation of IκBα, which sequesters NF-κB in the cytoplasm, and subcellular localization of p65. IκBα was assayed by Western blotting of Mutu-I, Kem-I, and Sav-I cells after different periods of TGF-β1 treatment. As shown in Fig. 1A, in the three BL cell lines, the IκBα protein was no longer detectable after 10 min of TGF-β1 treatment. Addition of the proteasome inhibitor MG262 abrogated the degradation of IκBα, showing that treatment with TGF-β1 induces proteasome-dependent IκBα degradation. This should lead to NF-κB activation. To confirm this, the subcellular localization of RelA in Mutu-I, Kem-I, and Sav-I cells after treatment with 2 ng/ml of TGF-β1 for various time periods was investigated. Cell fractionation and an ELISA for estimation of p65 concentration were performed as recommended by the manufacturer. The loads of nuclear and cytoplasmic proteins were monitored by Western blotting of HP1γ or tubulin, respectively. The results are shown in Fig. 1B. In Mutu-I, Sav-I, and Kem-I cells, as soon as 30 min after addition of TGF-β1, the p65 subunit of NF-κB was translocated to the nucleus (Fig. 1B). In all three cell lines, when nuclear p65 was increased in the nucleus, its concentration in the cytoplasm decreased (Fig. 1C). However, the concentration of p65 in the nucleus decreased at 2 h after TGF-β1 treatment, and simultaneously the concentration of RelA protein in the cytoplasm increased. These results show that TGF-β1 induces a quick and transient activation of NF-κB. NF-κB activation was not a consequence of LMP1 expression, since this protein was not expressed during the Lat I program and in TGF-β1-treated BL cells as investigated by RT-PCR and Western blotting (data not shown).

NF-κB activation does not occur through the canonical TGF-β1 signaling pathway. Canonical signaling of TGF-β1 involves the activin receptor-like kinase 5 (ALK5) receptor (4); therefore, involvement of the ALK5 receptor in triggering TGF-β1-mediated activation of NF-κB was examined. Prior to 30 min of treatment with TGF-β1, Mutu-I cells were exposed for 1 h to SB-431542, which has been shown to specifically inhibit ALK5 (6, 20). This inhibitor blocked ALK5-dependent Smad pathway activation as judged by the total inhibition of Smad2 phosphorylation, but it did not alter the TGF-β1-induced subcellular localization of RelA in TGF-β1-treated Mutu-I cells (see Fig. S1 in the supplemental material). As a

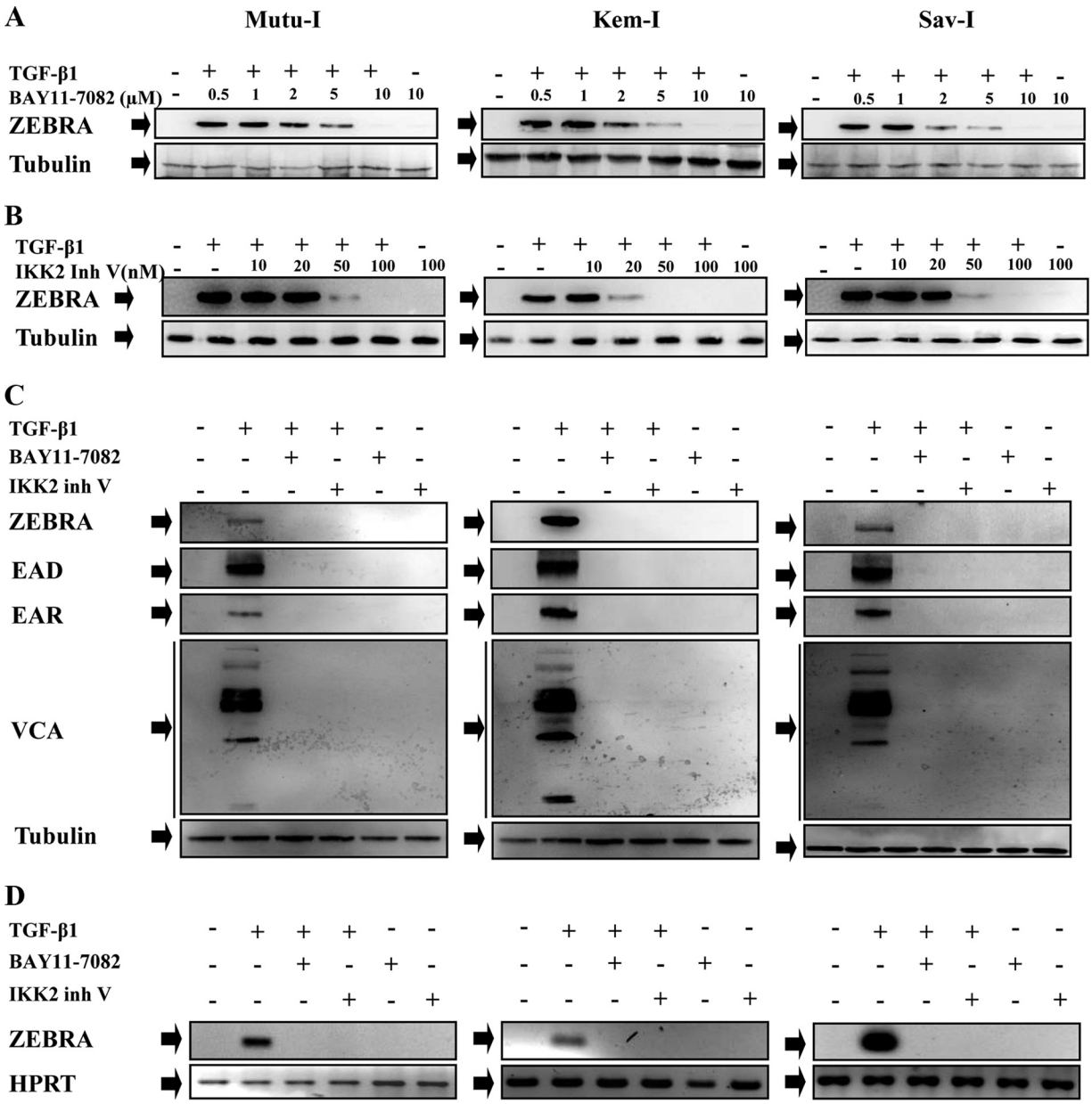

FIG. 2. Inhibitors of the NF-κB signaling pathway abolish TGF-β1-induced EBV reactivation. (A and B) Mutu-I, Kem-I, and Sav-I cells were treated with increasing concentrations of BAY11-7082 (0.5, 1, 2, 5, or 10 μM) (A) or IKK inhibitor V (10, 20, 50, or 100 nM) (B) for 1 h prior to incubation with TGF-β1 (2 ng/ml). Seventeen hours later, cells were harvested and lysed. Equal amounts of protein were separated by SDS-PAGE and analyzed by Western blotting with antibodies to ZEBRA and tubulin. (C) Total lysates from Mutu-I, Kem-I, and Sav-I cells were pretreated or not with 10 μM BAY11-7082 or with 100, 50, or 100 nM IKK inhibitor V for 1 h. They were then stimulated with TGF-β1 (2 ng/ml) for 17 h, separated by SDS-PAGE, and analyzed by Western blotting using antibodies against ZEBRA, EAD, EAR, VCA, and tubulin. (D) RT-PCR assay of ZEBRA was performed; 3 μg of total RNA from Mutu-I, Kem-I, and Sav-I cells was pretreated with 10 μM BAY11-7082 or 100, 50, or 100 nM IKK inhibitor V for 1 h, stimulated with TGF-β1 (2 ng/ml) for 17 h, and reverse transcribed. cDNA coding for ZEBRA was then analyzed by PCR; cDNA of HPRT was used as the internal control.

control, pretreatment with BAY11-7082 (a specific inhibitor of NF-κB) completely abolished p65 translocation. This result indicates that a noncanonical signaling pathway, independent of ALK5, was used to activate NF-κB.

Inhibition of the NF-κB pathway prevents TGF-β1-induced progression of the EBV lytic cycle. The contribution of the NF-κB pathway to the balance between the latent and lytic phases of the EBV life cycle was addressed using two different pharmacological inhibitors of NF-κB activation. We observed that low levels of BAY11-7082 inhibited TGF-β1-mediated ZEBRA expression in a dose-dependent manner (Fig. 2A). In Mutu-I and Kem-I cells, 5 μM BAY11-7082 drastically reduced ZEBRA expression, and total inhibition was observed with 10 μM BAY11-7082. In Sav-I cells, 2 μM BAY11-7082 severely reduced ZEBRA expression, and total inhibition was observed with a 10 μM concentration of the inhibitor. No

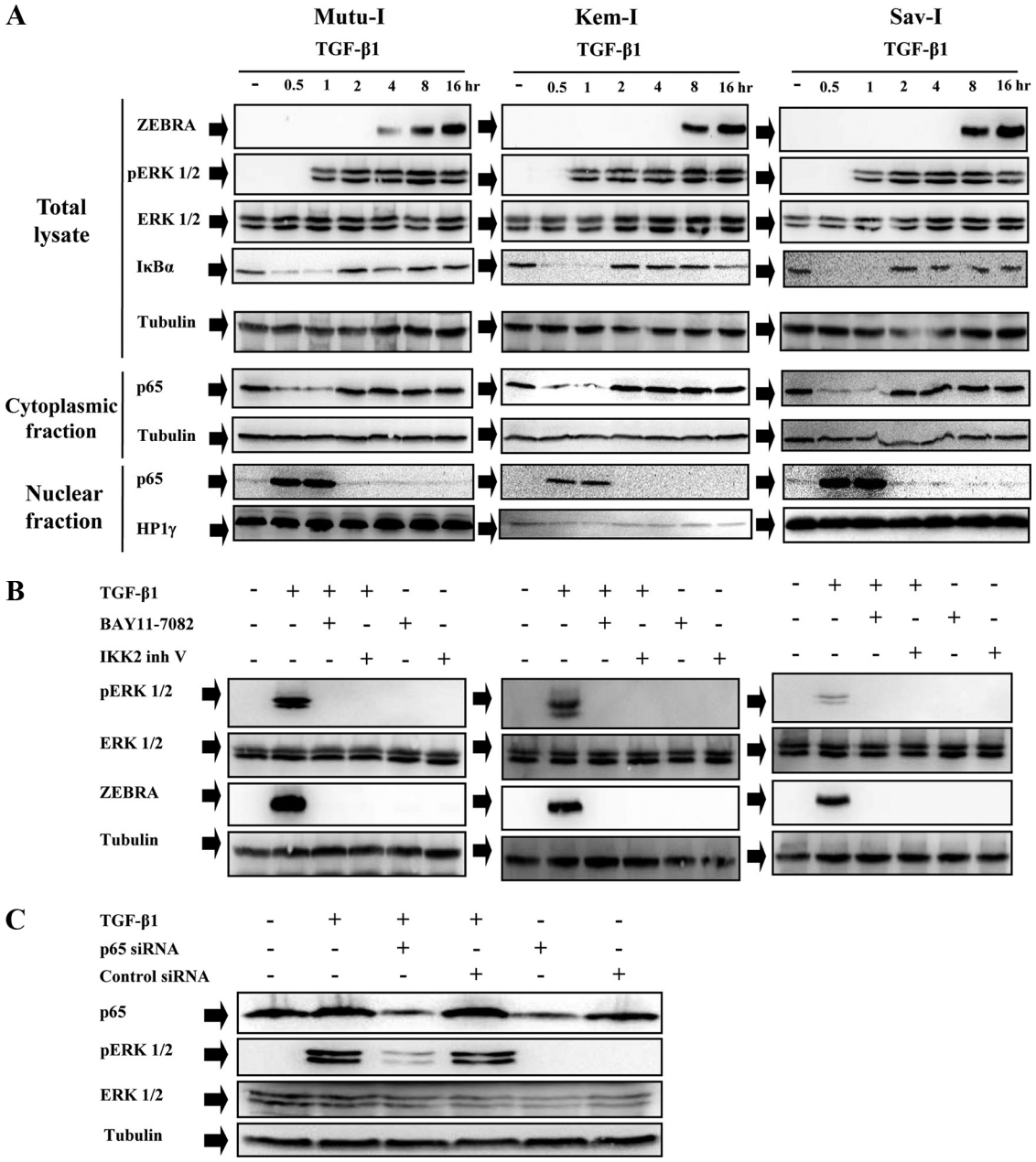

FIG. 3. Inhibitors of the NF-κB signaling pathway abolish TGF-β1-induced ERK 1/2 phosphorylation. (A) Time course of TGF-β1-induced ERK 1/2 phosphorylation in EBV-positive BL cells. Mutu-I, Kem-I, and Sav-I cells were treated with TGF-β1 (2 ng/ml) for various periods of time. At the indicated time points, cells were harvested and lysed. Equal amounts of protein were separated by SDS-PAGE and analyzed by Western blotting with antibodies to ZEBRA, phospho-ERK 1/2, ERK 1/2, IκBα, and tubulin. Cytosolic and nuclear extracts were prepared as described in Materials and Methods. Equal amounts of each extract were fractioned by SDS-PAGE, and the p65 content was determined by Western blotting using anti-p65 antibody. Loading of cytosolic and nuclear fractions was assayed by blotting with antibodies to tubulin (for the cytosolic fraction) and HP1G (for the nuclear fraction). (B) Mutu-I, Kem-I, and Sav-I cells were treated or not with 10 μM BAY11-7082 or with 100, 50, or 100 nM IKK inhibitor V for 1 h and then stimulated with TGF-β1 (2 ng/ml). Cells were harvested, washed, and resuspended in Laemmli sample buffer. Cell extracts were analyzed by Western blotting against phospho-ERK 1/2, ERK 1/2, ZEBRA, and tubulin. (C) DG75 cells were transfected or not with siRNA specific to p65 protein and then stimulated with TGF-β1 (2 ng/ml). Cells were harvested, washed, and resuspended in Laemmli sample buffer. Cell extracts were analyzed by Western blotting against p65, phospho-ERK 1/2, ERK 1/2, and tubulin.

significant effects of the inhibitor on cell viability were observed (see Fig. S2A in the supplemental material).

IKK2 inhibitor V is known to selectively block IκBα phosphorylation and prevent NF-κB p65 nuclear translocation (22, 34). TGF-β1-induced production of ZEBRA in Mutu-I, Kem-I, and Sav-I cells was reduced by treatment with this inhibitor in a dose-dependent manner (Fig. 2B). In Mutu-I and Sav-I cells, 50 nM IKK2 inhibitor V dramatically diminished ZEBRA expression, and complete inhibition was observed with 100 nM. For Kem-I cells, 20 nM IKK2 inhibitor V dra-

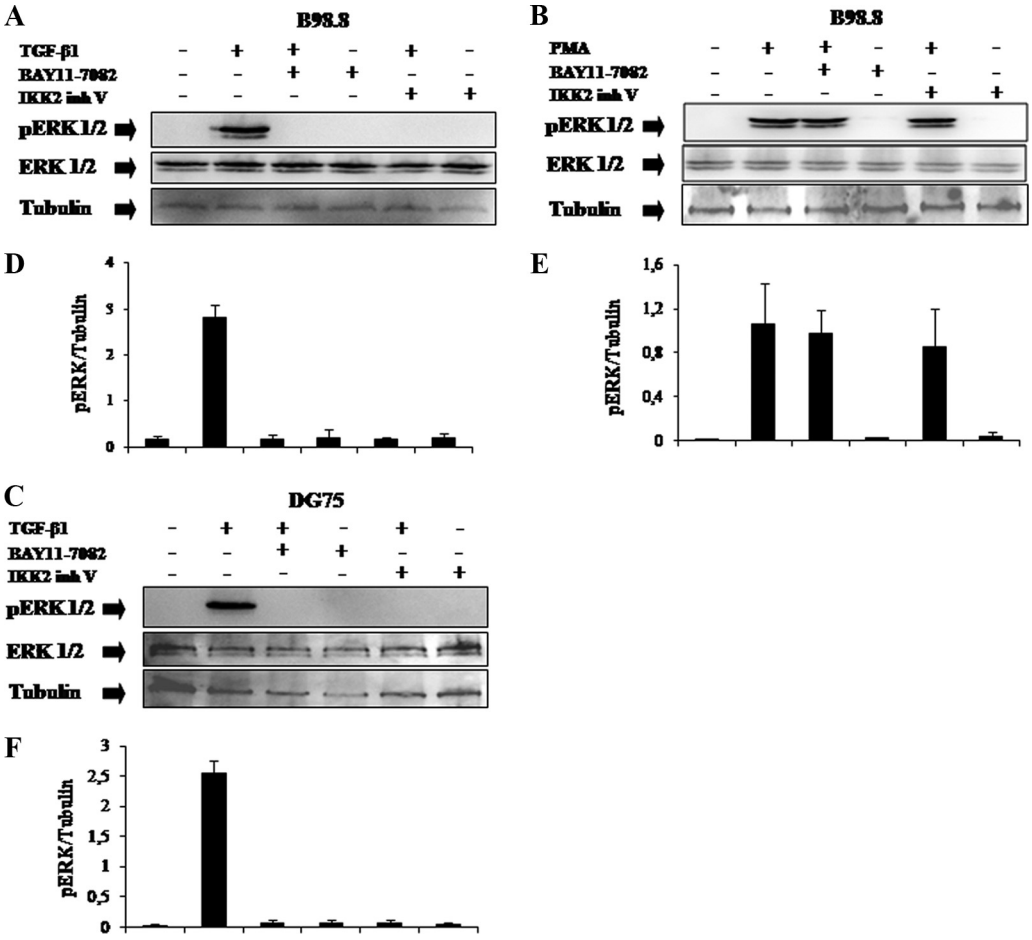

FIG. 4. Effect of NF-κB pathway inhibitors on PMA-, TGF-β1-, or anti-IgG-induced ERK 1/2 phosphorylation in B cells. (A to C) B95-8 (A and B) and DG75 (C) cells were pretreated for 1 h with BAY11-7082 (10 μM) or IKK2 inhibitor V (100 nM) before the addition of the appropriate ERK 1/2 pathway inducer agent (PMA [20 ng/ml] or TGF-β1 [2 ng/ml]). One hour later, cells were harvested and resuspended in Laemmli sample buffer. Equal amounts of protein were separated by SDS-PAGE and analyzed by Western blotting with anti-phospho-ERK 1/2, anti-ERK 1/2, and antitubulin antibodies. The signal was quantified with the ImageJ software. (D to F) Means and standard deviations calculated from two independent experiments.

matically diminished ZEBRA expression, and complete inhibition was observed with 50 nM (Fig. 2B). No significant effects of the inhibitor on cell viability were observed (see Fig. S2B in the supplemental material). In Mutu-I, Kem-I, and Sav-I TGF-β1-stimulated cells, the whole EBV lytic protein pattern, i.e., ZEBRA, EAD, EAR, and VCA, was expressed, showing that the virion proteins were produced. NF-κB inhibitors (BAY11-7082 and IKK2 inhibitor V) abrogated this expression (Fig. 2C).

Expression of the transcripts encoding ZEBRA was investigated by RT-PCR (Fig. 2D). Pretreatment with NF-κB inhibitors (BAY11-7082 or IKK2 inhibitor V) prevents amplification of BZLF1 cDNA. Consequently, inhibition of ZEBRA expression might be the result of inhibited production of transcripts encoding the protein. These results suggest that transient activation of NF-κB is required for TGF-β1-induced ZEBRA expression.

TGF-β1 induces NF-κB-dependent ERK 1/2 phosphorylation. We already established a key role of ERK 1/2 MAPK in the TGF-β1 signaling pathway (11). Given that transient NF-κB activation was also shown to be required for triggering ZEBRA expression upon TGF-β1 simulation, we focused on the possible interaction between the ERK 1/2 and NF-κB pathways. A detailed time course study of the kinetics of ERK 1/2 pathway activation in response to TGF-β1 stimulation indicated that activation of ERK 1/2 (1 h after addition of TGF-β1) occurred after IKBα degradation and nuclear p65 translocation (30 min) (Fig. 3A). The possible role of NF-κB in TGF-β1-induced ERK 1/2 signaling activation was then investigated in Mutu-I, Kem-I, and Sav-I cells. Those cells were treated or not with 10 μM BAY11-7082 or with 100, 50, or 100 nM IKK inhibitor V for 1 h and were then stimulated with TGF-β1 (2 ng/ml). Total proteins were analyzed for phosphorylated forms of ERK 1/2 by Western blotting with phospho-specific antibodies. The membranes were reprobed to evaluate total ERK 1/2 expression. Figure 3B shows that treatment with each NF-κB inhibitor completely inhibited ERK 1/2 phosphorylation and consequently ZEBRA expression. This result was confirmed by examining the effect of transfection a small interfering oligonucleotide RNA (siRNA) specific for p65 in

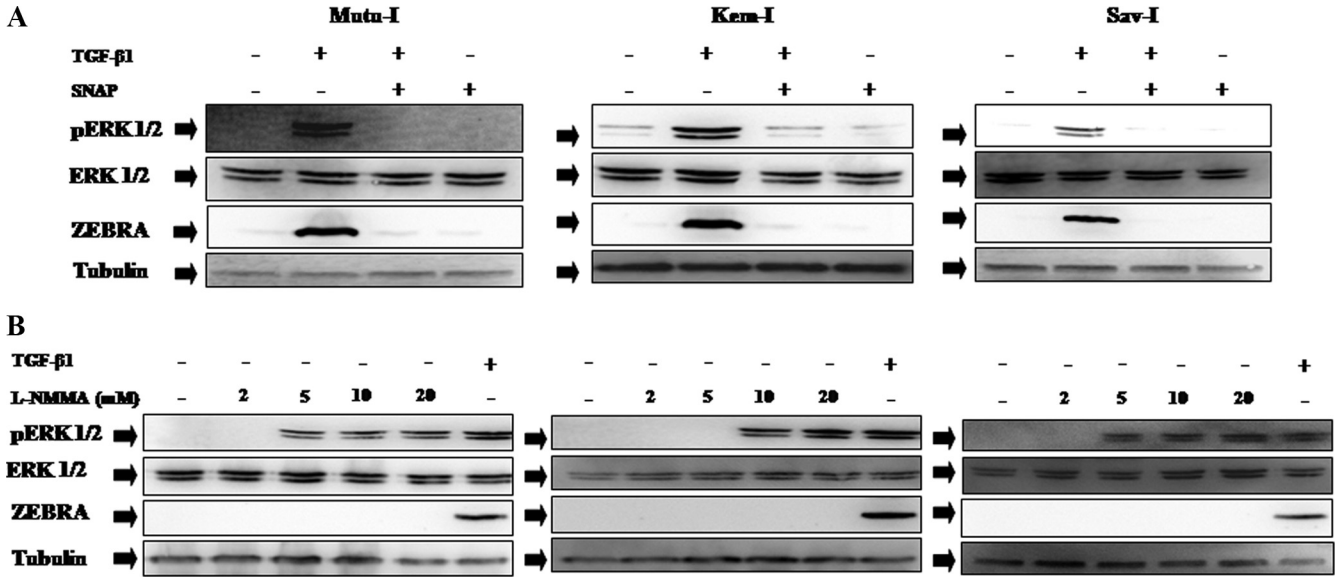

FIG. 5. Nitric oxide modulates TGF-β1-induced ERK 1/2 pathway activation. Mutu-I, Kem-I, and Sav-I cells were treated or not with 1 mM SNAP (A) or with increasing concentrations of L-NMMA (2, 5, 10, or 20 mM) (B) for 1 h prior to incubation with TGF-β1 (2 ng/ml) for 8 h. Cells were then harvested and lysed. Equal amounts of protein were separated by SDS-PAGE and analyzed by Western blotting with antibodies to phosphorylated-ERK 1/2, ERK 1/2, ZEBRA, and tubulin.

DG75 cells. This transfection dramatically reduced p65 expression as well as ERK 1/2 phosphorylation (Fig. 3C). As expected, inhibition of ERK 1/2 phosphorylation by the specific inhibitor U0126 did not affect TGF-β1-induced NF-κB activation (see Fig. S3 in the supplemental material). These data are in accordance with the hypothesis of NF-κB-dependent ERK 1/2 activation on TGF-β1 stimulation of BL cells.

NF-κB-dependent ERK 1/2 phosphorylation triggered by TGF-β1 also occurs in an LCL with a latency III pattern of EBV expression. TGF-β1-treated EBV-positive lymphoblastoid cell line (LCL) B95-8 cells also exhibited NF-κB-dependent ERK 1/2 phosphorylation, as pretreatment with either BAY11-7082 or IKK2 inhibitor V completely abrogated TGF-β1-induced ERK 1/2 phosphorylation as shown by Western blotting with antibodies to phospho-ERK 1/2 (Fig. 4A). Thus, in either latency I (Fig. 3B) or latency III (Fig. 4A), EBV-positive cells exhibited NF-κB-dependent ERK 1/2 activation.

However, when B95-8 cells were treated with PMA, although ERK 1/2 phosphorylation was observed, this activation was NF-κB independent since inhibition of this pathway did not affect PMA-induced ERK 1/2 phosphorylation (Fig. 4B). Thus, NF-κB-mediated ERK 1/2 phosphorylation was TGF-β1 dependent.

NF-κB-dependent ERK 1/2 phosphorylation triggered by TGF-β1 also occurs in EBV-negative BL cells. DG75 cells were treated with TGF-β1 for 1 h; ERK 1/2 was phosphorylated, and pretreatments with NF-κB inhibitors (BAY11-7082 or IKK2 inhibitor V) abolished TGF-β1-induced ERK 1/2 phosphorylation (Fig. 4C). This suggests that the effect of TGF-β1-mediated ERK 1/2 activation that occurs through NF-κB is independent of EBV.

NO inhibits TGF-β1-induced ERK 1/2 phosphorylation. It has been shown that inducible nitric oxide synthase (iNOS) activity is involved in maintaining EBV latency through down-regulation of ZEBRA expression (26). We analyzed whether the nitric oxide (NO) donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) had an effect on ERK 1/2 phosphorylation and EBV reactivation in Mutu-I, Kem-I, and Sav-I cells. These cells were treated (or not) with 1 mM SNAP (Fig. 5A). ERK 1/2 phosphorylation (as well as ZEBRA expression) was inhibited by the NO donor treatment. This suggests that inhibition of ZEBRA expression by NO was mediated by this MAPK pathway.

The NOS inhibitor N^G-monomethyl-L-arginine acetate salt (L-NMMA) was used to investigate the effect of NOS activity on ERK 1/2 phosphorylation. Mutu-I, Kem-I, and Sav-I cells were treated for 1 h with increasing concentrations of L-NMMA (2, 5, 10, or 20 mM), and cell lysates were assayed for ERK 1/2 phosphorylation and ZEBRA expression. As shown in Fig. 5B, 5 mM NOS inhibitor induced ERK 1/2 phosphorylation in Mutu-I and Sav-I cells, whereas 10 mM was efficient for Kem-I cells. However, although ERK 1/2 was phosphorylated under L-NMMA treatment, ZEBRA expression was not induced (Fig. 5B); this suggests that activation of this MAPK pathway is insufficient, even though it is necessary, to provoke EBV reactivation in the cells explored. No change in cell viability was observed under treatments with either SNAP or L-NMNA (see Fig. S4 in the supplemental material). Activity of the promoter directing expression of the ZEBRA protein could be achieved in DG75 cells by coexpression of Smad2 to -4 with Ras (which induced ERK 1/2 phosphorylation). This suggests that expression from Zp necessitated both ERK 1/2 MAPK and Smad activation (see Fig. S5 in the supplemental material).

TGF-β1 mediates inhibition of nitrite production through NF-κB activation. As NO influences ERK 1/2 phosphorylation, TGF-β1-treated BL cells were assayed for nitrite produc-

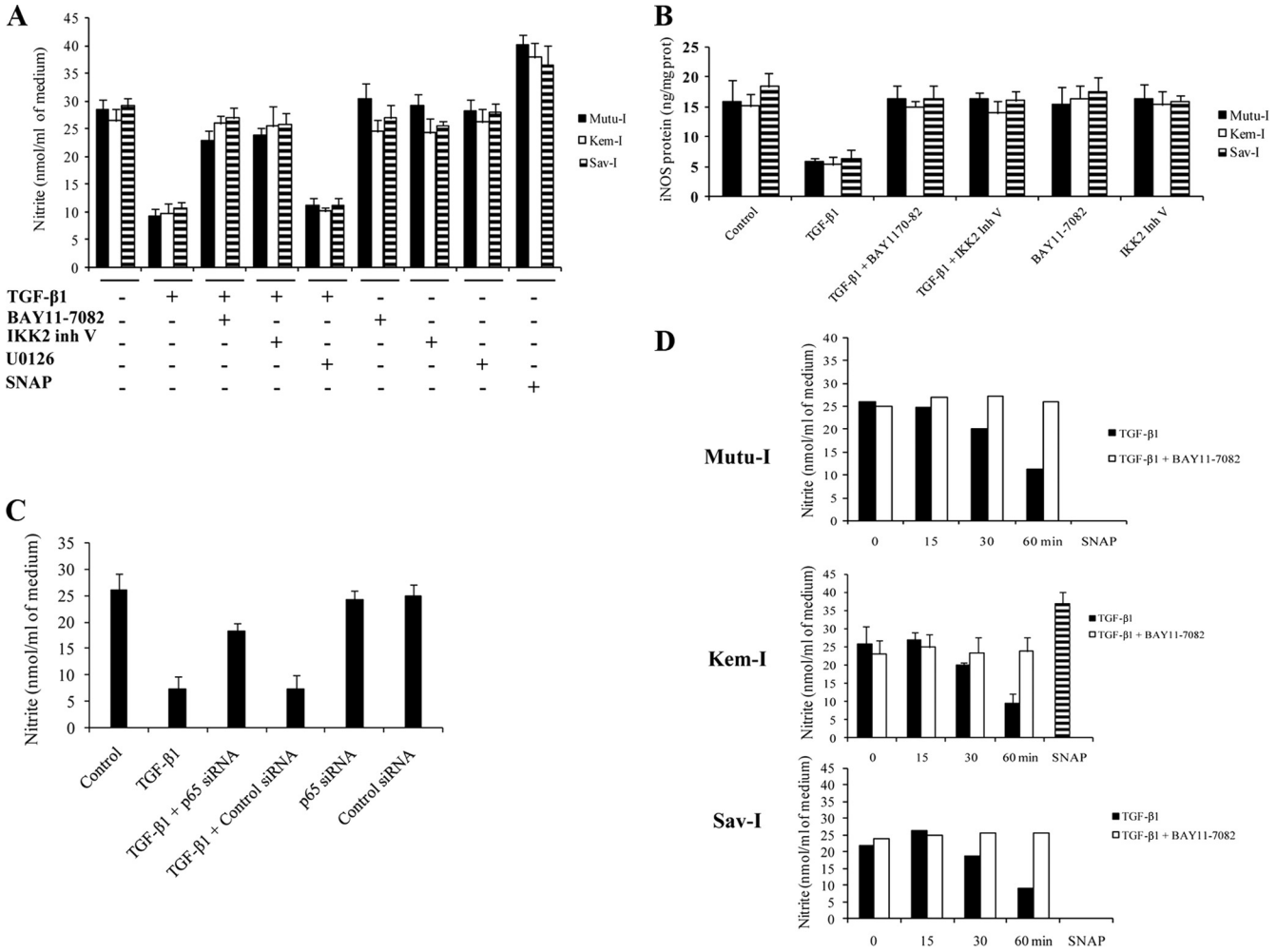

FIG. 6. TGF-β1 downregulates both nitric oxide and iNOS protein in BL cells through the NF-κB pathway. (A) Mutu-I, Kem-I, and Sav-I cells were pretreated or not with 10 μM BAY11-7082, 100, 50, or 100 nM IKK inhibitor V, 20 μM U0126, or 1 mM SNAP for 1 h prior to stimulation or not with TGF-β1 (2 ng/ml). Culture supernatants were collected 1 h later. The production of nitric oxide was measured with a nitrate/nitrite assay kit (Cayman). (B) Mutu-I, Kem-I, and Sav-I cells were pretreated or not with 10 μM BAY11-7082, 100, 50, or 100 nM IKK inhibitor V, or 10 mM L-NMMA for 1 h prior to stimulation or not with TGF-β1 (2 ng/ml). After 1 h of incubation, cells were collected, washed, and lysed and the amount of iNOS protein was measured with a Quantikine iNOS ELISA kit (R&D). (C) DG75 cells were transfected or not with siRNA specific to p65 protein and then stimulated with TGF-β1 (2 ng/ml). Culture supernatants were collected 1 h later and analyzed with a nitrate/nitrite assay kit (Cayman). (D) Mutu-I, Kem-I, and Sav-I cells were pretreated or not with 10 μM BAY11-7082 prior to incubation with TGF-β1 (2 ng/ml) for various periods of time. At the indicated time points, the production of nitric oxide was measured with a nitrate/nitrite assay kit (Cayman). Supernatants of Mutu-I, Kem-I, and Sav-I cultures cells treated with 1 mM SNAP for 1 h were used as positive controls.

tion in the culture medium. The results, presented in Fig. 6A, show that nitrite production was drastically diminished with TGF-β1 treatment. This reduction was dependent on NF-κB, as it was abolished by the NF-κB inhibitors BAY11-7082 and IKK inhibitor V. This result suggested that NF-κB inhibited the process of nitrite production, while, as expected, no effect on the decrease of nitrite production was observed when the BL cells were pretreated with U0126. The iNOS protein, explored in an ELISA, was shown to be diminished by treatment with TGF-β1 (Fig. 6B). This decrease was dependent on NF-κB, as specific inhibitors of this pathway abrogate this effect (Fig. 6B).

This result was confirmed by examining the effect of transfection of a RelA siRNA in DG75 cells. This transfection restored the level of nitrite production that had been decreased by TGF-β1 treatment (Fig. 6C). These results again show that activation of NF-κB is required for TGF-β1-mediated inhibition of nitrite production. The drastic TGF-β1-induced drop in nitrite production is observed mostly at 60 min posttreatment. This decrease in nitrite production was abolished by the NF-κB inhibitor BAY11-7082 (Fig. 6D) thus showing again that NF-κB is required to achieve this effect. However, when DG75 cells were transiently transfected with an expression plasmid for p65 (pEGFP-p65), no effect on nitrite production or on ERK 1/2 phosphorylation was observed (Fig. 7). Despite NF-κB activation, nitrite production was not decreased; thus, NF-κB alone was not sufficient to downregulate iNOS, demonstrating that another, uncharacterized event(s) is involved in this pathway.

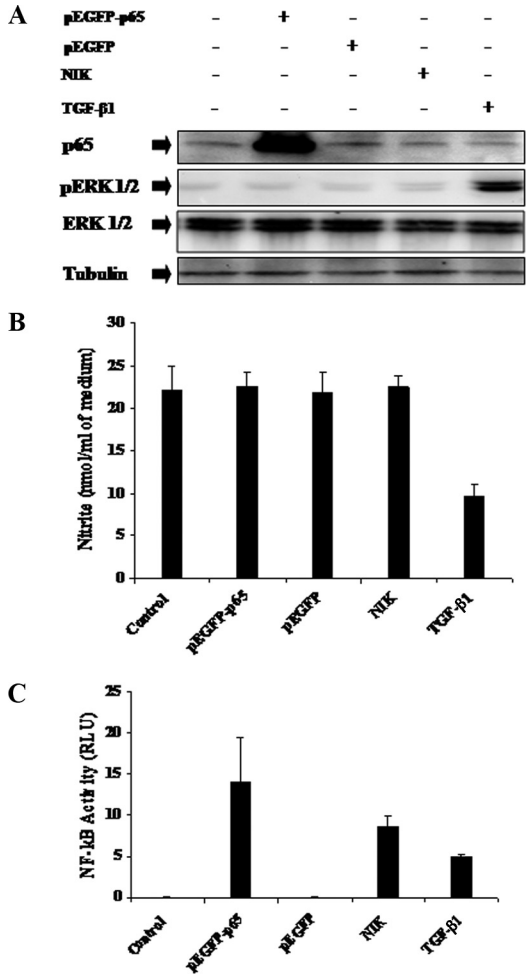

FIG. 7. The activated NF-κB pathway alone is not sufficient for TGF-β1-mediated downregulation of nitric oxide production. (A) DG75 cells were transiently transfected with 5 μg of the luciferase reporter plasmid pNF-κB-Luc and 100 ng of internal control plasmid pRL-TK or with a combination of expression plasmids for p65 (pEGFP-p65), NIK (positive control), or an empty vector (pEGFP) or were treated (or not) with TGF-β1 (2 ng/ml). Cells were harvested and lysed, and equal amounts of protein separated by SDS-PAGE were then Western blotted with antibodies against p65, phospho-ERK 1/2, ERK 1/2, and tubulin. (B) The production of nitric oxide was measured by a nitrate/nitrite assay (Cayman). (C) NF-κB-dependent luciferase activity (luminescence) was measured using a dual-luciferase assay system (Promega, Madison, WI); relative light units (RLU) were calculated by normalizing firefly luciferase activity against *Renilla* luciferase activity.

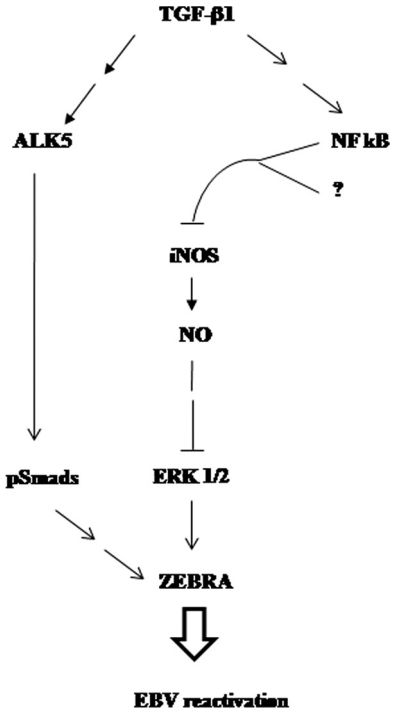

FIG. 8. Schematic description of the TGF-β1-mediated pathway that leads to EBV reactivation in BL cells. First there is a quick and transient activation of NF-κB, and then there is a downregulation of iNOS activity that requires the participation of NF-κB, leading to ERK 1/2 phosphorylation. Although necessary, NF-κB alone is not sufficient to produce downregulation of iNOS, suggesting that another, uncharacterized event(s) is involved at this step. Simultaneously, TGF-β1 mediates Smad pathway activation through its receptor I/ALK5. However, the activated canonical pathway (involving Smad factors) and noncanonical pathways (involving NF-κB, iNOS, and ERK 1/2) in concert contribute to TGF-β1-mediated ZEBRA expression and consequently EBV reactivation in BL cells.

DISCUSSION

TGF-β1, produced by a wide range of cells, is an active component of the inflammatory process. We have shown that it triggered entry of EBV into the lytic cycle. Moreover, in EBV-infected BL cells in which the productive viral cycle is induced, endogenous TGF-β1 production was activated (9). This effect would amplify the TGF-β1-induced effects. In EBV-positive BL cells TGF-β1 upregulates antiapoptotic pathways such as activation of PI3K/Akt (36) and downregulates the expression of the BimEL antiapoptotic molecule (35); this might contribute to the biology of BL. We have demonstrated that exposure of different EBV-positive BL cells to TGF-β1 resulted in the expression of ZEBRA protein (11) through the MAPK pathway. The molecular mechanisms underlying this TGF-β1-mediated ERK 1/2 activation remained unknown. In the present study we showed that upstream ERK 1/2 activation, a noncanonical pathway, included activation of NF-κB and modulation of iNOS. This work (summarized in Fig. 8) allowed identification of several steps involved in TGF-β1-induced EBV reactivation in BL cells.

Many agents that activate MAPKs also activate NF-κB, suggesting that cross talk between these pathways occurs (20). Here, we provide evidence that NF-κB was quickly and transiently activated by treatment with TGF-β1 (Fig. 1) and that the NF-κB signaling pathway was involved in the early steps leading to the TGF-β1 effect on viral expression. Our data clearly demonstrate that low levels of different NF-κB inhibitors (IKK2 inhibitor V and BAY11-7082) completely abolished TGF-β1-induced ERK 1/2 phosphorylation in different cell lines. Furthermore, ERK 1/2 phosphorylation was dramatically decreased by transfection of p65 siRNA. These results show that the NF-κB pathway is involved in the early steps of the TGF-β1-mediated MAPK activation (Fig. 3), which in turn is required for expression of ZEBRA protein. NF-κB signaling

upregulation occurred transiently in minutes after TGF-β1 addition; a few more hours were needed to express ZEBRA protein. Progression within the lytic cycle is further modulated by NF-κB Asp65, which inhibits ZEBRA transactivation of several lytic gene promoters (17).

The effect on NF-κB-mediated ERK 1/2 phosphorylation was not restricted to EBV-infected cells, as DG75 cells (EBV-negative BL cells) responded to TGF-β1 treatment by ERK 1/2 activation depending on NF-κB. However, other treatments that lead to ERK 1/2 phosphorylation, such as treatment with PMA or anti-Ig, did not signal through NF-κB, regardless of whether or not the cells were infected by EBV (Fig. 4). This suggests that the signaling pathway described in this study might be more restricted to TGF-β1. However, Gao et al. (12) showed that NF-κB was an important mediator in regulating EBV reactivation after tetradecanoyl phorbol acetate (TPA) treatment in gastric cells.

It has been shown that EBV reactivation of Akata cells leads to induction of expression of latent genes (25, 49), including the LMP1 EBV oncogene. In TGF-β1-treated Mutu-I cells, no LMP1 expression occurred, showing that NF-κB activation did not come from LMP1 signaling. Moreover, Yuan et al. (49) showed that in anti-Ig-treated-Akata cells, expression of LMP1 took place after DNA replication because it was phosphonoacetic acid (PAA) sensitive. In TGF-β1-treated Mutu-I cells, NF-κB activation occurred within 30 min of treatment; this time period was not compatible with the progression of the EBV cycle after DNA replication.

Mechanisms of NF-κB-mediated ERK 1/2 activation need to be characterized. Mannick et al. (26) have shown that NO inhibits EBV reactivation. NO is an antiviral effector of the innate immune system which can inhibit replication of herpesviruses. In this study, we have investigated the effect of NF-κB on iNOS activity. In many cell types, NF-κB induces iNOS (8, 21, 28). We have shown here, for the first time, that NF-κB is implicated in iNOS downregulation and that this step is crucial for ERK 1/2 activation. The mechanisms by which this phenomenon occurs need to be further investigated. Possible hypotheses can be put forward: interaction of NF-κB with transcription factors (for a review, see reference 15) or with nonhistone chromosomal proteins may modulate target gene expression (1). Mechanisms for TGF-β1-mediated suppression of NO release have been suggested: reduction of iNOS translation, a decrease in iNOS transcript stability, and increased degradation of iNOS proteins (45). Furthermore, different microRNAs have been shown to regulate iNOS expression (7, 47), and NF-κB may also alter gene expression through modulation of microRNA (52). The possible role of microRNA in the cascade of events downstream of TGF-β1 is currently under investigation.

We show here that NO abolishes ERK 1/2 phosphorylation (Fig. 5); this would explain that NO inhibits ZEBRA expression (26) through downregulation of ERK 1/2 activation (this paper). Low levels of iNOS are constitutively expressed in EBV-infected B cells, and NO downregulates EBV reactivation in those cells; downregulation of iNOS would prevent inhibition of EBV reactivation in TGF-β1-treated cells. iNOS plays a critical role in the eradication of intracellular pathogens. Complete EBV reactivation would give rise to particle progeny; thus, inhibition of iNOS will prevent EBV particle eradication by the host cell and thus will participate in the modulation of the innate immune system. Moreover, as low levels of NO promote cell growth (for a review, see reference 5), inhibition of iNOS during the process of EBV reactivation might contribute to tumorigenesis.

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