NF-κB activation upon interaction of HIV-1 envelope glycoproteins with cell surface CD4 involves IκB kinases

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Abstract Human immunodeficiency virus type-1 envelope glycoprotein (gp120^{env}) binding to cell surface CD4 receptor triggers a broad range of intracellular effects leading to T cell activation and cell cycle entry. Among these effects we and others previously reported on the nuclear translocation of the nuclear factor-κB (NF-κB) transcription factor. The present work further investigates the signal transduction pathways involved in gp120^{env}-induced NF-κB activation. We demonstrate that gp120^{env}–CD4 interaction stimulates the hyperphosphorylation of IκB-α inhibitory protein. Conversely, overexpression of a dominant-negative IκB-α transgene mutated at S32 and S36 residues, abolishes gp120^{env}-induced NF-κB activation. IκB kinases (IKKs) activity was found to be selectively enhanced following CD4 engagement with gp120^{env} and to mediate the phosphorylation of IκB-α while co-transfection experiments using dominant-negative forms of IKKs inhibited gp120^{env}-induced NF-κB activation. Taken together, these results confirm that IKKs complex play a key role in gp120^{env}-induced NF-κB activation. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Human immunodeficiency virus type-1; CD4; IκB kinase; Nuclear factor-κB; IκB

1. Introduction

Nuclear factor-κB (NF-κB) transcription factor is a family of related proteins that includes potent transactivator subunits (p65, c-Rel and RelB) and DNA-binding subunits (p50 and p52). Homo- and heterodimers formed by association of various members of the NF-κB protein family are important signal transduction mediators that regulate a broad range of genes involved in T cell activation, inflammatory processes, oncogenesis and activation of anti-apoptotic genes [1–3]. NF-κB also participates in the regulation of human immunodeficiency virus type-1 (HIV-1) gene expression by interacting with two tandemly repeated NF-κB sites located in the U3 region (−109 to −79) within the 5′-long terminal repeat (LTR) of the virus.

In unstimulated T lymphocytes, NF-κB is retained in the cytoplasm through reversible association with an inhibitory protein belonging to the IκB family, mainly represented by IκB-α, β, γ, ε (for review see [4,5]). In response to various stimuli including mitogen, stress factor, inflammatory cytokines [6], bacterial or viral gene products, activated NF-κB translocates to the nucleus as a result of proteolytic degradation of IκB proteins [7]. We previously demonstrated that interactions between HIV-1 gp120^{env} and the cell surface CD4 molecule were sufficient to stimulate NF-κB DNA-binding activities in CD4$^+$ CEM lymphoblastoid cell lines and in HeLa P4p56 cells, a non-lymphoid human cell line stably expressing CD4 and p56^{lck} and primary T lymphocytes [8–11].

Recent observations made in different cellular systems provided new possibilities to gain insight into the molecular mechanisms leading to NF-κB activation. It has been previously reported in the literature that subsequent to exogenous stimuli of resting cells, IκB inhibitory proteins are inducibly phosphorylated at residues S32 and S36, and correspondingly at positions S19 and S23 for IκB-β [12,13] and that serine kinases IκB kinase IKK1/α and IKK2/β are responsible for IκB phosphorylation [14–16]. Although IκB phosphorylation by IKKs is thought to be the common mechanism by which exogenous stimuli trigger NF-κB nuclear translocation, the expected role of IKKs in NF-κB activation induced by gp120^{env} binding to CD4 has not been investigated so far.

The aim of the present study was to determine the respective involvement of IκB-α, IκB-β, IKKs in the signaling pathways leading to NF-κB activation after gp120^{env} binding to CD4$^+$ cells. We demonstrate here that the cell surface-expressed gp120^{env} binding to the CD4 receptor on target cells induces phosphorylation and degradation of IκB-α and p50/p65 NF-κB heterodimers translocation to the nucleus of CD4$^+$ HeLa P4p56 cells. Moreover, we provide direct evidence that IKKs act as upstream kinases involved in IκB-α hyperphosphorylation subsequent to gp120^{env} binding to CD4.

2. Materials and methods

2.1. Cells

Non-lymphoid HeLa P4p56, HeLa 243^{env} and HeLa 273Δ^{env} cell lines kindly provided by Dr. O. Schwartz (Institut Pasteur, Paris) were cultured at 37°C (in a 5% CO_2 atmosphere) in Dulbecco's modified Eagle's culture medium (DMEM) supplemented with antibiotics, glutamax (Life Technologies, France), 10% mycoplasma-free heat-inactivated fetal bovine serum (FBS; Eurobio, Les Ulis, France). The medium was supplemented with 1 mg/ml G418 (Life Technologies) and 200 μg/ml hygromycin (Sigma Aldrich, Saint Louis, MO, USA). The CD4$^+$ lymphoblastoid CEM and 8E5 cell lines were cultured at a density of 5×10^5 cells/ml in RPMI 1640 (Eurobio) supplemented with antibiotics, glutamax, 10% mycoplasma-free heat-inactivated FBS.

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Abbreviations: IKK, IκB kinase; WT, wild type; DN, dominant negative; LTR, long terminal repeat

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2.2. Expression vectors

Plasmids pRc-CMV IκB-α wild type (WT), pRc-CMV IκB-α A32 A36, pRc-CMV IκB-βWT, pRc-CMV IκB-β A19 A23 provided by R. Weil (Institut Pasteur, Paris) have been previously described [12]. The N-Flag-CHUK, $_{K44A}$N-Flag-CHUK, pRK5 IKKβ C-Flag and pRK5 $_{K44A}$IKKβ C-Flag encoding, respectively, WT or kinase inactive IKKα or IKKβ [14,17] were provided by Tularik (South San Francisco, CA, USA). pcDNA3-RSK1-HA and pcDNA3-$_{D205N}$ RSK1-HA plasmids encoding, respectively, WT and dominant negative (DN) $p90^{rsk}$ kinase were provided by Dr. Zantema (Sylvius Laboratories, Leiden, The Netherlands) [18]. The pACβ1 plasmid (β-galactosidase-containing reference plasmid) was used to normalize transfection experiments.

2.3. Monoclonal antibodies, reagents and recombinant proteins

Anti-IκB-α, anti-IκB-β, anti-IKKα, anti-IKKβ rabbit polyclonal sera, anti-p50 and anti-p65 NF-κB subunit polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The expression of Flag-epitope tagged constructs was monitored with an anti-Flag M2 monoclonal antibody purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Anti-actin mAb was from ICN (Costa Mesa, CA, USA). GST-IκB-α was obtained from Santa Cruz Biotechnology. MG132 proteasome inhibitor was purchased from Affiniti (Exeter, UK). Recombinant tumor necrosis factor-α (TNF-α) was purchased from R&D System Corporation (Abingdon, UK). Recombinant soluble gp120 was obtained from Advanced Biotechnologies (Columbia, MD, USA). Cycloheximide and phorbol myristate acetate (PMA) were purchased from Sigma Aldrich. Staurosporine and H-7 (Santa Cruz) were used at a final concentration of 1 and 40 μM, respectively.

2.4. Oligonucleotides

Double stranded oligonucleotides NF-κB (NF-κB sequence from HIV-1) (sense strand only: 5′-GCTGG GGACT TTCCA GGGAG GCGT-3′) purchased from Eurogentec (Seraing, Belgium) were used for electrophoretic mobility shift assay (EMSA).

2.5. Detection of β-galactosidase activity

HeLa P4p56 (1×10^6) cells were cultured in the presence of medium alone or supplemented with additives, or co-cultivated in the presence of 5×10^5 CEM ($gp120^{env}$-negative) or 8E5 ($gp120^{env}$-positive) cells. HeLa P4p56 cells were extensively washed to remove non-adherent cells, harvested and lysed. β-Galactosidase activities were determined as previously described [19]. β-Galactosidase activities were normalized according to total protein contents of the samples (Bio-Rad Protein Assay).

2.6. Western blotting analysis

Cell lysates were electrophoresed in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto polyvinylidene difluoride membranes (Millipore, St. Quentin en Yvelines, France). The blots were incubated for 1 h at 20°C with the appropriate mAb, washed three times with phosphate-buffered saline (PBS), 0.05% Tween-20 and incubated for 30 min with 1/3000 dilution of goat anti-mouse or goat anti-rabbit Ig peroxidase conjugate (Immunotech, Marseilles, France). Bound mAbs were detected by incubating the membrane for 1 min with enhanced chemoluminescence reagent (Amersham, Orsay, France) and exposed to Hyperfilms (Amersham). Band intensities were quantified by using the NIH Image 1.61 program after scanning of the autoradiograms with a SnapScan 600 scanner (Agfa, Mortsel, Belgium).

2.7. EMSA and supershift experiments

Nuclear extracts were prepared according to the published method [20]. The mobility shift assays were performed using 2 μg protein of nuclear extract, 1×10^5 cpm of a ^{32}P-labeled oligonucleotide corresponding to the NF-κB sequence binding site from the HIV-1 and 100 mM KCl, 1 mM dithiothreitol (DTT), 1 mM $ZnSO_4$, 20% glycerol, 0.01% Nonidet P-40 and 50 mM HEPES pH 7.9, supplemented with bovine serum albumin (BSA), tRNA and poly(dI:dC). After 20 min at room temperature, the mixture was separated on a 10% polyacrylamide gel. The supershift experiments were performed by incubating nuclear extracts with 1 μl of serum to p50, or p65 followed by incubation with ^{32}P-labeled NF-κB oligonucleotide. The resulting DNA–protein complexes were analyzed by EMSA as described above.

2.8. Flow cytometry detection of cell surface-expressed HIV-1 $gp120^{env}$

Cells (1×10^6) were washed in PBS and incubated for 1 h at 4°C with PBS containing 0.2% BSA (PBS-BSA) or PBS-BSA supplemented with 1/50 dilution of pool of sera from HIV-infected patients. After incubation, the cells were washed twice in ice-cooled PBS-BSA and incubated for an additional 30 min in the presence of 1/50 dilution of goat anti-human fluorescein isothiocyanate (FITC)-labeled secondary antibodies (Immunotech). After three washes, the cells were resuspended in PBS and fluorescence intensity was recorded on the log mode on an Epics XL4 flow cytofluorometer (Coulter, Coultronics, Margency, France).

2.9. Transfection experiments

HeLa P4p56 cells grown in exponential phase were transfected by using the polyethyleneimine transfection technique with a ratio of 2 μl of Exgen 500 transfection reagent (Euromedex, Soufflemeyerseheim, France) per 1 μg of DNA. 36 h post-transfection HeLa P4p56 cells were used in stimulation experiments. Transfection efficiency was assessed by quantification of luciferase activity in total cell extracts. Briefly, an aliquot of the cells was lysed in passive lysis buffer (Promega, Charbonnières, France) and luciferase activities were determined from 20 μl total cell extract in the presence of buffer containing 20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 530 μM ATP, 470 μM luciferin with a Lumat LB9507 luminometer (Berthold, Evry, France).

CEM cells were transfected by electroporation. Briefly, 1×10^7 cells were resuspended in 400 μl RPMI and incubated for 10 min at room temperature in the presence of 10 μg DNA plasmids and electroporated. After shocking, the cells were resuspended in RPMI medium containing 20% fetal calf serum at a density of 5×10^5 cells/ml. Transfected cells were used in stimulation experiments 36 h post-transfection.

2.10. Immunoprecipitation of IKKs and in vitro kinase assays

300 μg of cell extract were immunoprecipitated with 4 μg M2 anti-Flag mAb for 1 h at 4°C before incubation with A-Sepharose beads for another 30 min at 4°C. The beads were washed three times in lysis buffer and resuspended in 25 μl kinase buffer containing 20 M HEPES, pH 7.9, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 10 mM NaF, 10 mM β-glycerophosphate, 30 μM $NaVO_3$, 2 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM DTT with 5 μM ATP, 5 μCi [γ-^{32}P]ATP and 1 μg GST-IκB-α. The kinase reaction was performed for 20 min at 30°C and stopped by addition of 2× Laemmli buffer. Phosphorylated proteins were separated on a 15% SDS–PAGE, transferred onto Immobilon P membrane (Millipore) and examined by autoradiography.

3. Results

3.1. $gp120^{env}$ binding to CD4 activates NF-κB-dependent HIV-1 LTR transactivation by stimulating p50/p65 complexes nuclear translocation

We have previously shown that multimerized soluble HIV-1 envelope glycoprotein $gp120^{env}$ induces NF-κB nuclear translocation and HIV-1 genes transcription in $CD4^+$ primary T lymphocytes and T lymphoblastoid cell lines [8,21]. NF-κB activation signals can also be delivered following interaction between multimerized $gp120^{env}$ and CD4 present at the surface of non-lymphoid HeLa P4p56 cells, a cell line stably expressing the CD4 and the $p56^{lck}$ molecules and an integrated copy of a β-galactosidase reporter gene driven by the HIV-1 LTR promoter [19,22].

In the present study we tested the capacity of cell surface-expressed $gp120^{env}$ to stimulate NF-κB activation and HIV-1 LTR driven reporter gene transcription in HeLa P4p56 cells. To this end, 8E5 cells, a derivative of CEM T cells infected with a defective uninfectious HIV-1 strain expressing $gp120^{env}$ at the cell surface (Fig. 1C), were co-cultured for various times with HeLa P4p56 cells and transactivation of HIV-1 LTR was assessed by quantification of β-galactosidase activities in total

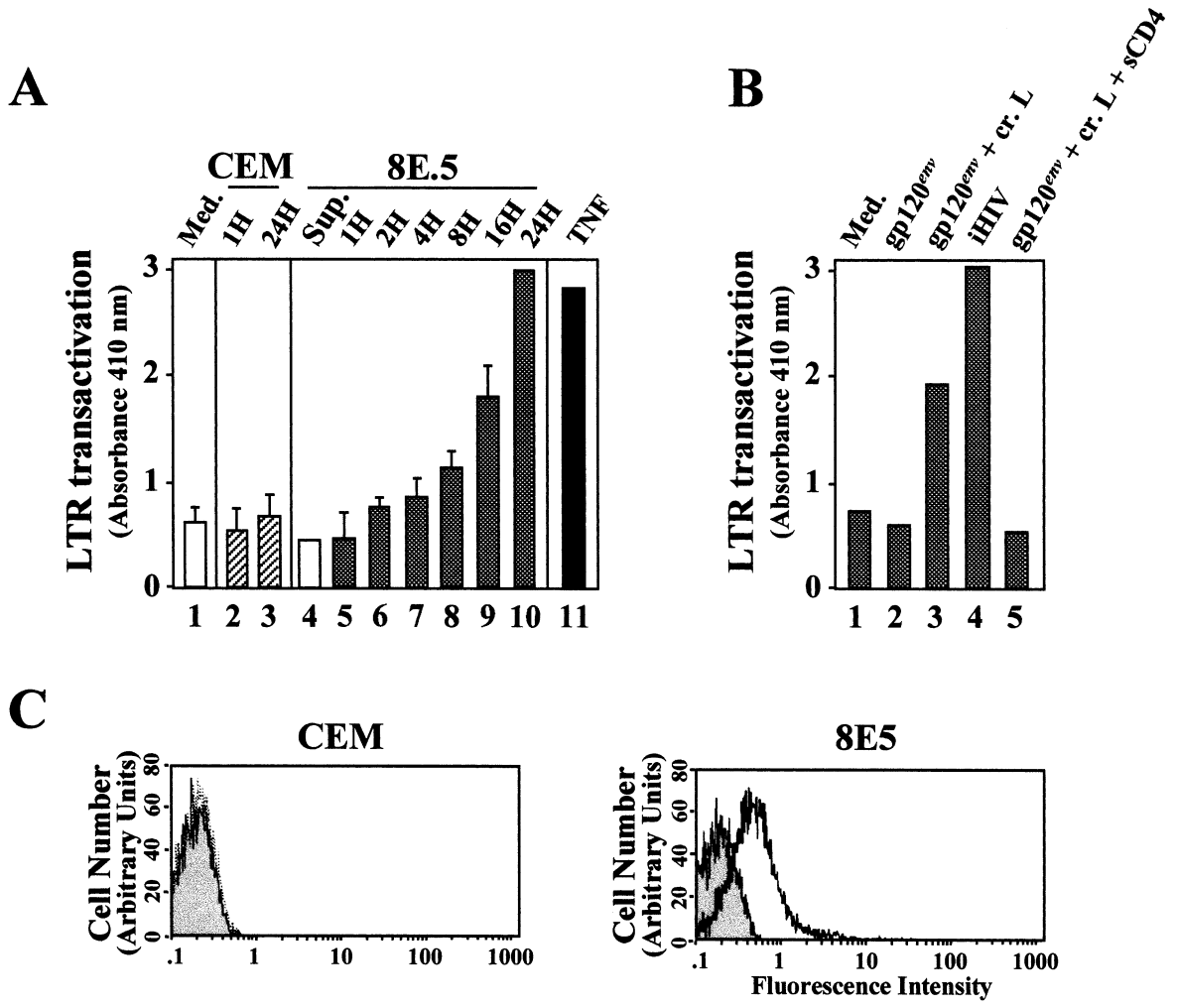

Fig. 1. Cell surface expression of HIV-1 envelope glycoprotein gp120^{env} on 8E5 cells stimulates viral LTR transactivation in HeLa P4p56 indicator cells. A: Transactivation of HIV-1 LTR by cell surface-expressed gp120^{env}. 1×10^6 HeLa P4p56 cells were cultivated in DMEM alone (Med.) (lane 1) or maintained in the presence of 5×10^5 gp120^{env}-negative CEM cells for 1 or 24 h (lane 2 and 3) or 5×10^5 gp120^{env}-positive 8E5 cells for 1–24 h at 37°C (lanes 5–10). In control experiments, cells were cultured in medium supplemented with 100 ng/ml TNF-α (lane 11) or in filtered culture supernatant from 8E5 cells (Sup.) (lane 4). LTR transactivation was measured by quantification of β-galactosidase activity in total cell lysates in the presence of ONPG substrate in appropriate buffer followed by measuring absorbance at 410 nm. Absorbances were normalized according to the amount of proteins contained in cell lysates. B: Multimeric gp120^{env} specifically stimulates LTR transactivation in HeLa P4p56 cells. LTR transactivation was observed when HeLa P4p56 cells were cultured for 24 h in the presence of recombinant gp120^{env} protein (5 μg/ml) complexed with anti-gp120^{env} antibodies (gp120^{env}+cr. L) (lane 3), or heat-inactivated HIV-1 (iHIV) (lane 4), but not when the cells were incubated either with monomeric gp120^{env} (5 μg/ml) (lane 2) or when gp120^{env}–anti-gp120^{env} immune complexes were preincubated with 10 μg/ml sCD4 for 2 h at 4°C prior to addition to cells (lane 5). C: Characterization of HIV-1 gp120^{env} expression at the surface of 8E5 cells by flow cytometry. CEM cells (left panel) or CEM-derived HIV-1 infected 8E5 cells (right panel) were incubated with a pool of sera from HIV-1-infected patients with gp120^{env} reactivity. Bound immunoglobulins were revealed by incubating the cells with anti-human-FITC secondary antibodies and fluorescence was recorded on the log mode using an Epics XL4 cytofluorometer (Coulter) (white histograms). Isotypic controls (gray histograms) were performed by direct incubation of the cells with the secondary antibodies.

HeLa P4p56 cell extracts. Transcription of the β-galactosidase reporter gene was induced from 4 to 24 h in HeLa P4p56 cells co-cultivated with gp120^{env}-positive 8E5 cells (Fig. 1A), indicating that viral LTR transactivation has occurred. In contrast, no β-galactosidase activity was observed in HeLa P4p56 cells co-cultivated with gp120^{env}-negative CEM cells. In such co-cultivation experiments LTR transactivation cannot be ascribed to cell-free defective virus particles or soluble Tat transactivation of LTR-β-galactosidase reporter gene since cell-free supernatant of 8E5 cells did not stimulate β-galactosidase gene expression (Fig. 1A, lane 4). Induction of β-galactosidase gene transcription in response to TNF-α proinflammatory cytokine is shown as a positive control (Fig. 1A, lane 11).

The contribution of cell surface-expressed gp120^{env} in LTR transactivation was ascertained by incubating HeLa P4p56 cells with soluble gp120^{env} or with gp120^{env}–anti-gp120^{env} immune complexes or with heat-inactivated HIV-1 (Fig. 1B). As previously reported for peripheral blood mononuclear cells [9], LTR transactivation was observed upon stimulation of HeLa P4p56 cells with multimeric gp120^{env} resulting from gp120^{env}–anti-gp120^{env} immune complexes or heat-inactivated HIV-1, but not when the cells were stimulated with monomeric recombinant gp120^{env} or when immune complexes were preincubated with sCD4 before addition to the HeLa P4p56 cells.

The implication of NF-κB DNA-binding proteins in LTR

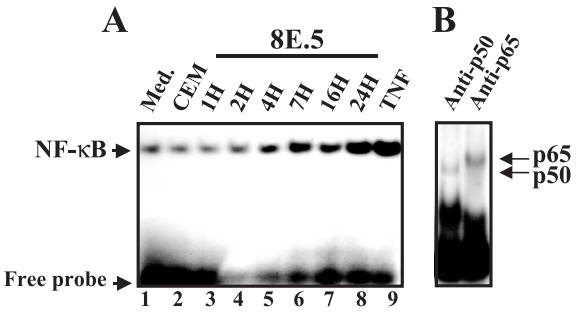

Fig. 2. Cell surface-expressed gp120^{env} stimulates nuclear translocation of p50/p65 NF-κB complexes. A: Induction of NF-κB DNA-binding activity in HeLa P4p56 cells co-cultivated with gp120^{env}-positive 8E5 cells was tested in gel shift experiments. Nuclear extracts prepared from HeLa P4p56 cells cultivated in DMEM alone (Med.) (lane 1) or supplemented with 100 ng/ml TNF-α (lane 9) or co-cultivated either with 5×10^5 gp120^{env}-negative CEM cells for 16 h (lane 2), or with 5×10^5 gp120^{env}-positive 8E5 cells for various times (lanes 3–8) were reacted with radiolabelled double-stranded NF-κB oligonucleotide probe (HIV-1_{Lai} LTR sequence). The samples were electrophoresed and analyzed by autoradiography. B: Characterization by supershift experiments of NF-κB complexes activated in gp120^{env}-stimulated HeLa P4p56 cells. Nuclear extracts of HeLa P4p56 cells co-cultivated for 24 h in the presence of gp120^{env}-positive 8E5 cells were reacted with NF-κB subunit specific mAbs (anti-p50 or anti-p65) before incubation with the radiolabelled NF-κB oligonucleotide probe.

transactivation was next analyzed by EMSA. Nuclear proteins extracted from HeLa P4p56 cells maintained for various times in the presence of gp120^{env}-negative CEM cells or gp120^{env}-positive 8E5 cells were reacted with radiolabelled oligonucleotides containing a consensus binding site for NF-κB factor. As shown in Fig. 2A, an increase in shifted oligonucleotide probe was observed when HeLa P4p56 cells were co-cultured for 4–24 h with 8E5 cells as compared with HeLa P4p56 cells maintained for 16 h in the presence of CEM cells (lane 2) or medium alone (lane 1), indicating that cell surface-expressed gp120^{env} stimulates NF-κB binding activity in HeLa P4p56 cells. TNF-α stimulation is shown as a positive control for NF-κB activation (Fig. 2A, lane 9). The nature of the NF-κB complexes stimulated after gp120^{env}–CD4 interaction was then investigated by supershift experiments in the presence of antiserum raised either to p50 or to p65 subunits (Fig. 2B). Slower migration patterns were detected when nuclear extracts of cells co-cultivated with 8E5 cells for 16 h were preincubated with monoclonal antibodies to p50 or p65. Although these results do not exclude the possibility that other members of the NF-κB family are present in the DNA-binding complex induced by cell surface-expressed HIV-1 gp120^{env}, our data indicate that the NF-κB complex consists at least in part of p50/p65 proteins.

3.2. Phosphorylation and degradation of IκB-α inhibitory protein is targeted by cell surface-expressed HIV-1 gp120^{env} binding to CD4

Having determined that DNA-binding activity of p50/p65 NF-κB complexes is enhanced in HeLa P4p56 cells following co-cultivation with gp120^{env}-positive 8E5 cells, we next analyzed the consequences of such activation on phosphorylation and degradation of IκB inhibitors. Total cell extracts prepared from HeLa P4p56 cells after different co-cultivation times either in the presence of CEM or 8E5 cells or in medium supplemented with TNF-α were probed in Western blotting experiments with anti-IκB-α or anti-IκB-β mAbs (Fig. 3) or with an anti-actin antibody to control the amounts of proteins present in each sample. Co-cultivation experiments were performed in the presence of cycloheximide in order to prevent de novo synthesis of IκB inhibitory proteins during the incubation periods. As expected, stimulation of HeLa P4p56 with TNF-α induced a rapid and complete degradation of IκB-α (Fig. 3, upper panel). IκB-α degradation was found to be simulated in HeLa P4p56 cells maintained for 2 h in the presence of gp120^{env}-positive 8E5 cells, with a complete degradation after 4 h co-cultivation. In contrast, no significant modification was observed in extracts of HeLa P4p56 cells maintained for 4 h in the presence of CEM cells. The half-life of IκB-α deduced after scanning of immunoblotting experiments was estimated to be approximately 120 min in extracts of HeLa P4p56 cells co-cultivated with CEM cells and was lowered to approximately 60 min when the cells were co-cultured with 8E5 cells. In contrast, IκB-β inhibitor was not found degraded upon stimulation of HeLa P4p56 cells with gp120^{env}-positive 8E5 cells.

We next investigated whether this degradation is linked to IκB-α phosphorylation on critical serine residues. To this end, total cellular extracts of HeLa P4p56 cells co-cultured with CEM or 8E5 cells for different time periods were analyzed by immunoblotting with an antibody specific for S32 phosphorylated forms of IκB-α (Fig. 3, lower panel). To avoid degradation of phosphorylated forms of IκB-α, co-cultivation experiments were performed in the presence of MG132 proteasome inhibitor. Phosphorylated IκB-α was clearly evidenced in HeLa P4p56 cells after 1 h co-cultivation with gp120^{env}-positive 8E5 cells. In contrast, phosphorylated IκB-α was not detected in HeLa P4p56 cells co-cultivated for 4 h in medium alone or maintained in the presence of CEM cells for an equivalent time period.

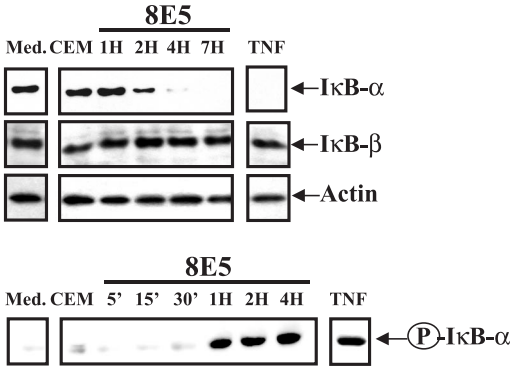

Fig. 3. Degradation of IκB inhibitory proteins in HeLa P4p56 cells after stimulation with cell surface-expressed gp120^{env}. A: Cell lysates from HeLa P4p56 cells co-cultivated in the presence of 5×10^5 gp120^{env}-negative CEM cells for 4 h or 5×10^5 gp120^{env}-positive 8E5 for different time periods were analyzed by immunoblotting with anti-IκB-α anti-IκB-β mAbs or anti-phospho-IκB-α mAbs (specific for S32 phosphorylated forms of IκB-α). Lysates from cells cultivated in DMEM (Med.) is shown to estimate the basal expression level of IκB-α in unstimulated cells and extracts from HeLa P4p56 cells cultivated in medium supplemented with 100 ng/ml of TNF-α are shown as positive control for activation. Immunoblotting of cell extracts with anti-actin mAb was performed to estimate the amount of total proteins in each lane. Stimulation experiments aimed at analyzing the degradation of IκB-α or IκB-β were performed in the presence of 50 μg/ml cycloheximide to block IκB proteins resynthesis. Lysates reacted with anti-phospho-IκB-α were obtained from cells activated in the presence of MG132 proteasome inhibitor.

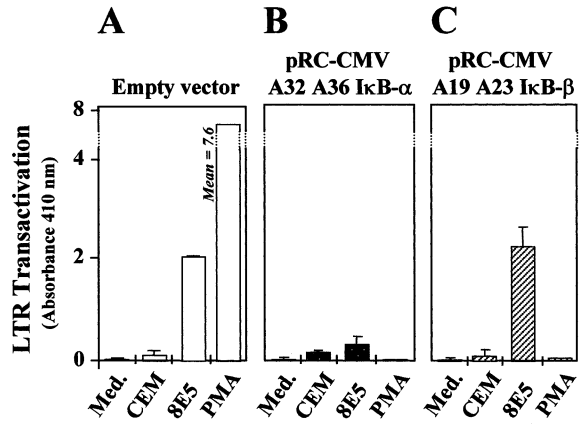

Fig. 4. Inhibition of gp120^{env}-induced LTR transactivation in HeLa P4p56 cells by expression of degradation resistant forms of IκB-α or IκB-β. HeLa P4p56 cells transiently transfected with empty vector or plasmids encoding A32 A36 IκB-α or A19 A23 IκB-β proteins were cultivated in DMEM medium (Med.) or in the presence of CEM or 8E5 cells or in medium containing 50 ng/ml PMA. Transactivation of HIV-LTR was measured by quantification of β-galactosidase activity in total cell lysates normalized according to the protein content. Each value is the mean of two representative independent experiments performed in duplicate and error bars indicate ± S.D.

The selective implication of IκB-α inhibitor degradation in gp120^{env}-induced NF-κB activation, was confirmed by stimulation experiments of HeLaP4p56 cells after transient transfection of plasmids encoding A32 A36 IκB-α and A19 A23 IκB-β DN forms of IκB-α or IκB-β. It is known from the literature that serine for alanine substitutions at residues 32 and 36 for IκB-α and 19 and 23 for IκB-β render IκBs resistant to hyperphosphorylation and subsequent degradation by the proteasome in response to TNF-α [13,23]. 24 h after vector transfection the cells were co-cultivated for 24 h with 8E5 cells or CEM cells. Expression of A32 A36 IκB-α dramatically reduced the level of β-galactosidase activity induced in HeLa P4p56 by cell surface-expressed gp120^{env} as compared to data obtained from cells transfected with empty control vector (Fig. 4). Despite the fact that A19 A23 IκB-β was capable of blocking PMA-induced cell activation in HeLa P4p56 cells, β-galactosidase activity was unaffected, indicating that expression of a DN form of IκB-β unable to phosphorylate and to undergo degradation does not affect gp120^{env}-induced NF-κB activation.

3.3. IKK complex regulates activation of NF-κB induced by cell surface-expressed gp120^{env} binding to CD4

IKKs complex has been shown to mediate IκB-α phosphorylation at residue S32 and S36 in response to proinflammatory cytokines and to participate in NF-κB subunits nuclear translocation [13,23,24]. To investigate a possible role of IKKα or IKKβ in transduction of gp120^{env}-induced NF-κB activation in HeLa P4p56 cells, transcription of LTR-β-galactosidase reporter gene was examined in transiently transfected HeLa P4p56 cells expressing WT or transdominant negative forms of either IKKα or IKKβ. A green fluorescent protein expression vector was used to normalize β-galactosidase activities for transfection efficiency. Over-expression of either WT IKKα or WT IKKβ proteins was found to stimulate gp120^{env}-induced β-galactosidase reporter gene transcription (Fig. 5). It is worth noting that transient expression of WT IKKβ stimulated β-galactosidase activity with a greater efficiency than observed for WT IKKα.

When the vector encoding DN form of IKKα (K44A IKKα referred to below as DN IKKα) was transfected in HeLa P4p56 cells, β-galactosidase activity induced in response to gp120^{env} stimulation was significantly decreased compared to cells transfected with empty vector. In the same fashion, β-galactosidase expression following gp120^{env} stimulation was also reduced when a transdominant negative form of IKKβ (K44A IKKβ referred to as DN IKKβ) was expressed in the indicator cell line. It is worth stressing that LTR transactivation is not totally abolished by transient expression of IKKs DN expression vectors. This observation suggests that signaling pathways independent from the IKKs complex may also contribute to NF-κB-dependent LTR transactivation following gp120^{env} ligation of cell surface CD4 receptor [25]. In addition, IκB-α half-life determined by Western blotting experiments in gp120^{env}-stimulated HeLa P4p56 cells expressing DN forms of either IKKα or IKKβ was significantly increased (data not shown).

Finally, the implication of IKKα and β kinases in gp120^{env}-induced NF-κB was investigated in an in vitro kinase assay (Fig. 6). HeLa P4p56 cells were transiently transfected with plasmids encoding Flag-epitope tagged WT IKKα or WT IKKβ proteins. Tagged kinases were immunoprecipitated with an anti-Flag monoclonal antibody and the kinase activity of immunoprecipitates was analyzed in an in vitro kinase reaction using GST-IκB-α as substrate. Epitope-tagged IKKs expression levels were ascertained by Western blotting of immunoprecipitates with anti-IKKs mAbs. The kinase activity of IKKα and IKKβ proteins immunoprecipitated was found to be increased after 30 min co-cultivation with gp120^{env}-positive 8E5 cells (Fig. 6). Altogether these data indicate that both IKKα and IKKβ likely contribute to the transduction of activation signals targeting NF-κB following gp120^{env} binding to CD4.

Besides IKKs, several kinases including p90^{rsk} and atypical isoforms of PKCs were demonstrated to phosphorylate IκBs

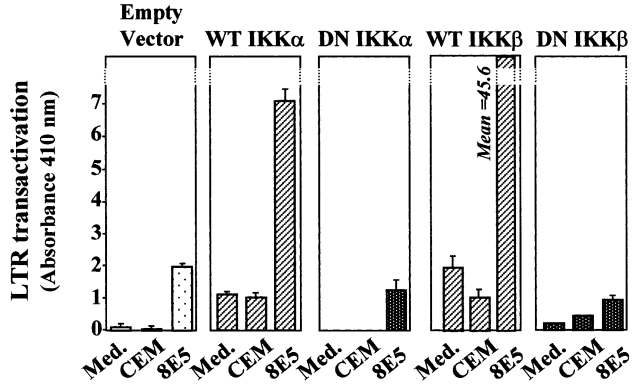

Fig. 5. gp120^{env}-induced IκB-α degradation and LTR transactivation is negatively regulated by expression of DN forms of IKKs. Expression of DN forms of IKKs abolish gp120^{env}-induced LTR transactivation. HeLa P4p56 cells were transiently transfected with empty vector or plasmids encoding WT or DN forms of IKKα or IKKβ proteins. 36 h post-transfection, cells were maintained in DMEM medium (Med.) or co-cultivated for an additional 24 h in the presence of gp120^{env}-negative CEM or gp120^{env}-positive 8E5 cells. LTR transactivation was estimated by quantification of β-galactosidase activities. Values are the mean of two independent experiments performed in duplicate.

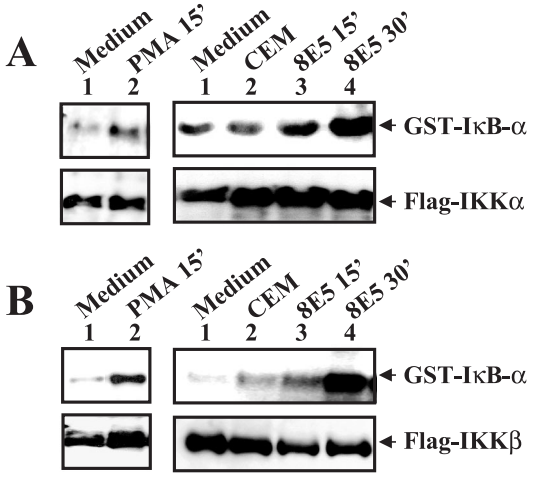

Fig. 6. gp120^{env} binding to CD4 stimulates IKKs activity in HeLa P4p56 cells. HeLa P4p56 cells were transiently transfected with WT Flag-epitope tagged forms of IKKα (A) or IKKβ (B). 24 h after transfection, the cells were incubated with gp120^{env}-negative CEM or gp120^{env}-positive 8E5 cells for 15 and 30 min or were maintained in DMEM medium for control experiments. IKKs complex was immunoprecipitated with anti-Flag epitope specific antibodies and the kinase activity of the immunoprecipitated complexes was determined in kinase assay experiments using GST-IκB-α as a substrate. Phosphorylation of IκB-α was visualized by autoradiography. In each experiment, expression levels of epitope-tagged IKKs were controlled in Western blotting experiments with anti-IKKα or IKKβ mAbs (lower panels).

[18,26–28]. Their contribution in gp120^{env}-induced activation was questioned in co-cultivation experiments of cells either transiently expressing DN forms of p90^{rsk} or incubated in the presence of staurosporine and H-7 synthetic inhibitor of PKCs. In both cases LTR transactivation induced by 8E5 co-cultivation was unaffected (data not shown), suggesting that p90^{rsk} and PKCs probably play no role or have a reduced contribution to gp120^{env}-induced IκB-α phosphorylation.

3.4. Implication of IKKs in gp120^{env}-induced activation of CD4$^+$ lymphoblastoid T cells

Despite the fact that we have previously demonstrated that HeLa P4p56 cells expressing the p56^{lck} tyrosine kinase associated with the cytoplasmic tail of CD4 represent an appropriate model for studying CD4 signaling [19], the physiological relevance of our results was next assessed in the context of CEM lymphoblastoid cells stimulated gp120^{env} expressed at the surface of HeLa 243^{env} cells. To this end, we tested the capacity of DN forms of IKKs to interfere with transcription of LTR-luciferase reporter gene in transiently transfected CD4$^+$ lymphoblastoid CEM cells stimulated for 24 h by HeLa 243^{env} gp120^{env}-expressing cells or by the appropriate control cell line HeLa 273Δ^{env} lacking gp120^{env} expression [29]. Luciferase activities were measured and normalized according to the expression of a β-galactosidase vector co-transfected in the cells. LTR transactivation was found to be strongly stimulated in CEM cells expressing either WT IKKα or WT IKKβ co-cultivated with the gp120^{env}-expressing HeLa 243^{env} cells (Fig. 7). In contrast, strong inhibition of LTR transactivation was measured when co-cultivations were performed between CD4$^+$ CEM transiently expressing the IKKs DN forms and HeLa 243 gp120^{env}-expressing cells. Furthermore, GST-IκB-α phosphorylation studied in in vitro kinase assays was found to be enhanced in response to gp120^{env} stimulation when CEM cells expressed WT IKKs while expression of IKKs DN forms strongly decreased GST-IκB-α phosphorylation induced after gp120^{env} stimulation of the cells (data not shown). These observations are very similar to those obtained using HeLa P4p56 cells and indicate that IKKs also play a critical role in gp120^{env}-dependent activation of NF-κB in cells from lymphoid origin.

4. Discussion

HIV-1 gp120^{env} binding to CD4 cellular receptor is known to profoundly modulate T cell function, a mechanism that is suspected to participate in AIDS pathogenesis [11,30]. Among the consequences of these interactions are T cell hyperactivation and apoptosis. Our previous papers focused on T cell activation triggered by gp120^{env} binding to the CD4 receptor and demonstrated that CD4 cross-linking with gp120^{env} stimulates both AP-1 and NF-κB DNA-binding activity. The consequences of such activation signals on reactivating latent proviruses harbored by primary T cells from HIV-1 infected T cells have also been described [31].

The aim of the present study was to identify some important members of the signaling pathways recruited by gp120^{env} binding to CD4 and leading to the stimulation of NF-κB. We have demonstrated by using the HeLa P4p56 cell line, that following cell surface-expressed gp120^{env} binding to CD4, IκB-α is phosphorylated and degraded, allowing translocation of p50/p65 NF-κB complexes to the nucleus. Experiments aimed at characterizing IKKs recruited for IκB-α phosphorylation and subsequent degradation have demonstrated that IKKs activity is enhanced upon gp120^{env} stimulation. Furthermore, NF-κB DNA-binding activity observed after gp120^{env} binding to the CD4 cell surface receptor was reduced by expression of transdominant negative forms of IKKα and IKKβ indicating that the IKKs complex participates in transduction

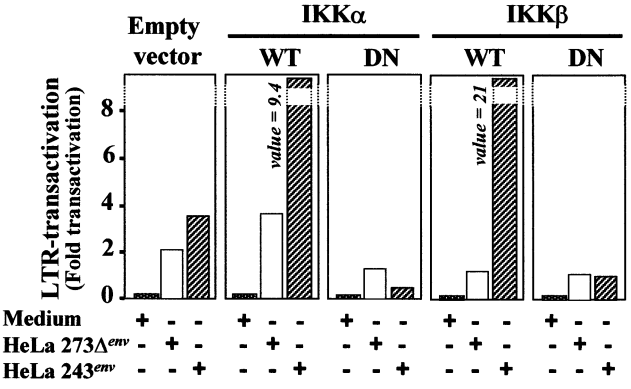

Fig. 7. IKKs complex is involved in gp120^{env}-induced LTR transactivation in CEM lymphoblastoid cell line. CD4$^+$ CEM cells were transfected with plasmid encoding LTR-luciferase reporter gene and plasmids encoding either WT or DN forms of IKKα or IKKβ proteins. 24 h after transfection, the cells were co-cultivated in the presence of gp120^{env}-negative 273 Δ^{env} cells or 243^{env} gp120^{env}-expressing cells for an additional 24 h. LTR transactivation was measured by quantification of luciferase activity in total cell extracts. Values were normalized according to the total protein content of the samples and to the level of expression of the pACβ plasmid co-transfected in each experiment.

of gp120^{env}-induced activation signals. It is worth noting that inhibition of gp120^{env}-induced NF-κB activation by transdominant negative form of IKKs was observed both in HeLa P4p56 cells and in CD4$^+$ lymphoblastoid cells. Indeed, marked effects were observed in CEM T cells which is a more physiologic cellular model.

Despite the fact that our data indicate as expected that HIV-1 gp120^{env} stimuli may recruit both IKKα and IKKβ, our observations suggest that IKKβ plays a major role in gp120^{env}-induced NF-κB activation. IκB-α degradation was found to be more profoundly altered by expression of kinase dead IKKβ and the kinase activity of IKKβ was more strongly stimulated under gp120^{env} stimulation than that of IKKα kinase. Evidence for a predominant role of IKKβ is consistent with previous literature reports demonstrating 50- to 60-fold greater activity for IKKβ as compared to IKKα in cells stimulated with TNF-α or interleukin-1-β [32].

Having demonstrated that the IKKs complex is involved in gp120^{env}-induced NF-κB activation, our data suggest that signals other than the IKKs mediated signal may also contribute to NF-κB activation. Our experiments indicate the implication of IKKs in early phosphorylation of IκB-α (enhanced IKKs activity was evidenced after 30 min co-cultivation with gp120^{env}-expressing cells), however, gp120^{env}-induced IκB-α phosphorylation was found to be maintained through 4 h of co-cultivation as evidenced in Western blotting experiments suggesting that signaling pathways independent of IKKs might participate in late NF-κB activation. We have previously reported observations indicating that expression of DN forms of MEK-1 and ERK-1 abolishes gp120^{env}-induced NF-κB activation, suggesting a possible molecular link between the classical MAPK-dependent pathway and the final steps of the NF-κB activation cascade [33]. The contribution of the p90^{rsk} protein kinase, which is known to phosphorylate IκB in response to ERK stimulation [34], and of some PKC isoforms was evaluated in gp120^{env}-induced NF-κB activation. Our results indicate that staurosporine and H-7-sensitive PKC isoforms poorly participate in gp120^{env}-induced NF-κB activation and p90^{rsk} was found to play a small or no role in the transduction of such activation signals. Recently, Yang et al. [35] demonstrated that ERK plays a central role in NF-κB activation induced by cytokine stimulation by activating AP-1 which subsequently physically interacts with and stimulates DNA-binding activity of NF-κB. These results indicate a new avenue for investigating the contribution of the ERK module in gp120^{env}-induced NF-κB-dependent gene expression.

Multiple studies have addressed the role played by NF-κB transcription factor in the reactivation of HIV from the latent state and in the control of viral persistence. We previously demonstrated that gp120^{env}-dependent activation of NF-κB has a significant relevance to the physiopathology of HIV infection by reactivating HIV-1 transcription in PBMCs from symptomatic infected patients [31]. Besides this, the direct interaction of gp120^{env} with a CXCR4 receptor was also reported by others to stimulate T cell apoptosis in a CD4-independent fashion [36]. Thus, the physiopathological consequences of HIV-1 envelope binding to its receptor/co-receptor appear to be complex, resulting both in activation of latently infected lymphocytes and programmed cell death leading to severe immunodeficiency and AIDS. Identification of the precise signaling pathways stimulated upon viral envelope binding to its receptor/co-receptors may offer new potential cellular targets for anti-retroviral interventions.

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