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ARTICLES

Glucocorticoid Can Reduce the Transcriptional Activation of HIV-1 Promoter Through the Reduction of Active NF-κB

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Abstract It is well documented that glucocorticoid (GC) can induce the transcription of the IκBα gene in several cell lines. GC treatment then increases IκBα protein levels and markedly reduces the amount of active NF-κB. NF-κB is a strong activator of HIV-1 promoter, but the mechanism by which GC influence the HIV-1 activities is not well documented. In the present study, MOLT4 and Jurkat cells were transfected with HIV-1-LTR-CAT DNA and treated with 0.2 mM H_2O_2 or 10 ng/ml of TPA. CAT activities of the transformed MOLT4 and Jurkat cells were greatly enhanced by these treatments, but the CAT activities were markedly reduced when cells were pretreated with GC. This reduction in activity correlated well with the reduction in active NF-κB and the accumulation of IκBα. These findings suggest that GC can reduce the transcription of HIV-1 promoter through the reduction of active NF-κB. J. Cell. Biochem. 76:13–19, 1999.

Key words: glucocorticoid; HIV-1; NF-κB, I-κB; transcriptional activation

It is well known that the transcriptional activation of cytokine genes is repressed by glucocorticoid (GC). Activation of these genes is critical for the activation of the immune and inflammation systems [Cupps and Fauci, 1982]. GC is used in the treatment of a variety of human immunodeficiency virus type 1 (HIV-1)-related disorders. Reduction of HIV-1 promoter activity by GC has also been reported [Mitra et al.,1995; Laurence et al., 1989]. In some cases, the GC can activate the promoter of HIV-1 [Markham et al., 1986; Mitra et al., 1995]. Recently, however, several studies have shown that GC inhibits the expression of some members of the NF-κB-rel transcription factor family [Ray and Prefontaine, 1994; Scheinman et al., 1995a] but activates the transcription of the IκBα gene [Scheinman et al., 1995b]. NF-κB motif elements are required for the function of many cytokine promoters [Grilli et al., 1993], including HIV-1 promoters [Nable and Baltimore, 1987; Kurata, 1996]. GC blocks the induction of active NF-κB by tumor necrosis factor-α (TNF-α) in HeLa cells and in monocytic THP1 cells treated with TNF-α [Scheinman et al., 1995]. Synthetic GC (DEX) treatment also reduces NF-κB-mediated gene expression, as measured by transfected reporter constructs [Scheinman et al., 1995; Auphan et al., 1995]. Inhibition of NF-κB by GC appears to result in a decrease in nuclear translocation of NF-κB. The decrease in active nuclear NF-κB is caused by the reassociation of active NF-κB with newly synthesized IκBα. Active NF-κB also activates the transcription of the HIV-1 gene [Nable and Baltimore, 1987; Kurata, 1994,1996], which has two NF-κB motifs in its promoter region. In this study, we examined the effect of DEX on the activation of HIV-1 promoter, and found that the transcription of HIV-1 gene activated by

Abbreviations used: GC, glucocorticoid; DEX, dexamethasone; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; TPA, phorbol 12-myristate 13-acetate.

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H_2O_2 or TPA was markedly reduced by treatment with DEX. The reduction in the transcription of HIV-1 may result from the reassociation of active NF-κB with newly synthesized IκBα.

MATERIALS AND METHODS

Transformation

MOLT4 and Jurkat cells were maintained in RPMI 1640 with 10% fetal bovine serum (FBS). About 5×10^6 cells were fused with *Escherichia coli* DH1 containing plasmid pCD12 (HIV-LTR-CAT, 10^9 cells [Okamoto and Wöng-Staal, 1986]) or mutant pCD12 (containing two mutant NF-κB motif in long terminal repeat [LTR]) [Kurata, 1994] plus DH-1 containing pSV2neo (5×10^7 cells) by the protoplast fusion method [Oi et al., 1983]. The medium was changed 18 h after protoplast fusion and cells further cultured for 2 days. Construction of mutant pCD12 was previously reported [Kurata, 1994]. Cells were then selected with G418 in RPMI 1640 medium. After selection with G418 for 10 days, transformed cells (MOLT4 and Jurkat cells transformed by pCD12) were obtained and named MOLT4CD, MOLT4CD* (transformed by mutant pCD12 and Jurkat CD) [Kurata, 1994]. MOLT4CD cells were also transiently transformed with 100 ng of CMV-S32/36AIκBα vectors and named MOLT4CDS32/36A.

H_2O_2 or TPA Treatment and CAT Assay

Transformed cells were maintained in normal RPMI 1640 and treated with synthetic glucocorticoid (DEX; 0, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M) for 5 h. During DEX treatment, some of the cells were treated with 10 ng/ml of TPA for 2 h or H_2O_2 (0.2 mM) for 1 h and maintained in normal RPMI 1640 for 24 h. After treatment, transformed MOLT4 cells were collected and washed with phosphate-buffered saline (PBS). Samples of 5×10^5 cells were suspended in 0.25 M Tris-HCl (pH 8.0), and cellular extracts prepared by five freeze (−80°C) and thaw cycles. Chloramphenicol acetyltransferase (CAT) activity was measured by incubating whole cell extracts with [^{14}C]-labeled chloramphenicol and 5 mM acetyl coenzyme A at 37°C for 18 h. Acetylated chloramphenicol was separated from non-acetylated chloramphenicol by ascending thin-layer chromatography (TLC)[Kurata et al., 1993]. Chromatograms were examined and quantitated with a Fuji image analyzer BA100.

Northern Blot Analysis

Samples of 5×10^6 transformed cell lines were collected after treatment with test compound, and RNAs were isolated by the guanidium-isothiocyanate-cesium chloride method. Extracted RNA (10 µg) was denatured, separated by formaldehyde-acrylamide gel electrophoresis, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled human IκBα probe [Scheinman et al., 1995a] for 24 h at 45°C. The filters were then washed with $2 \times$ SSC (0.3 M NaCl, 30 mM sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS) at 30°C, followed by $0.1 \times$ SSC containing 0.1% SDS at 65°C for 0.5 h. Blots were reprobed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the control. Filters were then exposed to X-ray film (Kodak-XAR) at −70°C for about 2 days. After autoradiography, the hybridized slots were removed and radioactivities quantitated with a liquid scintillation counter.

Binding of Nuclear Proteins With HIV-1-LTR DNA

HIV-1-LTR DNA was digested with *Sac*I and *Pvu*II to obtain a 120-base pair (bp) DNA fragment [Kurata et al., 1993]. This 120-bp fragment, which contained the two NF-κB motifs, was end labeled with [γ-^{32}P]ATP for binding with nuclear extracts. Nuclear extracts were prepared at intervals by the method of Dignam et al. [1983] after treatment of the transformed cell lines with H_2O_2 or TPA. Samples of 5 ng of end-labeled DNA fragments were bound with 3 mg of nuclear proteins in a solution of 20 mM Hepes buffer (pH 7.9), 100 mM KCl, 20% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM DTT, 10 mM $MgCl_2$, 125 mM spermidine, and 3 µg poly (dI-dC) for 20 min. The preparations were then separated by electrophoresis in 4% polyacrylamide gels in Tris-borate-EDTA buffer and autoradiographed. For competition assays, excess amounts of cold 42-mer fragments and a synthetic mutant sequence of the NF-κB motif (TCGACAGAATTCACTTTCCGAGAGGCTCGA) [Lenardo et al., 1989] were used for binding assays. For supershift assays, 10-fold diluted rabbit antiserum against NF-κB (p65) (Santa Cruz Biotech.) was added to the binding reaction. The complexes of NF-κB motif DNA, nuclear protein, and antibody were identified by electrophoresis as previously described [Mezger et al., 1993].

Western Blotting

Western blotting and detection of immunocomplexes were performed as described by Tranckner et al. [1994]. In short, MOLT4CD, MOLT4CD*, and MOLT4CDS32/36A cells extracts were boiled in sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were equibirated in ice-cold blotting buffer (TBST; 50 mM Tris-HCl, 40 mM glycine 15% methanol, 0.037% SDS). Proteins were transferred onto Immobilon P membranes by semidry blotting appratus. Nonspecific binding site were blocked at 4°C by immersing the membrane in Tris-buffered saline (150 mM NaCl, 0.1% Tween-20, 10 mM Tris-HCl, pH 8.0) containing 5% dried milk powder. After short wash in TBST, the membranes were incubated in 1:1,500 diluted primary antibody in blocking solution for 1 h at room tempereture. After extensive washing with TBST, bound antibody was reacted peroxidase conjugate for 30 min. The immunocomplex was detected by using enhanced chemiluminescence (ECL) Western blotting reagents and exposed to Kodak XAR-5 firms for about 30 s.

RESULTS AND DISCUSSION

It is well known that DEX can inhibit the HIV-1 promoter activity [Mitra et al.,1995; Laurence et al., 1989], but the mechanism of this inhibition has not been elucidated. NF-κB is one of the most important transcriptional regulators of HIV-1 gene activation. It is also well established that DEX can increase the IκBα levels and reduce the amount of active NF-κB translocated into the nucleus in HeLa cells after treatment with TNF-α [Ray and Prefontaine, 1994; Scheinman et al., 1995a,b]. This reduction in NF-κB translocation might be critical for inhibition of the HIV-1 promoter by DEX treatment. Then we examined the HIV-1 promoter activity in pCD12 plasmid (HIV-1 LTR-CAT DNA) transformed MOLT4 and Jurkat cells after DEX treatment.

Effects of DEX Treatment on CAT Activity

Transformed MOLT4 and Jurkat cells were obtained by the protoplast fusion method as described [Kurata, 1996]. The transformed cells (MOLT4CD, JurkatCD, MOLT4CD* and MOLT4CDS32/36A) were treated with H_2O_2 (0.2 mM for 1 h) or TPA (10 ng/ml for 2 h) after pretreatment with DEX for 3 h. The cells were then washed with normal medium (RPMI1640, 10% FBS) and cultured for 24 h. Samples of 5×10^5 cells were used for measurement of CAT activity by thin-layer chromatography (TLC) (Fig. 1 Aa,Ba). CAT activity was measured as a percentage conversion of the acetylated form of chroramphenicol from extracts derived of transformed cells. The CAT activities of H_2O_2-treated MOLT4CD cells pretreated with 0, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} M DEX were 63.2%, 0%, 5.3%, 19.2%, 32.3%, and 65.8%, respectively (cf. Fig. 1Ab). Almost the same results were obtained from TPA-treated MOLT4CD cells pretreated with DEX. Almost the same results were also obtained from DEX-pretreated Jurkat CD cells. By contrast, in MOLT4* CD cells (transformed by mutant NF-κB motif HIV-1 LTR CAT DNA) H_2O_2, TPA, and DEX treatment do not influence CAT activity (Fig. 1Ba,b). The same experiments were also carried out using MOLT4CDS32/36A cells (MOLT4CD cells retransformed by CMV-S32/36AIκBα [Baeurle et al. 1995]). CAT activity was found to remain at a low level in H_2O_2- and TPA-treated MOLT4CDS32/36A cells pretreated with DEX (Fig. 1Ba, b). In summary, we found that TPA and H_2O_2 treatment resulted in marked activation in CAT activity in normal pCD12-transformed Jurkat and MOLT4 cells; further, these activations were greatly inhibited by 10^{-5} M DEX pretreatment. And the inactivation of HIV-1 promoter and NF-κB may be correlated with the expression of IκBα (cf. Fig. 1B; MOLT4CDS32/36A). In some cell lines (e.g., U937), GC does not repress the transcriptional activation of cytokine genes. When U937 cells transformed by pCD12 were treated with DEX, marked inactivation of CAT activity was not observed (data not shown). The percentage conversion of the acetylated form of [^{14}C]chloramphenicol is expressed in Figure 1Ab, Bb.

Reduction of the NF-κB DNA Motif-Binding Protein in DEX-Treated Transformed Cells

Binding proteins with the NF-κB DNA motif were detected by a bandshift assay. A *Sac*I-*Pvu*II fragment (120 bp) containing the two NF-κB binding DNA motifs [Kurata et al., 1993] in HIV-1 LTR was isolated, end-labeled with [γ-^{32}P]-ATP, and incubated with nuclear proteins from the transformed cell lines pretreated with various concentrations of DEX. The DNA

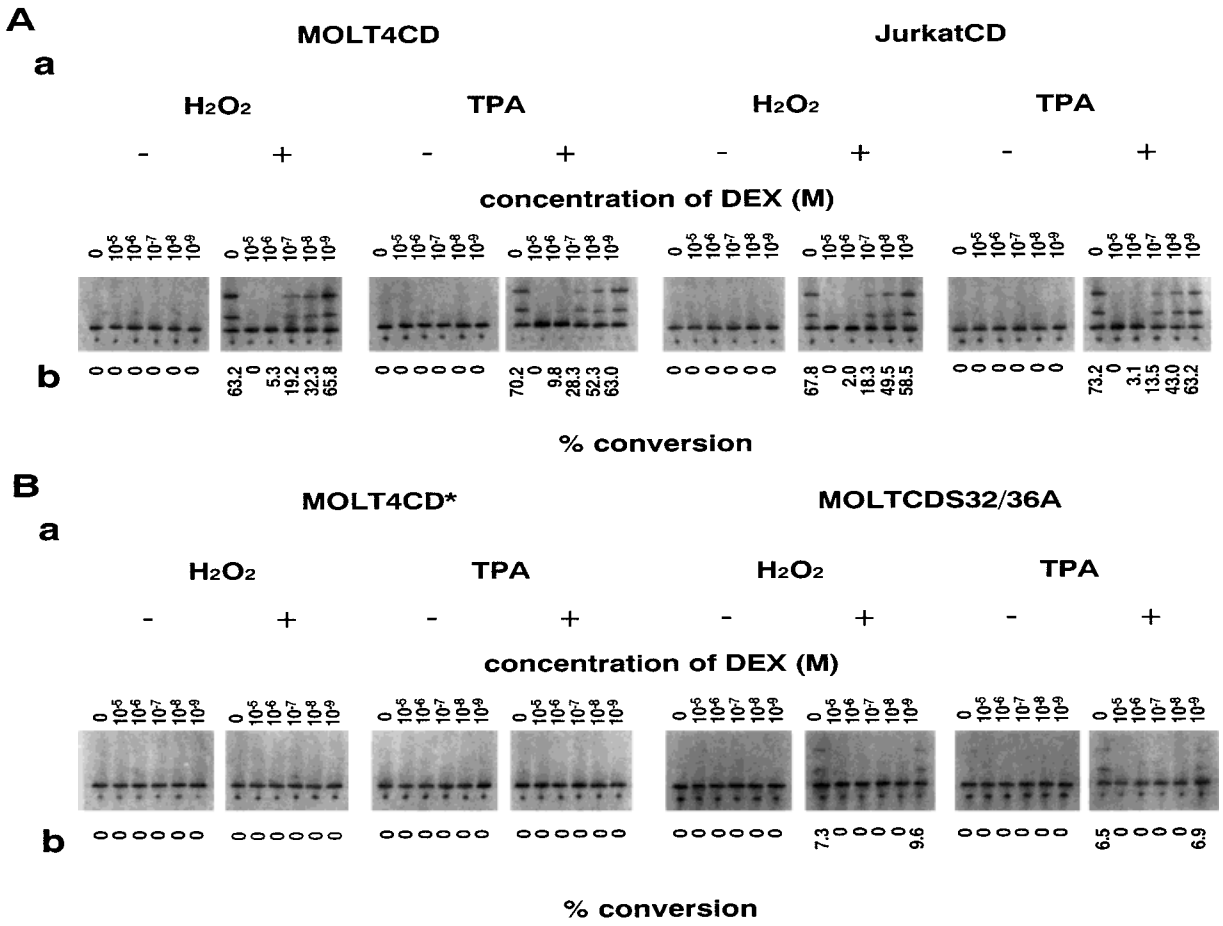

Fig. 1. Chloramphenicol acetyltransferase (CAT) assay of transformed cells lines. Samples of 5×10^5 cells treated with several concentrations of DEX for 3 h or for the last 2 h with 0.2 mM, or both H_2O_2 or 10 ng/ml of TPA was collected and the CAT activity measured. After separation by thin-layer chromatography, the percentage conversion of the acetylated form of chloramphenicol was calculated as follows: % conversion = (counts/min) of converted spot/total (counts/min) $\times$ 100 **Aa:** CAT assay of DEX pretreated MOLT4CD (transformed by pCD12 DNA) and JurkatCD (transformed by pCD12 DNA) cells on H_2O_2 or TPA treatment. **Ab:** Percentage conversion of CAT activity. **Ba:** CAT assay of DEX pretreated MOLT4CD* (transformed by mutant NF-κB motif pCD12 DNA) and MOLT4CDS32/36A (MOLT4CD cells retransformed by double mutant IκBα S32/36A) cells on H_2O_2 or TPA treatment. **Bb:** Percentage conversion of CAT activity.

fragments were separated by electrophoresis and their mobility shifts were examined (Fig. 2). Bandshift decreased with the addition of a 50-fold excess of unlabeled 42-mer DNA fragments, but not on addition of a 50-fold excess of an unlabeled mutant NF-κB motif. Shifted bands from nuclear extracts of H_2O_2- or TPA-treated cells were also supershifted by antisera against NF-κB p65 protein, which produced several supershift bands (Fig. 2). It was found that the percentage shift of the 120-bp HIV-1-LTR DNA fragment highly corresponded with CAT activity in the MOLT4CD and JurkatCD cell lines (Fig. 2Aa; cf. Fig. 1Aa). In spite of the very low CAT activities, same bandshift results were obtained in MOLT4CD* cells (Fig. 2Ba). It is interesting to note that only the faint bandshifts were observed in MOLT4CDS32/36A cells (Fig. 2Ba), and the percentage shift showed high correspondence with CAT activity in this cell line (Fig. 1Aa). The percentage shift of the complex of 120-bp DNA and nuclear proteins were expressed in Figure 2Ab,Bb. After the bandshift assay, shifted bands (except supershifted bands) were eluted from the gel and the DNA-protein complexes immunoprecipitated with anti-NF-κB p65 rabbit serum. At least 80% radioactivity from the shifted bands was recovered. These results strongly suggest that after DEX treatment transformed cells suppress both an active NF-κB and CAT activity through the expression of IκBα.

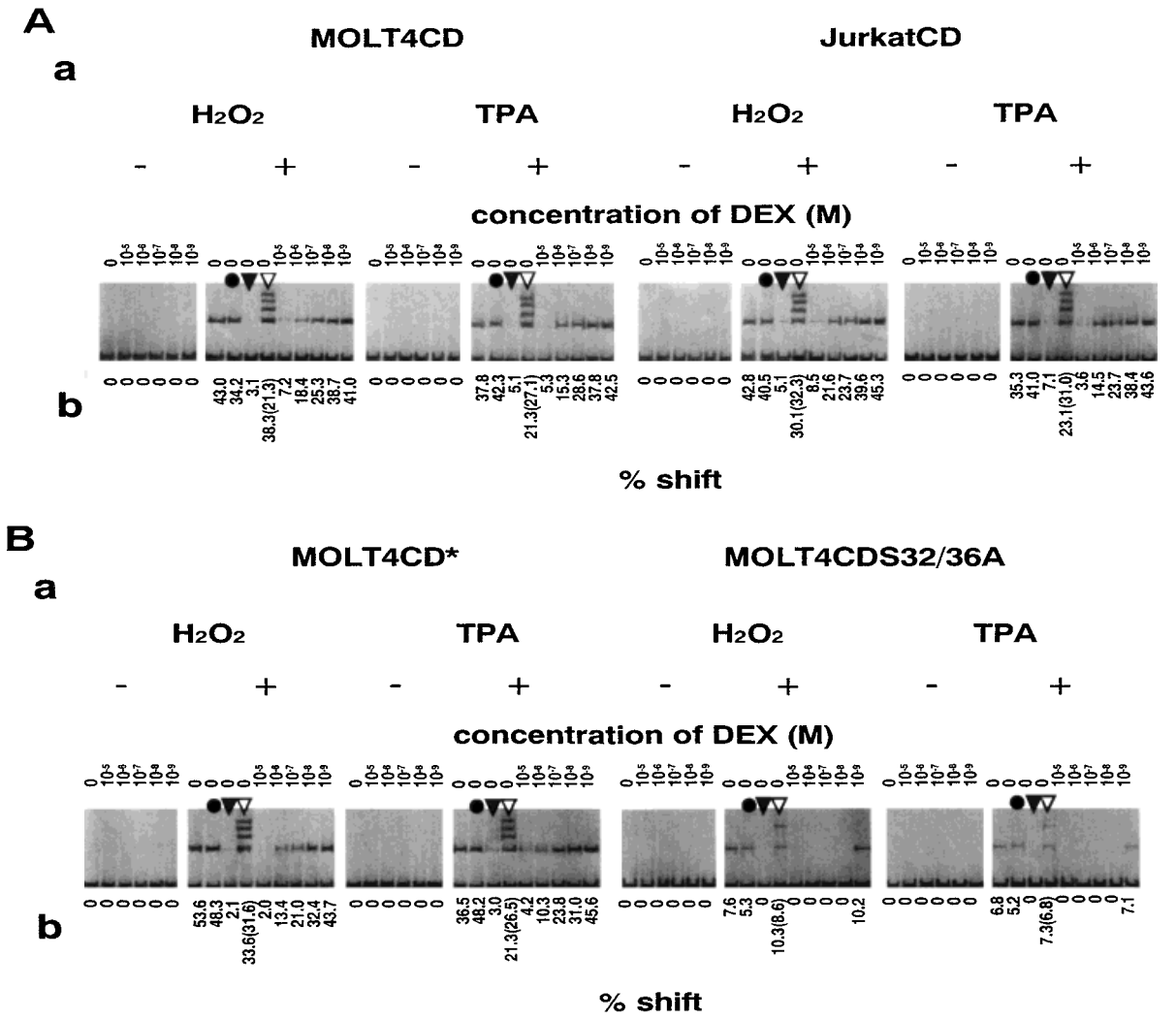

Fig. 2. Binding of NF-κB motif DNA in HIV-1-LTR with nuclear proteins from transformed cell lines. **Aa:** Binding of a *Sac*I-*Pvu*II fragment of HIV-1-LTR DNA (120 bp) with nuclear proteins from DEX pretreated MOLT4CD and JurkatCD cells on H_2O_2 or TPA treatment. **Ab:** Percentage shift of the complex of 120-bp DNA and nuclear proteins. **Ba:** Binding of a 120-bp fragment with nuclear proteins from DEX-pretreated MOLT4CD* and MOLT3CDS32/36A cells on H_2O_2 or TPA treatment. **Bb:** Percentage shift of 120-bp DNA. The percentage shift of the DNA-protein complex was calculated as follows: % shift = (counts/min) of shifted band/(total counts/min) × 100 (●) with a 50-fold excess of unlabeled mutant NF-κB motif added during the bandshift assay. ▼, with a 50-fold excess of unlabeled 120-bp DNA added during the bandshift assay. ▽, supershift assay of the complex of 120-bp DNA with nuclear proteins.

Amounts of IκBα mRNA on DEX Treatment

During DEX treatment, the increase in IκBα protein is preceded by an increase in IκBα mRNA [Scheinman et al., 1995b]. Therefore, we performed Northern blot analysis on mRNA of transformed cells treated with several concentrations of DEX. DEX was found to induce a marked increase in IκBα mRNA (Fig. 3). The decrease in CAT activity after DEX treatment was also found to be highly correlated with an increase in IκBα mRNA in H_2O_2- or TPA-treated MOLT4CD and JurkatCD cells (Fig. 3Aa; cf. Fig. 1Aa). Despite the low CAT activities, the same results were obtained in MOLT4CD* cells (Fig. 3Ba; cf. Fig. 1Ba). Thus, it is likely that, in the absence of DEX, H_2O_2 or TPA treatment results in the transient loss of IκBα, which allows NF-κB to activate and translocate to the nucleus [cf. Laurence et al., 1989; Ray and Prefontaine, 1994]. We then tried to transfect MOLT4CD cells by double mutant IκBα (CMV-S32/36AIκBα) DNA. This double mutant prevents the transient loss (degradation) of IκBα [Baeurle et al. 1995]. This tran-

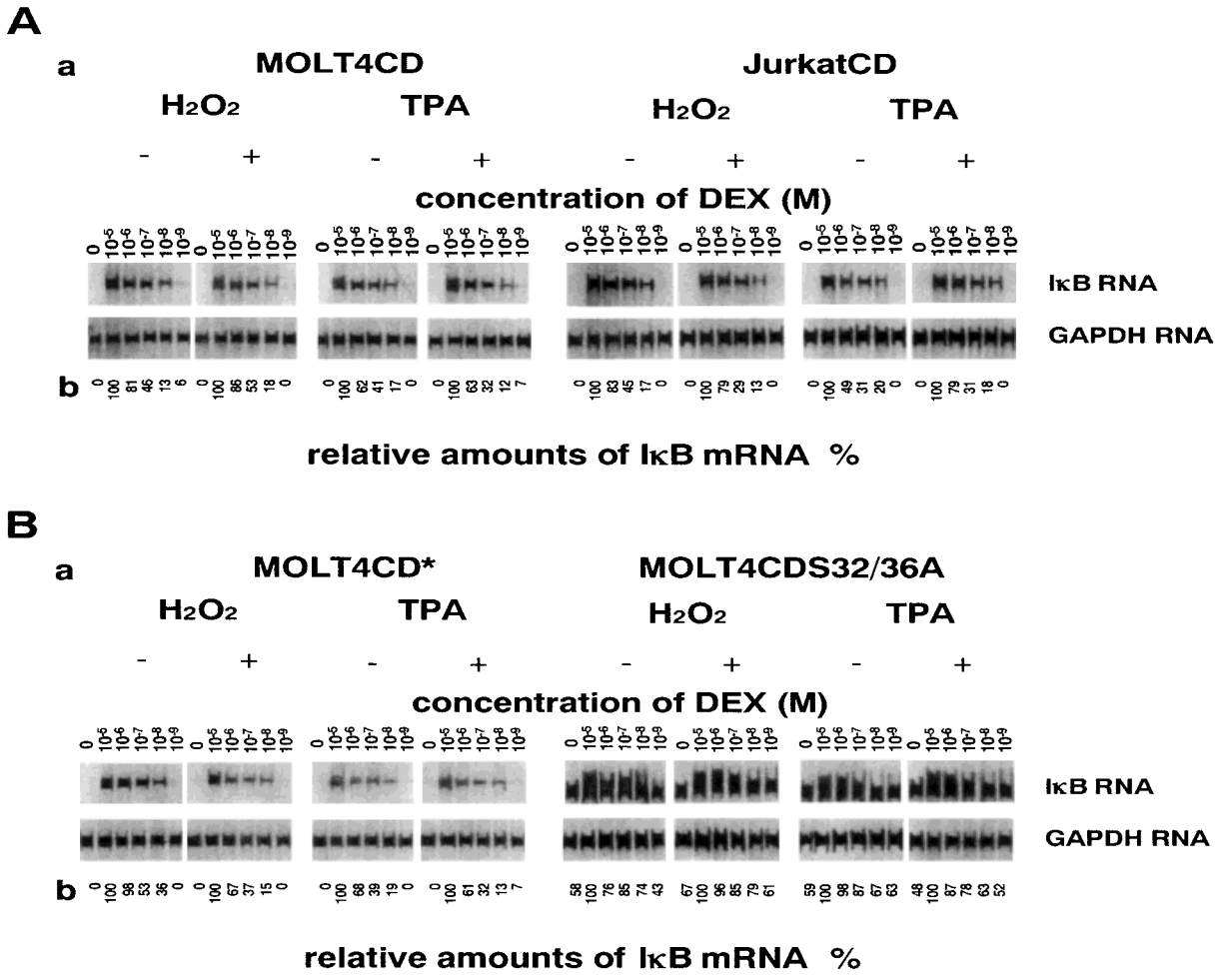

Fig. 3. DEX induces IκBα mRNA accumulation. **Aa:** Northern blot analysis of DEX pretreated MOLT4CD and Jurkat CD cells on H_2O_2 or TPA treatment. **Ab:** Densitometry data are expressed as a percentage of activation by 10^{-5} M DEX treatment. **Ba:** Northern blot analysis of DEX-pretreated MOLT4CD* and MOLT4CDS32/36A cells on H_2O_2 or TPAtreatment. **Bb:** Densitometry data are expressed as a percentage of activation by 10^{-5} M DEX treatment.

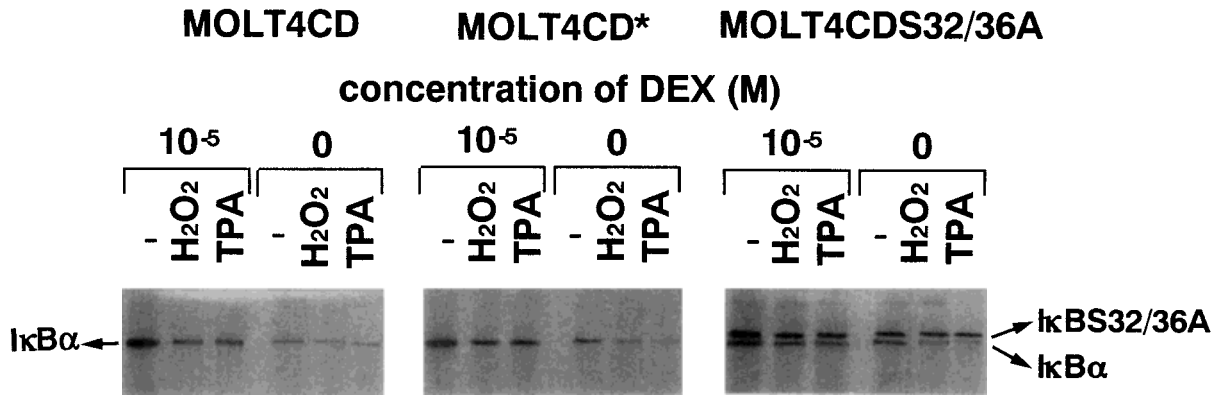

Fig. 4. Western blot analysis of IκBα. The amounts of IκBα and the effect of a S32/36A mutation on IκBα in response to DEX treatment were examined by using MOLT4CD, MOLT4CD*, and MOLT4CDS32/36A cell lines. To achieve higher resolution, proteins were separated 7.5% sodium dodecyl sulfate (SDS) polyacrylamide gel. A section an anti-IκBα stained Western blot is shown. The positions of endogenous IκBα and of slowly migrating S32/36A mutant are indicated.

sient transformant cell produces large amounts of IκBα mRNA (Fig. 3Ba). These results suggest that DEX treatment induces the transcription of the IκBα gene and synthesis of IκBα protein, and this increase in IκBα concentration may cause association of IκBα protein with NF-κB. In the presence of an activator of NF-κB such as H_2O_2 or TPA, IκBα produced by DEX treatment may reassociate with active NF-κB and reduce the amount of active NF-κB translocating to the nucleus [cf. Scheinman et al., 1995b]. The relative amounts of IκBα mRNA are expressed in Figure 3Ab,Bb.

Amounts of IκBα on DEX Treatment

S32/36A double mutant product of IκBα gene no longer degrades in response to stimulation in cells [Baeurle et al., 1995]. MOLT4CD cells were then transformed by CMV-S32/36AIκBα, and equal amounts of cell extracts were analyzed by Western blotting. The endogenous IκBα were significantly degraded in response to TPA and H_2O_2 treatment of cells. This reduction was suppressed by 10^{-5} M DEX treatment (Fig. 4), whereas the S32/36A mutant protein remained intact (Fig. 4) and CAT activity remained at a low level (cf. Fig. 1Ba). These results strongly indicate that GC induces the transcription and translation of IκBα, and this increased IκBα may reduce the activation of HIV-1 promoter through the reduction of active NF-κB. It is interesting to note that the MOLT4CDS32/36A cells decreased the shifted band and only faint shifted bands were observed (cf. Fig.2Ba).

These results strongly suggest that GC can induce IκBα and this IκBα accumulation may cause association of IκBα and NF-κB. Then the HIV-1 promoter activation through NF-κB should be reduced by GC. GC has been used to suppress the immunoresponse. Acquired immunodeficiency syndrome (AIDS) is one of the most well known immunosuppressive diseases. Examination of the effect of GC in HIV-1 activation is needed [Markham et al., 1986].

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