B-Oligomer of Pertussis Toxin Inhibits HIV-1 LTR-Driven Transcription through Suppression of NF-κB p65 Subunit Activity

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The binding subunit of pertussis toxin (PTX-B) has been shown recently to inhibit the entry and postentry events in HIV-1 replication in primary T lymphocytes and monocyte-derived macrophages. While the effect of PTX-B on HIV-1 entry was shown to involve CCR5 desensitization, the mechanism of postentry inhibition remained unclear. In T lymphocytes, PTX-B affected transcription or stability of Tat-stimulated HIV-1 mRNAs. In this study, we sought to identify the mechanism of postentry inhibition of HIV-1 replication by PTX-B in U-937 promonocytic cells. We demonstrate that in these cells PTX-B inhibits expression of luciferase reporter gene controlled by the HIV-1 LTR promoter. This effect is Tat-independent and is not restricted to the HIV-1 LTR promoter. Instead, PTX-B activity is mediated through suppression of the cellular transcription factor, NF-κB. PTX-B inhibits phosphorylation and nuclear translocation of the p65 subunit of NF-κB. This effect is independent of the cytoplasmic NF-κB inhibitor, IκBα, as PTX-B stimulates phosphorylation and subsequent degradation of this protein. The suppressive activity of PTX-B on NF-κB p65 phosphorylation and nuclear translocation is delayed, suggesting that PTX-B signaling might initiate synthesis and cytoplasmic accumulation of a p65 phosphorylation inhibitor.
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INTRODUCTION

Pertussis toxin, the major virulence factor of *Bordetella pertussis,* is a 105-kDa noncovalently linked heterohexameric protein composed of the A (active)-promoter and the B (binding)-oligomer. The A-promoter is an enzymatically active subunit exhibiting ADP ribosyltransferase activity responsible for ribosylation and inactivation of G_i-like proteins (Katada *et al.,* 1983). The pentameric B-oligomer is composed of S2-S4 and S3-S4 dimers held together by the S5 subunit (Latif *et al.,* 2001; Wang and Oppenheim, 1999). The main function of the PTX-B is binding of pertussis toxin to target cells (Wong and Rosoff, 1996). However, PTX-B activity is not limited to the binding function; it also initiates signaling events in target cells (Alfano *et al.,* 1999; Li and Wong, 2001; Rosoff *et al.,* 1987; Stewart *et al.,* 1989; Thom and Casnellie, 1989). This signaling activity is mediated by an as yet unidentified receptor. Possible candidates include a 43-kDa protein in T lymphocytes (Rogers *et al.,* 1990), the CD11b-CD18 integrin (Wong *et al.,* 1996; Wong and Luk, 1997), and the CD14 molecule in myelomonocytic cells (Li and

Wong, 2000, 2001). PTX-B signaling activity produces multiple effects, including chemotaxis and cell proliferation (Alfano *et al.,* 1999; Gray *et al.,* 1989; Kolb *et al.,* 1990). Recently, it was shown that a highly purified S3-S4 dimer induces proliferation of T cells and probably accounts for the mitogenic activity of the B-oligomer (Latif *et al.,* 2001). PTX-B signaling also intensifies Th1-dependent immune response through up-regulation of IFN-γ and IL-2 (Ryan *et al.,* 1997; Stewart *et al.,* 1989) and inhibition of prostaglandin production (O'Neill *et al.,* 1992).

Recently, we demonstrated that PTX-B, through its signaling activity, induces selective heterologous desensitization of the major HIV-1 chemokine coreceptor, CCR5, in activated primary CD4+ T lymphocytes (Alfano *et al.,* 1999). CCR5 desensitization correlated with suppression of entry of the CCR5-dependent HIV-1 strains (Alfano *et al.,* 1999). However, PTX-B also inhibited replication of the X4 HIV-1 strains in peripheral blood mononuclear cells (PBMC) (Alfano *et al.,* 1999, 2000). This activity was independent of the PTX-B effect on HIV-1 coreceptors and suggested additional, postentry mechanisms of suppression (Alfano *et al.,* 2000). Indeed, analysis of HIV-1 RNA production demonstrated that PTX-B suppressed HIV-1 RNA synthesis in cultures of X4 HIV-1-infected PBMC and reduced Tat-dependent expression of the luciferase reporter gene controlled by the HIV-1

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long terminal repeat (LTR) (Alfano *et al.,* 2000). Therefore, in T cells PTX-B inhibits entry of R5 HIV-1 strains and postentry events for both R5 and X4 viruses. Analysis of PTX-B anti-HIV activity was recently extended to monocytes/macrophages (Alfano *et al.,* 2001). This study demonstrated that, similar to T lymphocytes, PTX-B inhibits both entry and postentry steps of R5 HIV-1 infection. PTX-B also strongly inhibited virus expression in TNF-α- and IL-6-stimulated promonocytic cells chronically infected with HIV-1. These results suggest that the mechanisms of HIV-1 inhibition by PTX-B might be similar in both T cells and macrophages. While mechanisms of entry inhibition have been analyzed in detail (Alfano *et al.,* 1999, 2000), little is known about PTX-B effects on the postentry step of HIV-1 replication. This postentry activity most likely involves inhibition of transcription or destabilization of Tat-dependent HIV-1 RNAs (Alfano *et al.,* 2000).

The above-referenced study (Alfano *et al.,* 2001) showed that PTX-B suppressed TNF-α-stimulated HIV-1 replication in promonocytic cells. Since TNF-α is a classical transcriptional stimulator of viral expression, mainly operating via activation of the cellular transcription factor NF-κB (Antoni *et al.,* 1994; Griffin *et al.,* 1989; Osborn *et al.,* 1989; Vicenzi *et al.,* 1997), it appears likely that NF-κB is involved in the mechanism of suppression of HIV-1 LTR-driven transcription by PTX-B. NF-κB is the main cellular transcription factor regulating HIV-1 expression (Nabel and Baltimore, 1987). The HIV-1 LTR contains two binding sites for cellular proteins belonging to the NF-κB/Rel family (Fig. 1A). This protein family includes p65 (RelA), p50, p52, RelB, and c-Rel polypeptides, which form homo- and heterodimers (Baeuerle and Baltimore, 1996). The most abundant of these dimers is the NF-κB p65/p50 heterodimer, although the homodimers of p65 and p50 are also found in different cell types (Haskill *et al.,* 1991; Siebenlist *et al.,* 1994; Simeonidis *et al.,* 1997; Thompson *et al.,* 1995). In the cytoplasm, NF-κB p65/p50 heterodimers exist as a complex with the inhibitor protein IκBα, which inactivates the NF-κB heterodimers by masking their nuclear localization sequences (Ganchi *et al.,* 1992; Henkel *et al.,* 1992; Phelps *et al.,* 2000a). Activation of signaling pathways by such extracellular signals as TNF-α, IL-1, phorbol esters, or lipopolysaccharide leads to phosphorylation of IκBα at two amino-terminal serine residues (S32, S36) (reviewed in Ghosh *et al.,* 1998; Karin and Ben Neriah, 2000). Phosphorylation of these targets stimulates rapid proteolytic degradation of IκBα via the 26 S proteasome (Baeuerle and Baltimore, 1996), allowing active NF-κB to translocate into the nucleus (Mercurio *et al.,* 1997; Roff *et al.,* 1996; Scherer *et al.,* 1995; Yaron *et al.,* 1997) and to activate transcription from a wide variety of promoters, including the HIV-1 LTR (Griffin *et al.,* 1989; Moses *et al.,* 1994). Another mechanism regulating the transcriptional activity of NF-κB is through phosphorylation of the p65 subunit (Mercurio *et*

al., 1999; Wang and Baldwin, 1998; Zhong *et al.,* 1997). Extracellular stimuli, such as TNF-α, are believed to induce p65 phosphorylation (Mercurio *et al.,* 1997, 1999; Schwabe *et al.,* 2001; Wang and Baldwin, 1998), which occurs in the cytoplasm prior to p65 nuclear translocation (Sakurai *et al.,* 1999). Phosphorylation of p65 is considered to be an important step in the up-regulation of the transcriptional activity of NF-κB, independent of its DNA-binding activity (Schwabe *et al.,* 2001).

In this study, we investigated possible mechanisms of PTX-B inhibitory effect on HIV-1 transcription in promonocytic cells U-937. Our results suggest that PTX-B targets the p65 subunit of the NF-κB transcription factor.

RESULTS

PTX-B inhibits luciferase reporter gene expression through suppression of NF-κB activity

Recently, it was demonstrated that PTX-B effectively inhibits HIV-1 replication in U-937 cells at a postentry level of infection, as well as virus expression in chronically infected U1 cells stimulated with proinflammatory cytokines (TNF-α, IL-6) (Alfano *et al.,* 2001). This inhibitory activity of PTX-B was hypothesized to target either transcription, or splicing, or stability of viral mRNAs.

To analyze the mechanisms of PTX-B suppressing activity, we generated a series of pLTR–luciferase constructs containing the luciferase reporter gene controlled by the complete 5′ LTR$_{LAI}$ plus the leader region up to the beginning of the GAG open reading frame (nt 1–789, where nt + 1 is the start of the U3 region in the 5′ LTR) and carrying mutations in the TAR region and in different transcription factor binding sites of the 5′ LTR (Fig. 1A). The five resulting plasmids were designated pLTRluc-wt, pLTRluc-ΔTAR, pLTRluc-mut-Sp1(I,II,III), pLTRluc-mut-AP-1(I,II,III)/NF-AT, and pLTRluc-mut-κB (Fig. 1A). These plasmids were transiently transfected into U-937 cells. Incubation of transfected cells with 2.5 nM PTX-B for 48 h resulted in approximately 60% suppression of luciferase activity in cell lysates with the pLTRluc-wt construct (Fig. 1B). Unlike Jurkat cells (a T-cell line), where PTX-B inhibitory effect was specific only for Tat-dependent transcription (Alfano *et al.,* 2000), the effect in U-937 promonocytic cell line was Tat-independent. Indeed, the inhibitory effect of PTX-B on luciferase expression from HIV-1 LTR was similar in the presence or absence of Tat (coexpressed from pcDNA1/Tat plasmid) (Fig. 1B). Furthermore, *Renilla* luciferase expression from the CMV promoter was also suppressed (Fig. 1B, pRL-CMV).

In order to check whether the inhibitory activity of PTX-B was transcription-associated and to identify the transcription factor(s) involved, we used recombinant plasmids encoding the luciferase gene controlled by HIV-1 LTR promoter with point mutations in several cellular transcription factor binding sites and in the TAR region (Fig. 1A). These experiments demonstrated that
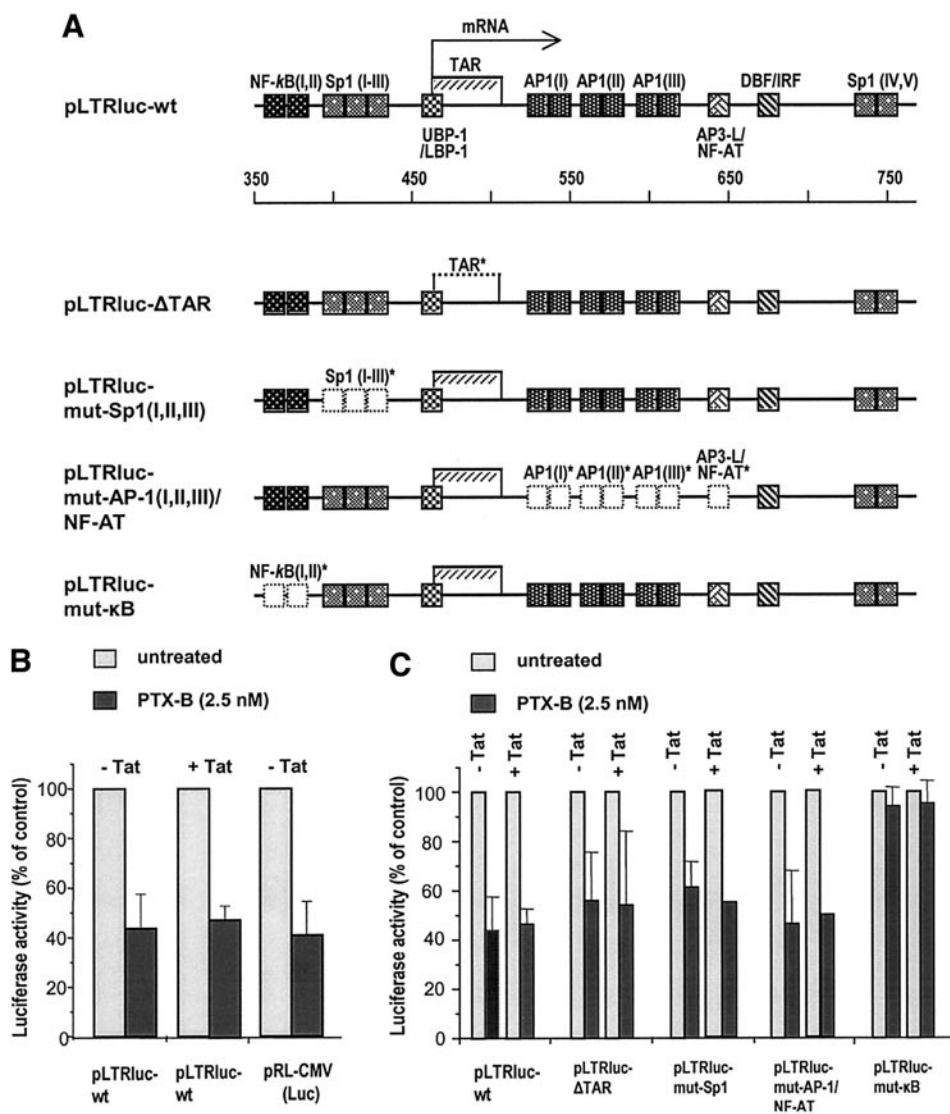
FIG. 1. PTX-B inhibits NF-κB-mediated luciferase reporter gene expression. (A) Schematic representation of the LTR promoter region in the constructs used for this study. The LTR is derived from the HIV-1 LAI strain and was cloned upstream of the firefly luciferase reporter gene in the pGL2-Basic vector. Point mutations were introduced into indicated transcription factor binding sites and in the TAR sequence. Mutated sites are marked with asterisks. (B) PTX-B activity is not HIV-1 LTR-specific. Triplicate cultures of U-937 cells were transfected with pLTRluc-wt, pRL-CMV (encoding Renilla luciferase under control of the CMV promoter), or cotransfected with pLTRluc-wt and the HIV-1 Tat expression vector pcDNA1/Tat. Three hours posttransfection, cells were treated or not for 48 h with PTX-B and lysed for luciferase activity analysis. Luciferase activity is presented as percentage of activity in PTX-B-untreated cells. Results are shown for one representative experiment of three total and are presented as mean ± SD. (C) PTX-B activity is NF-κB-dependent. U-937 cells were cotransfected with the various constructs shown in (A) with or without pcDNA1/Tat. PTX-B treatment and luciferase activity measurement was performed as described in (B). Results are mean ± SD of three replicates, except cotransfections of pcDNA1/Tat with pLTRluc-mut-Sp1 and pLTRluc-mut-AP-1/NF-AT that were performed one time.

the PTX-B inhibitory effect on luciferase gene expression was not reduced by inactivating mutations in the TAR region, in the three Sp1 sites (I, II, III), in the three AP-1 sites (I, II, III), or NF-AT binding sites of the HIV-1 LTR region (Fig. 1C). In contrast, mutation in the two κB sites of the HIV-1 enhancer (pLTRluc-mut-κB) resulted in a complete loss of sensitivity of luciferase expression to PTX-B inhibitory activity (Fig. 1C). These results suggest that the suppressing effect of PTX-B on luciferase gene expression is transcription-associated and is mediated by inhibition of NF-κB activity. Binding sites for this uni-

versal transcription activator can be found in both the HIV-1 LTR and the CMV early enhancer/promoter sequences.

PTX-B reduces the level of nuclear NF-κB p65 subunit

To investigate the link between the PTX-B effect and NF-κB, we cotransfected U-937 cells with pLTRluc-wt and with expression vectors for the NF-κB subunit p65 or p50 (pRSV-p65 or/and pRSV-p50) and treated them with PTX-B (Fig. 2A). The transfection approach provides an
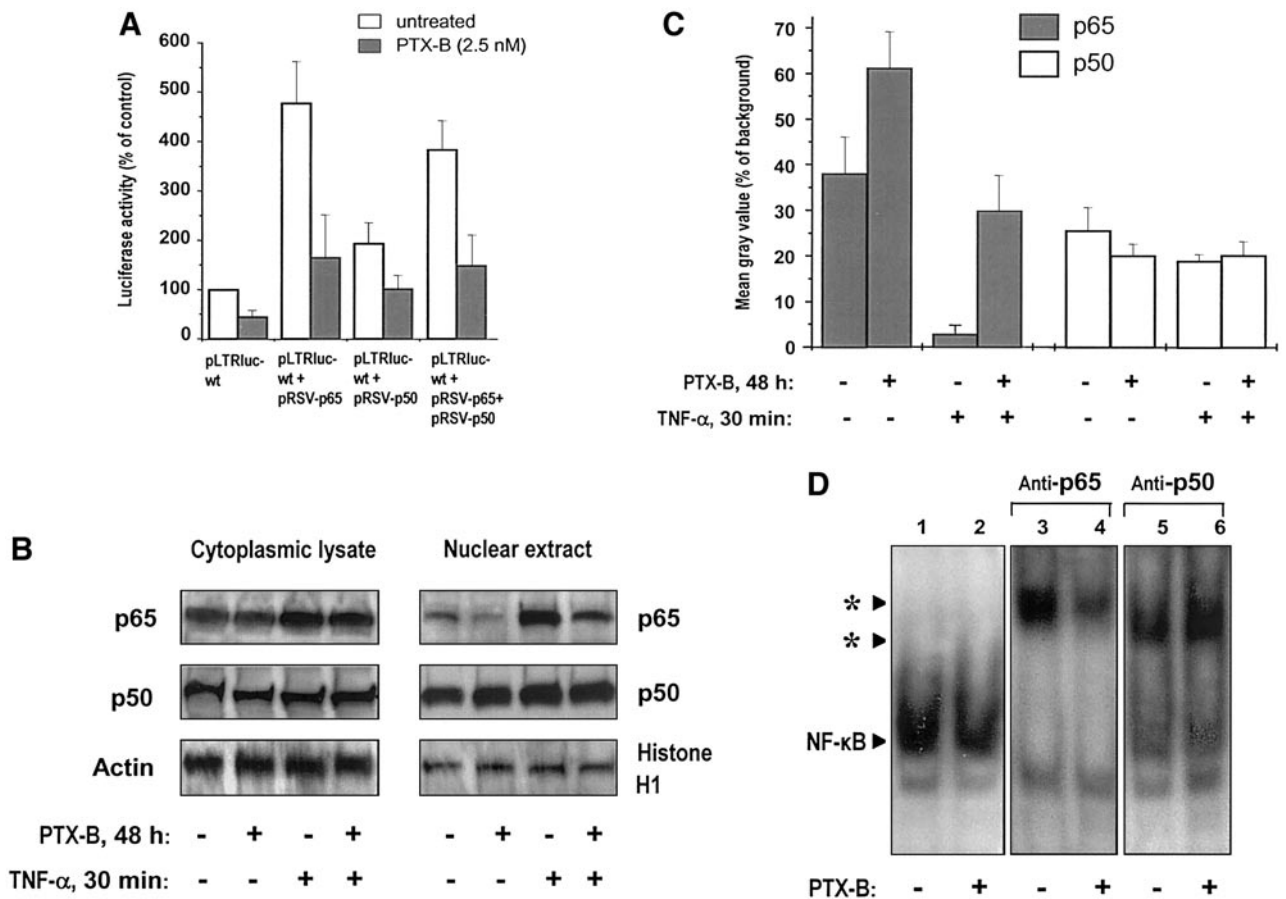
FIG. 2. PTX-B inhibits nuclear accumulation and activity of NF-κB. (A) Analysis of PTX-B effect on transcriptional activity of NF-κB p65 and p50 subunits. U-937 cells were either transfected with pLTRluc-wt (1 μg) or cotransfected with pLTRluc-wt (1 μg) and pRSV-p65 (0.01 μg), pLTRluc-wt (1 μg) and pRSV-p50 (0.01 μg), or pLTRluc-wt (1 μg), pRSV-p65 (0.005 μg), and pRSV-p50 (0.005 μg). Three hours posttransfection, cells were treated or not for 48 h with PTX-B and lysed for luciferase activity analysis. Luciferase activity is shown as percentage of activity in PTX-B-untreated cells transfected with pLTRluc-wt (control). Results are mean ± SD of three replicates. One representative experiment of two performed is shown. (B) PTX-B inhibits nuclear accumulation of NF-κB p65 subunit. U-937 cells were treated or not with 2.5 nM PTX-B and incubated for 48 h. Thirty minutes prior to harvesting, cells were stimulated or not with TNF-α (0.1 ng/ml). Cytoplasmic lysates and nuclear extracts were analyzed by Western blotting using rabbit anti-p65 (C-20) or anti-p50 (H-119) polyclonal antibodies. Mouse anti-actin (AC-40) and anti-histone H1 (AE-4) monoclonal antibodies were used to normalize protein concentration in the preparations. (C) Quantification of Western blotting results. Western blotting data from three independent experiments examining nuclear p65 and p50 (representative experiment is shown in (B)) were analyzed using UTHSCSA Image Tool Version 3.0 program. Results show mean gray values of the bands ± SE and are presented as percentage of the background. (D) Analysis of PTX-B effects on TNF-α-induced activation of NF-κB subunits. U-937 cells were incubated or not with 2.5 nM PTX-B for 48 h and then stimulated with TNF-α (0.1 ng/ml) for 30 min. Nuclear extracts were prepared and tested for NF-κB subunit activity by EMSA (lanes 1, 2) and supershift (lanes 3–6) assays, as described under Materials and Methods. Bands corresponding to supershifted NF-κB complexes are marked by asterisks.

opportunity to investigate the effect of PTX-B on individual subunits of NF-κB. Expression of the recombinant p65 subunit resulted in a fivefold increase of HIV-1 LTR-driven luciferase transcription, whereas p50 subunit initiated only a twofold stimulation (Fig. 2A). Coexpression of p50 and p65 subunits led to a fourfold increase in luciferase expression (Fig. 2A). It should be noted that the amount of pRSV-p65 and pRSV-p50 used in the cotransfection experiment was only half of the amount used for individual expression of these factors, thus explaining the lower activation effect of the p65/p50 combination compared to p65 alone. These data are consistent with a generally accepted view that p65 is a transcriptionally active member of the NF-κB family,

whereas p50 primarily serves as a DNA-binding subunit (reviewed in Karin and Ben Neriah, 2000; Liou and Baltimore, 1993). Incubation of transfected cells with PTX-B for 48 h resulted in suppression of luciferase activity; however, the rate of suppression varied. Maximal suppression was observed with pRSV-p65 (more than threefold inhibition, Fig. 2A). Less than a twofold inhibition was detected with pRSV-p50 (Fig. 2A). The inhibitory effect in cells cotransfected with p65 and p50 subunits was slightly lower than in p65-transfected cells.

NF-κB is maintained in a complex with IκBα in the cytoplasm of resting cells and enters the nucleus to bind specific DNA sites in response to various stimuli, including viral proteins, ultraviolet irradiation, and proinflam-

matory cytokines such as TNF-α and IL-1 (Mercurio *et al.,* 1997; Phelps *et al.,* 2000a,b). In order to evaluate possible effects of PTX-B on nuclear localization and DNA-binding activity of NF-κB, we analyzed p65 and p50 NF-κB subunits in the nuclear and cytoplasmic fractions of U-937 cells preincubated with PTX-B and activated or not with TNF-α. In our previous study (Alfano *et al.,* 2001), we determined that optimal stimulation time to detect TNF-α-induced NF-κB in U1 cells (derivatives of U-937) was 30 min. Western blotting analysis of U-937 cells using anti-p65 and anti-p50 antibodies demonstrated that PTX-B slightly (approximately 1.6-fold, Fig. 2B and 2C) reduced the nuclear, but not the cytoplasmic, NF-κB p65 subunit in nonactivated cells, and substantially (7.5-fold) diminished the nuclear p65 level in TNF-α-stimulated cells. In contrast, the amount of p50 subunit in both cytoplasmic and nuclear compartments did not change after PTX-B treatment (Figs. 2B and 2C). PTX-B-induced reduction of nuclear p65 in TNF-α-treated cells was also demonstrated by the supershift assay (Fig. 2D, lanes 3 and 4). Consistent with the results presented in Figs. 2A–2C, this assay did not detect reduction of nuclear p50 in PTX-treated cells (Fig. 2D, lanes 5 and 6). The p50 subunit likely accounts for the binding activity observed in PTX-B-treated cells by EMSA (Fig. 2D, lanes 1 and 2).

Taken together, our results suggest that the p65 subunit of NF-κB provides stronger activation of HIV-1 LTR-driven transcription than p50 in U-937 promonocytic cells. The inhibitory effect of PTX-B appears to be achieved by reduction of the steady-state nuclear levels of the p65, but not the p50, subunit. PTX-B-induced reduction of luciferase activity in pRSV-p50-transfected cells (Fig. 2A) can be explained by the effect of PTX-B on the basic activity level of the endogenous p65 subunit.

Effect of PTX-B on nuclear p65 is IκBα-independent

One possible mechanism for the observed inhibitory effect of PTX-B on NF-κB p65 nuclear import is through suppression of IκBα phosphorylation and degradation. We therefore analyzed IκBα phosphorylation and degradation in the cytoplasmic fraction of PTX-B-treated and untreated U-937 cells activated or not with TNF-α (Fig. 3). We chose a 7-min stimulation time with TNF-α because previous study (Manna *et al.,* 2000) demonstrated that maximal effect on IκBα phosphorylation and degradation occurred between 7 and 15 min of TNF-α stimulation. Surprisingly, PTX-B-pretreated cells demonstrated more IκBα degradation than PTX-B-untreated cells (Fig. 3, upper panel). Despite low levels of total IκBα, antibody to phosphorylated IκBα detected amounts of this protein that were substantially higher than in untreated cells (bottom panel). Interestingly, there was considerably more phosphorylated IκBα in TNF-α-stimulated PTX-B-treated cells than in untreated cells, suggesting that PTX-B-induced phosphorylation of IκBα is very brisk.
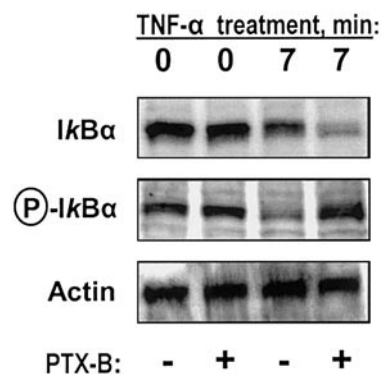

FIG. 3. PTX-B stimulates IκBα phosphorylation and degradation. U-937 cells were incubated or not with 2.5 nM PTX-B for 48 h and then were either stimulated with TNF (0.1 ng/ml) for 7 min or left unstimulated. Cytoplasmic lysates were analyzed by Western blotting using rabbit anti-IκBα or anti-phospho-IκBα (P-IκBα) polyclonal antibodies. Mouse anti-actin (AC-40) monoclonal antibody was used to normalize protein concentration in the cytoplasmic preparations.

These results suggest that PTX-B contributes to phosphorylation and subsequent degradation of IκBα and therefore is expected to stimulate nuclear accumulation of NF-κB. Since we observed not an increase but a decrease in the steady-state level of p65, we conclude that the inhibitory effect of PTX-B is independent of IκBα.

PTX-B effect on NF-κB p65 is delayed

Results presented above illustrate the analysis of U-937 cells incubated with PTX-B for 48 h. Previous study using EMSA and supershift assays of U1 cells pretreated with PTX-B for 20 min and 4 h did not reveal any PTX-B effects on NF-κB activity (Alfano *et al.,* 2001). However, a 20-min incubation of these cells with PTX-B followed by activation with TNF-α inhibited HIV-1 expression measured 72 h after stimulation, as well as HIV-1 RNA accumulation measured at 16 h. Taken together with our results, these data suggest that the effect of PTX-B is delayed.

To analyze the time course of PTX-B effect on LTR-driven transcription, we incubated pLTRluc-wt-transfected U-937 cells with PTX-B for 24, 48, and 72 h (Fig. 4A). Measurement of luciferase activity displayed maximal inhibitory effect at the 48-h time point (60% suppression). No suppression was observed with shorter incubation time (24 h). Longer incubation time (72 h) decreased the inhibitory effect to ~30%, probably as a result of culture media depletion. Consistent with previous results, no PTX-B inhibitory activity was observed with the pLTRluc-mut-κB plasmid (Fig. 4A).

To further explore the delayed effect of PTX-B, we incubated pLTRluc-wt-transfected U-937 cells with PTX-B for 2 and 24 h and then postincubated in culture media without the B-oligomer for 46 and 24 h, respectively. A time-dependent increase in PTX-B inhibitory activity was observed (Fig. 4B). This inhibitory effect partially corre-
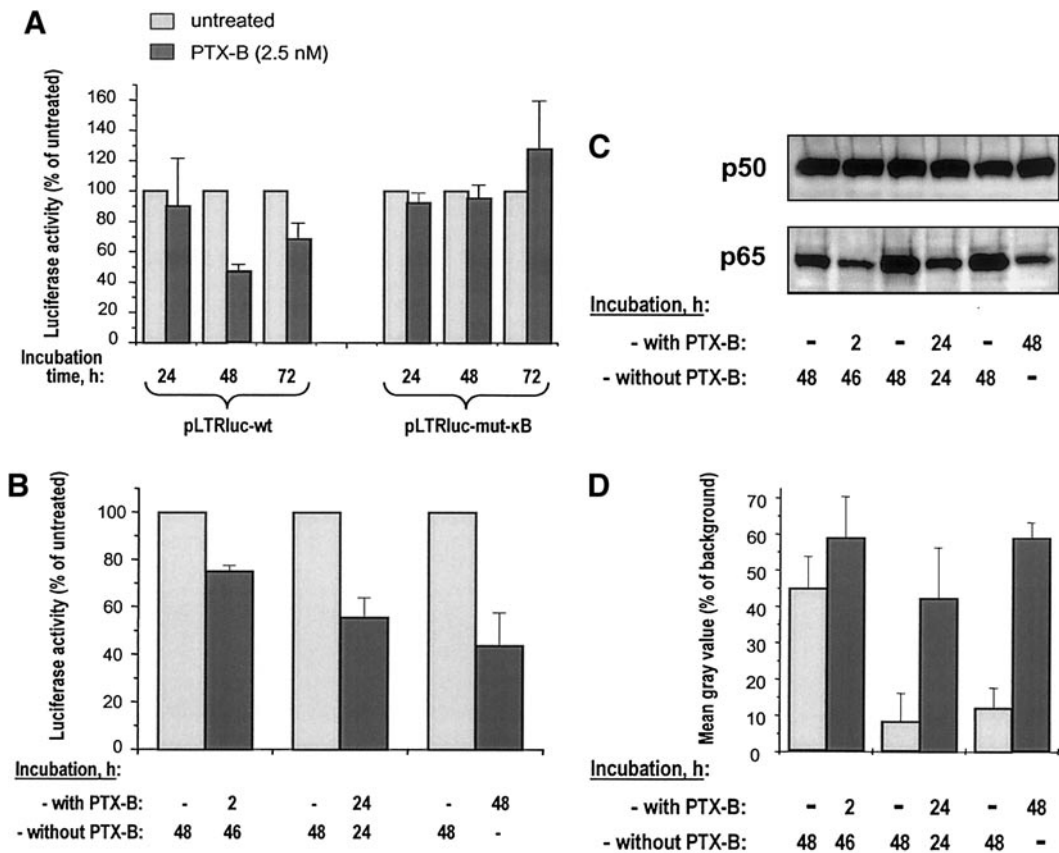
FIG. 4. Time course analysis of PTX-B effects on luciferase reporter gene expression and nuclear accumulation of NF-κB. (A) Triplicate cultures of U-937 cells were transfected with pLTRluc-wt or with pLTRluc-mut-κB. Cells were treated or not with PTX-B at 3 h posttransfection, incubated with PTX-B for 24, 48, or 72 h, and harvested. Luciferase activity was measured in total cell lysates and is presented as percentage of activity in PTX-B-untreated cells for each time point. Results are presented as mean ± SD. (B) Triplicate cultures of U-937 cells were transfected with pLTRluc-wt. Cells were treated or not with 2.5 nM PTX-B at 3 h posttransfection, incubated with PTX-B for 2, 24, or 48 h, washed, and cultured without PTX-B for 46, 24, or 0 h, respectively. Luciferase activity was measured as in (A). (C) The effect of PTX-B on nuclear translocation of NF-κB p65 subunit is delayed. Cells were treated or not with 2.5 nM PTX-B, incubated with PTX-B for 2, 24, or 48 h, washed, and cultured without PTX-B for 46, 24, or 0 h, respectively. At the 48-h time point, cells were stimulated with TNF-α (0.1 ng/ml) for 30 min. Nuclear extracts were analyzed by Western blotting as described in the legend to Fig. 2B. (D) Quantification of Western blotting results. Western blotting data from two independent experiments examining nuclear p65 (representative experiment is shown in (C)) were analyzed using UTHSCSA Image Tool Version 3.0 program. Results show mean gray values of the bands ± SE and are presented as percent of the background.

lated with the dynamics of nuclear accumulation of NF-κB p65 subunit in the treated cells (Figs. 4C and 4D). The maximal level of inhibition (approximately fivefold) was reached after 24 h incubation with the B-oligomer (Figs. 4C and 4D). Consistent with the report by Alfano et al. (2001), Western blot analysis of the nuclear fraction of U-937 cells incubated with PTX-B for 2 h, performed just after incubation (without long-time postincubation period), did not show any inhibition of the NF-κB p65 nuclear accumulation (data not shown). This delayed effect of PTX-B suggests involvement of an intracellular synthetic pathway initiated by PTX-B signaling that leads to IκBα-independent suppression of p65 nuclear accumulation and LTR-driven transcription. Although we cannot formally exclude the possibility that the observed delay in PTX-B activity reflects its intracellular accumulation, it appears unlikely, as there is no published evidence for

direct interaction between PTX-B and cellular signaling proteins.

PTX-B inhibits phosphorylation of NF-κB p65 subunit

PTX-B treatment of U-937 cells stimulates IκBα phosphorylation and degradation in response to TNF-α activation, but inhibits NF-κB p65 nuclear accumulation. Because p65 is also phosphorylated in response to TNF-α signaling and because this mechanism is believed to contribute to up-regulation of the transcriptional activity of NF-κB (Mercurio et al., 1997, 1999; Sizemore et al., 1999, 2002; Schwabe et al., 2001; Wang and Baldwin, 1998), we analyzed a possible effect of PTX-B on phosphorylation of NF-κB p65. For this analysis, we performed metabolic labeling of U-937 cells incubated or not with PTX-B (for 24 h) and TNF-α (for 7–40 min), and immunoprecipitated p65, p50, and their precursor, p105,
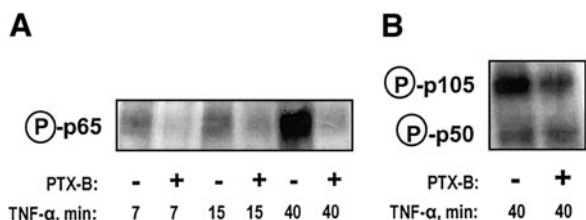
FIG. 5. Analysis of PTX-B effects on NF-κB phosphorylation. (A) PTX-B inhibits TNF-α-induced phosphorylation of NF-κB p65 subunit. U-937 cells were treated or not with PTX-B for 24 h followed by incubation in a phosphate-free medium without PTX-B for additional 24 h. Cells were then incubated with [³²P]orthophosphate for 2 h and then stimulated with TNF-α (0.1 ng/ml) for indicated time intervals. NF-κB p65 was immunoprecipitated from total cell lysates with anti-p65 antibody and analyzed by SDS–PAGE and autoradiography. P-p65 indicates a phosphorylated form of NF-κB p65. (B) PTX-B inhibits TNF-α-induced phosphorylation of the p105 precursor of NF-κB p50, but not of the p50 subunit itself. Analysis was performed as in (A), except that only a 40-min TNF-α stimulation was analyzed and anti-p50 antibody was used for immunoprecipitation. Note that this antibody precipitates both p50 and p105. P-p105 and P-p50 indicate phosphorylated forms of the proteins.

from the total cell lysates. It should be noted here that due to excess of cytoplasmic over nuclear NF-κB, this analysis mostly addresses cytoplasmic NF-κB phosphorylation. As shown in Fig. 5A, TNF-α-stimulated phosphorylation of p65 was suppressed in PTX-B-treated cells. The rate of phosphorylation of p65 molecules was gradually increasing during the time of TNF-α stimulation in PTX-B-untreated cells (Fig. 5A). In contrast, no increase was observed in PTX-B-treated U-937 cells (Fig. 5A). Interestingly, immunoprecipitation of the cell lysates with anti-p50 antibodies revealed significant levels of phosphorylation of the p50 protein and p105 precursor, the amino-terminal part of which forms the p50 subunit (Fig. 5B). Recent publications (Cohen et al., 2001; Heissmeyer et al., 1999; Orian et al., 2000) reported that chemokine-induced, IκB kinase-mediated phosphorylation of the p105 C-terminal domain leads to processing of the precursor, whereas phosphorylation of the p50 subunit contributes to the DNA-binding activity of NF-κB (Koul et al., 2001). Although we observed inhibition of p105 phosphorylation by PTX-B, we did not see PTX-B-initiated suppression of p50 phosphorylation and nuclear accumulation (Figs. 2B and 2C; Fig. 5B).

Taken together, these results indicate that PTX-B inhibits phosphorylation of the NF-κB p65 subunit. We suggest that this effect mediates the decrease of p65 steady-state levels and suppression of HIV-1 LTR-driven transcription inhibitory activity by PTX-B.

DISCUSSION

Mononuclear phagocytes are one of two major cell types infected during a natural course of HIV disease. They are known to play an important role both as viral reservoirs and as a source of replicating HIV during all stages of disease (Igarashi et al., 2001; Orenstein et al., 1997). Therefore, it is of paramount importance to identify mechanisms of HIV replication in these cells and agents that would inhibit infection of macrophages. Recently, it was demonstrated that the B-oligomer of PTX-B inhibits HIV-1 replication in monocyte-derived macrophages at both entry and postentry steps (Alfano et al., 2001). While inhibition of HIV-1 entry by PTX-B was shown to be mediated through CCR5 (Alfano et al., 1999), the mechanism of postentry effect was not identified. In the present study, we demonstrate that the PTX-B inhibits HIV-1 LTR-controlled transcription in U-937 promonocytic cells. This inhibitory activity is not limited to Tat-dependent transcripts, because PTX-B also suppressed transcription from the CMV promoter, as well as from the LTR promoter with mutated TAR or in the absence of the Tat-expressing vector. This effect is delayed and is mediated by inhibition of the nuclear accumulation of the NF-κB p65 subunit. The inhibitory effect of PTX-B on the NF-κB p65 subunit is IκBα-independent, because PTX-B activates phosphorylation and degradation of IκBα.

Our study confirms conclusions reached in our previous work (Alfano et al., 2001) that described the PTX-B effects on HIV-1 expression in a TNF-α- and IL-6-activated U1 cell line, which is an HIV-1 chronically infected U-937-derived line (Folks et al., 1987). Similar to results of this report, PTX-B activity in U1 cells was shown to be transcription-mediated. However, in the previous study we did not find any effect of PTX-B on NF-κB. This inconsistency with the results presented here is likely due to differences in the time of analysis. EMSA and supershift analyses in our previous study were performed shortly after PTX-B treatment (20 min and 4 h), while here we demonstrate that PTX-B stimulates a delayed response in U-937 cells, which is observed only after a prolonged (24–48 h) postincubation period. This delayed response suggests that the effect of PTX-B on NF-κB is indirect and might be mediated by induction of a synthetic pathway of a factor that inhibits p65 nuclear accumulation.

Our results indicate that PTX-B induces phosphorylation and subsequent degradation of IκBα in U-937 cells. Phosphorylation of IκB family members, when initiated by extracellular stimuli, is carried out by two related IκB kinases, IKKα and IKKβ. These kinases are found as homo- or heterodimeric IKK complexes that directly phosphorylate two N-terminal serine residues in IκBs (DiDonato et al., 1997; Mercurio et al., 1997; Zandi et al., 1997). Recently, it was shown that the IKKβ is mainly responsible for cytokine-induced IκB degradation (Hu et al., 1999; Takeda et al., 1999). PTX-B, through its signaling activity, might feed into this pathway and activate the components of the IKK complex, leading to phosphorylation and degradation of IκBα.

Recent publications elucidated the mechanism of

phosphorylation of the NF-κB family of proteins. The phosphorylation of these proteins is carried out by kinases belonging to the IKK complex, which also phosphorylates IκBα (Cohen *et al.,* 2001; Heissmeyer *et al.,* 1999; Koul *et al.,* 2001; Orian *et al.,* 2000; Sakurai *et al.,* 1999; Sizemore *et al.,* 1999, 2002; Wang and Baldwin, 1998). In particular, both IκBα and p105 (NF-κB p50 precursor) are phosphorylated predominantly by IKKβ (Orian *et al.,* 2000). However, while PTX-B stimulated IκBα phosphorylation, it inhibited phosphorylation of the p105 and NF-κB p65 subunit. Therefore, it appears that IKKβ is not the target of PTX-B.

Recent studies demonstrated that phosphorylation of the p65 subunit of NF-κB plays an essential role in up-regulation of the transcriptional activity of NF-κB, independent of its binding activity (Li *et al.,* 1999; Mercurio *et al.,* 1999; Sakurai *et al.,* 1999; Schwabe *et al.,* 2001; Vanden Berghe *et al.,* 1998; Wang and Baldwin, 1998). While the effect of phosphorylation on NF-κB nuclear transport has not been formally demonstrated, phosphorylation is a common mechanism for nuclear import and export regulation (Jans, 1995; Jans and Hubner, 1996). The p65 molecule has several potential phosphorylation sites for different kinases. The IKK complex induces phosphorylation of Ser-536 (Sakurai *et al.,* 1999), protein kinase A phosphorylates Ser-276 (Zhong *et al.,* 1998), and Ser-529 is phosphorylated by an undefined protein kinase in response to TNF-α (Wang and Baldwin, 1998). The IKKβ is required for p65 phosphorylation at the basal level of NF-κB activity, whereas IKKα phosphorylates p65 solely in response to cytokine induction (Sizemore *et al.,* 2002). Our results demonstrate PTX-B-induced suppression of NF-κB p65 activity in both TNF-α-activated and non-activated cells. Together with demonstrated activation of the IκB phosphorylation and the lack of a PTX-B suppressive effect on NF-κB p50 phosphorylation, these results suggest that the effect of PTX-B is not mediated by suppression of IKK activity.

What could then be a mechanism of NF-κB-directed PTX-B activity? The delayed character of the PTX-B suppressing effect on NF-κB p65 and p105 phosphorylation suggests that PTX-B signaling might initiate synthesis and cytoplasmic accumulation of a phosphorylation inhibitor, such as phosphatase. Inhibition of p65 phosphorylation might down-regulate its nuclear import, stimulate nuclear export, or stimulate degradation of the protein, thus reducing its steady-state nuclear concentration and transcriptional activity of p65/p65 homo- and p65/p50 heterodimers.

In conclusion, our results indicate that PTX-B specifically inactivates the p65, but not the p50, subunit of NF-κB. This effect appears to be mediated through a decrease of p65 nuclear steady-state level and correlates with suppression of p65 phosphorylation. In contrast, phosphorylation and nuclear accumulation of p50 is not affected by PTX-B. Since the p65 subunit of NF-κB is a strong transcriptional activator of HIV-1 LTR promoter activity, whereas p50 does not activate HIV-1 transcription (Pazin *et al.,* 1996), our results help explain the observed inhibitory activity of PTX-B on HIV-1 replication (Alfano *et al.,* 2001). It should be noted that we analyzed the effect of PTX-B on HIV-1 LTR-driven transcription of an extrachromosomal construct. This may explain the incomplete inhibition compared to results reported by Alfano *et al.* (2001). Indeed, p65 was shown to provide a much more potent stimulation of HIV-1 promoter activity in the context of a chromatin-organized template than in the context of a naked DNA template (Pazin *et al.,* 1996; West *et al.,* 2001).

Future studies will further investigate the molecular mechanisms underlying the observed inhibitory activity of PTX-B on NF-κB p65 phosphorylation and nuclear level. Understanding these mechanisms will help design a novel type of anti-HIV agent that targets intracellular signaling mechanisms in order to inhibit reactivation of the latent HIV-1 provirus. Such agents should further improve the efficiency of HAART and should help keep infection at bay when HAART has to be withdrawn.

EXPERIMENTAL PROCEDURES

Reagents

Pertussis toxin B-oligomer (Calbiochem, San Diego, CA) was dissolved in sterile PBS to a final concentration of 50 μg/ml and stored at 4°C. TNFα, rabbit anti-IκBα, and anti-phospho IκBα antibodies were also from Calbiochem. Mouse anti-histone H1 (AE-4) and anti-p65 NF-κB (F-6)X monoclonal, and rabbit anti-p65 NF-κB (C-20), anti-p50 NF-κB (H-119), and (H-119)X polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-actin (AC-40) monoclonal antibodies were from Sigma (St. Louis, MO). Luciferase and dual-luciferase assay systems were from Promega (Madison, WI). RPMI 1640 phosphate-free medium was from Specialty Media (Phillipsburg, NJ).

Cell cultures

U-937 human monoblastic leukemia cells were purchased from ATCC (Rockville, MD) and maintained at 37°C in 5% CO_2 in RPMI 1640 supplemented with 10% (v/v) FCS (HyClone), 100 units/ml penicillin, and 100 units/ml streptomycin.

Plasmid constructs

A 789-bp fragment containing the HIV-1$_{LAI}$ 5′ LTR (nt 1–789; numbering is according to the LAI provirus where nt + 1 is the start of U3 in the 5′ LTR) was prepared from pLTR-CAT (Van Lint *et al.,* 1994) by digestion with *Pst*I, blunt-ending of the 3′ overhang with T4 DNA polymerase, and digestion with *Xba*I successively, and this fragment was then cloned in pGL2-Basic (Promega) digested

with *Acc*65I, blunted with Klenow polymerase, and digested with *Nhe*I. The resulting plasmid was designated pLTRluc-wt. To construct pLTRluc-mut-κB, pLTRluc-wt was used as a substrate for mutagenesis of the two κB sites by the Quick Change Site-Directed Mutagenesis method (Stratagene). Mutations were generated with the following pair of mutagenic oligonucleotide primers (mutations are highlighted in bold and κB sites are underlined on the coding strand primer): CV211/CV212: 5′-CGAGCTTGCTACA<u>ACTCACTTTCC</u>GCT<u>GCTCACTTT-CC</u>AGGGAGG-3′. To construct pLTRluc-mut-Sp1(I,II,III), pLTRluc-wt was used as a substrate for mutagenesis of the three Sp1 sites by the Quick Change Site-Directed Mutagenesis method (Stratagene). Mutations were generated with the following pair of mutagenic oligonucleotide primers (mutations are highlighted in bold and Sp1 sites are underlined on the coding strand primer): CV421/CV422: 5′-GGGGACTTTCCAGG<u>GATTCGTGGCC</u>TGTTC-<u>GGGACTGGTTAGTGGC</u>GAGCCCTC-3′. To construct pLTRluc-ΔTAR, pLTRluc-wt was used as a substrate for the mutagenesis of the TAR region by the Transformer Site-Directed Mutagenesis method (Clontech, Palo Alto, CA). The 3-nt bulge of TAR (nt 476–478) was deleted with the following mutagenic oligonucleotide (CV61): 5′-G^{nt494}AGAGCTCCCAGGCTCTCTGGTCTAACC-3′. The oligonucleotide (CV68) 5′-CGGTCGACGGTACCAGACAT-GATAAG-3′, changing a unique *Bam*HI restriction site in pLTRluc-wt into a *Kpn*I site (highlighted in bold), was used for selection during mutagenesis. All mutated constructs were fully resequenced after identification by cycle sequencing using the Thermosequenase DNA sequencing kit (Amersham). The reporter construct pLTRluc-mut-AP-1(I,II,III)/NF-AT was previously described (Van Lint *et al.,* 1997).

Plasmids pRSV-p50 and pRSV-p65 were obtained from Dr. Gary Nabel and Dr. Neil Perkins through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH).

Transient transfection and luciferase assays

Transfections of U-937 cells with recombinant plasmids were performed using FuGene 6 Transfection Reagent (Roche, Indianapolis, IN). Suspensions of 2×10^6 exponentially growing cells were transfected with 1 μg of recombinant plasmid DNA encoding firefly luciferase controlled by HIV-1 LTR sequences either alone or together with 0.1 μg of HIV-1 Tat-encoding plasmid pcDNA1/Tat, kindly provided by Dr. Chiara Bovolenta. Also, as a control, we used *Renilla* luciferase-encoding plasmid pRL-CMV (Promega). For experiments with the expression vectors for p65 and p50, 2×10^6 U-937 cells were cotransfected with a pLTRluc-wt construct (1 μg) and pRSV-p65 or pRSV-p50 individually (0.01 μg each) or together (0.005 μg each). Transfected cells were cultured in the presence or absence of PTX-B (2.5 nM) for 24, 48,

or 72 h. Cells were harvested and washed twice with PBS. Luciferase activity was measured using the Luciferase (for firefly luciferase-encoding plasmids) and Dual-Luciferase (for pRL-CMV plasmid) assay systems (Promega) as suggested by the manufacturer.

Cell fractionation and extraction of nuclear proteins

Cytoplasmic and nuclear fractions were prepared from U-937 cells preincubated with or without PTX-B (2.5 nM) and TNF-α (0.1 ng/ml). Cells were washed twice with cold phosphate-buffered saline and lysed in low-salt buffer A (20 mM HEPES, pH 7.9, 5 mM KCl, 0.5 mM MgCl$_2$, 0.5 mM DTT, and Roche protease inhibitor cocktail), supplemented with 0.5% Triton X-100. Nuclei were precipitated at 1000 g and washed with cold buffer A; cytoplasmic fractions were separated and cleared at 20,000 g for 15 min. Nuclear proteins were extracted from nuclear precipitates by incubation in high-salt buffer B (20 mM HEPES, pH 7.9, 500 mM KCl, 0.5 mM MgCl$_2$, 0.5 mM DTT, and Roche protease inhibitor cocktail) for 3 h at 4°C. Protein concentrations were determined by Lowry method using DC Protein Assay kit (Bio-Rad, Hercules, CA).

Western blot analysis

The cytoplasmic lysates and nuclear extracts were separated by SDS–PAGE, transferred to PVDF membranes, and analyzed using above-listed antibodies. Specific bands were visualized by ECL (Amersham, Buckinghamshire, England).

Analysis of NF-κB phosphorylation *in vivo*

Cells were preincubated with 2.5 nM PTX-B in complete RPMI 1640 media for 24 h. Following double washing in PBS, cells were incubated in phosphate-free RPMI 1640 for 24 h and then incubated with 1 mCi/ml of [^{32}P]orthophosphate (Amersham Pharmacia Biotech, Piscataway, NJ) for 2 h. After stimulation with TNF-α (0.1 ng/ml) for various time intervals, cells were harvested, washed in PBS, and lysed in RIPA buffer (50 mM Tris–HCl, pH 8.0; 100 mM NaCl; 1 mM MgCl$_2$; 1% Triton X-100). The NF-κB subunits were immunoprecipitated with anti-p65 and anti-p50 antibodies, separated on 10–20% SDS–PAG and autoradiographed.

Electrophoretic mobility shift assay (EMSA) and supershift assay

Assays were performed according to previously published protocol (Alfano *et al.,* 2001). In brief, the oligonucleotide corresponding to HIV-1 LTR NF-κB binding sites, 5′-GCTACAAGGGACTTTCCGCTGGGGACTTTCCAGG-3′, was annealed to its complementary strand and labeled with [γ^{32}P]ATP (Amersham Pharmacia Biotech) to 50,000 cpm/ng using polynucleotide kinase (MBI Fermentas,

Amherst, NY). Equal amounts (10 μg) of nuclear extracts of the PTX-B treated/untreated U-937 cells in buffer B (see above) were added to 10 μl of a binding reaction buffer (20 mM HEPES, pH 7.9, 25% glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and Roche protease inhibitor cocktail) supplemented with 1 μl (5 mg/ml) of poly(dI:dC) (Roche) and 1 μl (10 mg/ml) of BSA (Sigma). For super-shift analysis, the aliquots of the same nuclear extracts were incubated with 1 μl (2 mg/ml) of anti-p65 NF-κB (F-6)X or anti-p50 NF-κB (H-119)X antibodies (Santa Cruz Biotechnology) for 20 min at room temperature. One microliter (0.5 ng) of [γ^{32}P]ATP-labeled double-strand oligonucleotide probe was added to all samples, and mixtures were incubated for 45 min at room temperature and then run on a nondenaturing 5% PAG in 1 $\times$ TBE. Gels were dried, and radioactive bands were analyzed by autoradiography.

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REFERENCES

Alfano, M., Pushkarsky, T., Poli, G., and Bukrinsky, M. (2000). The B-oligomer of pertussis toxin inhibits human immunodeficiency virus type 1 replication at multiple stages. *J. Virol.* **74,** 8767–8770.

Alfano, M., Schmidtmayerova, H., Amella, C. A., Pushkarsky, T., and Bukrinsky, M. (1999). The B-oligomer of pertussis toxin deactivates CC chemokine receptor 5 and blocks entry of M-tropic HIV-1 strains. *J. Exp. Med.* **190,** 597–605.

Alfano, M., Vallanti, G., Biswas, P., Bovolenta, C., Vicenzi, E., Mantelli, B., Pushkarsky, T., Rappuoli, R., Lazzarin, A., Bukrinsky, M., and Poli, G. (2001). The binding subunit of pertussis toxin inhibits HIV replication in human macrophages and virus expression in chronically infected promonocytic U1 cells. *J. Immunol.* **166,** 1863–1870.

Antoni, B. A., Rabson, A. B., Kinter, A., Bodkin, M., and Poli, G. (1994). NF-kappa B-dependent and -independent pathways of HIV activation in a chronically infected T cell line. *Virology* **202,** 684–694.

Baeuerle, P. A., and Baltimore, D. (1996). NF-kappa B: Ten years after. *Cell* **87,** 13–20.

Cohen, S., Orian, A., and Ciechanover, A. (2001). Processing of p105 is inhibited by docking of p50 active subunits to the ankyrin repeat domain, and inhibition is alleviated by signaling via the carboxyl-terminal phosphorylation/ubiquitin-ligase binding domain. *J. Biol. Chem.* **276,** 26769–26776.

DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M.

(1997). A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature* **388,** 548–554.

Folks, T. M., Justement, J., Kinter, A., Dinarello, C. A., and Fauci, A. S. (1987). Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. *Science* **238,** 800–802.

Ganchi, P. A., Sun, S. C., Greene, W. C., and Ballard, D. W. (1992). I kappa B/MAD-3 masks the nuclear localization signal of NF-kappa B p65 and requires the transactivation domain to inhibit NF-kappa B p65 DNA binding. *Mol. Biol. Cell* **3,** 1339–1352.

Ghosh, S., May, M. J., and Kopp, E. B. (1998). NF-kappa B and Rel proteins: Evolutionarily conserved mediators of immune responses. *Annu. Rev. Immunol.* **16,** 225–260.

Gray, L. S., Huber, K. S., Gray, M. C., Hewlett, E. L., and Engelhard, V. H. (1989). Pertussis toxin effects on T lymphocytes are mediated through CD3 and not by pertussis toxin catalyzed modification of a G protein. *J. Immunol.* **142,** 1631–1638.

Griffin, G. E., Leung, K., Folks, T. M., Kunkel, S., and Nabel, G. J. (1989). Activation of HIV gene expression during monocyte differentiation by induction of NF-kappa B. *Nature* **339,** 70–73.

Haskill, S., Beg, A. A., Tompkins, S. M., Morris, J. S., Yurochko, A. D., Sampson-Johannes, A., Mondal, K., Ralph, P., and Baldwin, A. S., Jr. (1991). Characterization of an immediate-early gene induced in adherent monocytes that encodes I kappa B-like activity. *Cell* **65,** 1281–1289.

Heissmeyer, V., Krappmann, D., Wulczyn, F. G., and Scheidereit, C. (1999). NF-kappaB p105 is a target of IkappaB kinases and controls signal induction of Bcl-3-p50 complexes. *EMBO J.* **18,** 4766–4778.

Henkel, T., Zabel, U., van Zee, K., Muller, J. M., Fanning, E., and Baeuerle, P. A. (1992). Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-kappa B subunit. *Cell* **68,** 1121–1133.

Hu, Y., Baud, V., Delhase, M., Zhang, P., Deerinck, T., Ellisman, M., Johnson, R., and Karin, M. (1999). Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. *Science* **284,** 316–320.

Igarashi, T., Brown, C. R., Endo, Y., Buckler-White, A., Plishka, R., Bischofberger, N., Hirsch, V., and Martin, M. A. (2001). Macrophage are the principal reservoir and sustain high virus loads in rhesus macaques after the depletion of CD4+ T cells by a highly pathogenic simian immunodeficiency virus/HIV type 1 chimera (SHIV): Implications for HIV-1 infections of humans. *Proc. Natl. Acad. Sci. USA* **98,** 658–663.

Jans, D. A. (1995). The regulation of protein transport to the nucleus by phosphorylation. *Biochem. J.* **311**(Pt. 3), 705–716.

Jans, D. A., and Hubner, S. (1996). Regulation of protein transport to the nucleus: Central role of phosphorylation. *Physiol. Rev.* **76,** 651–685.

Karin, M., and Ben Neriah, Y. (2000). Phosphorylation meets ubiquitination: The control of NF-κB activity. *Annu. Rev. Immunol.* **18,** 621–663.

Katada, T., Tamura, M., and Ui, M. (1983). The A promoter of islet-activating protein, pertussis toxin, as an active peptide catalyzing ADP-ribosylation of a membrane protein. *Arch. Biochem. Biophys.* **224,** 290–298.

Kolb, J. P., Genot, E., Petit-Koskas, E., Paul-Eugene, N., and Dugas, B. (1990). Effect of bacterial toxins on human B cell activation. I. Mitogenic activity of pertussis toxin. *Eur. J. Immunol.* **20,** 969–976.

Koul, D., Yao, Y., Abbruzzese, J. L., Yung, W. K., and Reddy, S. A. (2001). Tumor suppressor MMAC/PTEN inhibits cytokine-induced NFkappaB activation without interfering with the IkappaB degradation pathway. *J. Biol. Chem.* **276,** 11402–11408.

Latif, R., Kerlero, D. R., Amarant, T., Rappuoli, R., Sappler, G., and Ben Nun, A. (2001). Reversal of the CD4(+)/CD8(+) T-cell ratio in lymph node cells upon in vitro mitogenic stimulation by highly purified, water-soluble S3–S4 dimer of pertussis toxin. *Infect. Immun.* **69,** 3073–3081.

Li, H., and Wong, W. S. (2000). Mechanisms of pertussis toxin-induced

myelomonocytic cell adhesion: Role of CD14 and urokinase receptor. *Immunology* **100**, 502–509.

Li, H., and Wong, W. S. (2001). Pertussis toxin activates tyrosine kinase signaling cascade in myelomonocytic cells: A mechanism for cell adhesion. *Biochem. Biophys. Res. Commun.* **283**, 1077–1082.

Li, Q., Van Antwerp, D., Mercurio, F., Lee, K. F., and Verma, I. M. (1999). Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. *Science* **284**, 321–325.

Liou, H. C., and Baltimore, D. (1993). Regulation of the NF-kappa B/rel transcription factor and I kappa B inhibitor system. *Curr. Opin. Cell Biol.* **5**, 477–487.

Manna, S. K., Mukhopadhyay, A., and Aggarwal, B. B. (2000). Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: Potential role of reactive oxygen intermediates and lipid peroxidation. *J. Immunol.* **164**, 6509–6519.

Mercurio, F., Murray, B. W., Shevchenko, A., Bennett, B. L., Young, D. B., Li, J. W., Pascual, G., Motiwala, A., Zhu, H., Mann, M., and Manning, A. M. (1999). IkappaB kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. *Mol. Cell Biol.* **19**, 1526–1538.

Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997). IKK-1 and IKK-2: Cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science* **278**, 860–866.

Moses, A. V., Ibanez, C., Gaynor, R., Ghazal, P., and Nelson, J. A. (1994). Differential role of long terminal repeat control elements for the regulation of basal and Tat-mediated transcription of the human immunodeficiency virus in stimulated and unstimulated primary human macrophages. *J. Virol.* **68**, 298–307.

Nabel, G., and Baltimore, D. (1987). An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* **326**, 711–713.

O'Neill, L. A., Ikebe, T., Sarsfield, S. J., and Saklatvala, J. (1992). The binding subunit of pertussis toxin inhibits IL-1 induction of IL-2 and prostaglandin production. *J. Immunol.* **148**, 474–479.

Orenstein, J. M., Fox, C., and Wahl, S. M. (1997). Macrophages as a source of HIV during opportunistic infections. *Science* **276**, 1857–1861.

Orian, A., Gonen, H., Bercovich, B., Fajerman, I., Eytan, E., Israel, A., Mercurio, F., Iwai, K., Schwartz, A. L., and Ciechanover, A. (2000). SCF(beta)(-TrCP) ubiquitin ligase-mediated processing of NF-kappaB p105 requires phosphorylation of its C-terminus by IkappaB kinase. *EMBO J.* **19**, 2580–2591.

Osborn, L., Kunkel, S., and Nabel, G. J. (1989). Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc. Natl. Acad. Sci. USA* **86**, 2336–2340.

Pazin, M. J., Sheridan, P. L., Cannon, K., Cao, Z., Keck, J. G., Kadonaga, J. T., and Jones, K. A. (1996). NF-kappa B-mediated chromatin reconfiguration and transcriptional activation of the HIV-1 enhancer in vitro. *Genes Dev.* **10**, 37–49.

Phelps, C. B., Sengchanthalangsy, L. L., Huxford, T., and Ghosh, G. (2000a). Mechanism of I kappa B alpha binding to NF-kappa B dimers. *J. Biol. Chem.* **275**, 29840–29846.

Phelps, C. B., Sengchanthalangsy, L. L., Malek, S., and Ghosh, G. (2000b). Mechanism of kappa B DNA binding by Rel/NF-kappa B dimers. *J. Biol. Chem.* **275**, 24392–24399.

Roff, M., Thompson, J., Rodriguez, M. S., Jacque, J. M., Baleux, F., Arenzana-Seisdedos, F., and Hay, R. T. (1996). Role of IkappaBalpha ubiquitination in signal-induced activation of NFkappaB in vivo. *J. Biol. Chem.* **271**, 7844–7850.

Rogers, T. S., Corey, S. J., and Rosoff, P. M. (1990). Identification of a 43-kilodalton human T lymphocyte membrane protein as a receptor for pertussis toxin. *J. Immunol.* **145**, 678–683.

Rosoff, P. M., Walker, R., and Winberry, L. (1987). Pertussis toxin triggers

rapid second messenger production in human T lymphocytes. *J. Immunol.* **139**, 2419–2423.

Ryan, M. S., Griffin, F., Mahon, B., and Mills, K. H. (1997). The role of the S-1 and B-oligomer components of pertussis toxin in its adjuvant properties for Th1 and Th2 cells. *Biochem. Soc. Trans.* **25**, 126S.

Sakurai, H., Chiba, H., Miyoshi, H., Sugita, T., and Toriumi, W. (1999). IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. *J. Biol. Chem.* **274**, 30353–30356.

Scherer, D. C., Brockman, J. A., Chen, Z., Maniatis, T., and Ballard, D. W. (1995). Signal-induced degradation of I kappa B alpha requires site-specific ubiquitination. *Proc. Natl. Acad. Sci. USA* **92**, 11259–11263.

Schmitz, M. L., and Baeuerle, P. A. (1991). The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. *EMBO J.* **10**, 3805–3817.

Schwabe, R. F., Schnabl, B., Kweon, Y. O., and Brenner, D. A. (2001). CD40 activates NF-kappa B and c-Jun N-terminal kinase and enhances chemokine secretion on activated human hepatic stellate cells. *J. Immunol.* **166**, 6812–6819.

Siebenlist, U., Franzoso, G., and Brown, K. (1994). Structure, regulation and function of NF-kappa B. *Annu. Rev. Cell Biol.* **10**, 405–455.

Simeonidis, S., Liang, S., Chen, G., and Thanos, D. (1997). Cloning and functional characterization of mouse IkappaBepsilon. *Proc. Natl. Acad. Sci. USA* **94**, 14372–14377.

Sizemore, N., Lerner, N., Dombrowski, N., Sakurai, H., and Stark, G. R. (2002). Distinct roles of the Ikappa B kinase alpha and beta subunits in liberating nuclear factor kappa B (NF-kappa B) from Ikappa B and in phosphorylating the p65 subunit of NF-kappa B. *J. Biol. Chem.* **277**, 3863–3869.

Sizemore, N., Leung, S., and Stark, G. R. (1999). Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. *Mol. Cell Biol.* **19**, 4798–4805.

Stewart, S. J., Prpic, V., Johns, J. A., Powers, F. S., Graber, S. E., Forbes, J. T., and Exton, J. H. (1989). Bacterial toxins affect early events of T lymphocyte activation. *J. Clin. Invest.* **83**, 234–242.

Takeda, K., Takeuchi, O., Tsujimura, T., Itami, S., Adachi, O., Kawai, T., Sanjo, H., Yoshikawa, K., Terada, N., and Akira, S. (1999). Limb and skin abnormalities in mice lacking IKKalpha. *Science* **284**, 313–316.

Thom, R. E., and Casnellie, J. E. (1989). Pertussis toxin activates protein kinase C and a tyrosine protein kinase in the human T cell line Jurkat. *FEBS Lett.* **244**, 181–184.

Thompson, J. E., Phillips, R. J., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1995). I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B. *Cell* **80**, 573–582.

Van Lint, C., Amella, C. A., Emiliani, S., John, M., Jie, T., and Verdin, E. (1997). Transcription factor binding sites downstream of the human immunodeficiency virus type 1 transcription start site are important for virus infectivity. *J. Virol.* **71**, 6113–6127.

Van Lint, C., Ghysdael, J., Paras, P., Jr., Burny, A., and Verdin, E. (1994). A transcriptional regulatory element is associated with a nuclease-hypersensitive site in the pol gene of human immunodeficiency virus type 1. *J. Virol.* **68**, 2632–2648.

Vanden Berghe, W., Plaisance, S., Boone, E., De Bosscher, K., Schmitz, M. L., Fiers, W., and Haegeman, G. (1998). p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways are required for nuclear factor-kappaB p65 transactivation mediated by tumor necrosis factor. *J. Biol. Chem.* **273**, 3285–3290.

Vicenzi, E., Biswas, P., Mengozzi, M., and Poli, G. (1997). Role of pro-inflammatory cytokines and beta-chemokines in controlling HIV replication. *J. Leukoc. Biol.* **62**, 34–40.

Wang, D., and Baldwin, A. S., Jr. (1998). Activation of nuclear factor-kappaB-dependent transcription by tumor necrosis factor-alpha is mediated through phosphorylation of RelA/p65 on serine 529. *J. Biol. Chem.* **273**, 29411–29416.

Wang, J. M., and Oppenheim, J. J. (1999). Interference with the signaling capacity of CC chemokine receptor 5 can compromise its role as an

HIV-1 entry coreceptor in primary T lymphocytes. *J. Exp. Med.* **190,** 591–595.

West, M. J., Lowe, A. D., and Karn, J. (2001). Activation of human immunodeficiency virus transcription in T cells revisited: NF-kappaB p65 stimulates transcriptional elongation. *J. Virol.* **75,** 8524–8537.

Wong, W. S., and Luk, J. M. (1997). Signaling mechanisms of pertussis toxin-induced myelomonocytic cell adhesion: Role of tyrosine phosphorylation. *Biochem. Biophys. Res. Commun.* **236,** 479–482.

Wong, W. S., and Rosoff, P. M. (1996). Pharmacology of pertussis toxin B-oligomer. *Can. J. Physiol. Pharmacol.* **74,** 559–564.

Wong, W. S., Simon, D. I., Rosoff, P. M., Rao, N. K., and Chapman, H. A. (1996). Mechanisms of pertussis toxin-induced myelomonocytic cell adhesion: Role of Mac-1(CD11b/CD18) and urokinase receptor (CD87). *Immunology* **88,** 90–97.

Yaron, A., Gonen, H., Alkalay, I., Hatzubai, A., Jung, S., Beyth, S., Mer-curio, F., Manning, A. M., Ciechanover, A., and Ben Neriah, Y. (1997). Inhibition of NF-kappa-B cellular function via specific targeting of the I-kappa-B-ubiquitin ligase. *EMBO J.* **16,** 6486–6494.

Zandi, E., Rothwarf, D. M., Delhase, M., Hayakawa, M., and Karin, M. (1997). The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell* **91,** 243–252.

Zhong, H., Su Yang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997). The transcriptional activity of NF-kappaB is regulated by the IkappaB-associated PKAc subunit through a cyclic AMP-independent mechanism. *Cell* **89,** 413–424.

Zhong, H., Voll, R. E., and Ghosh, S. (1998). Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Mol. Cell* **1,** 661–671.