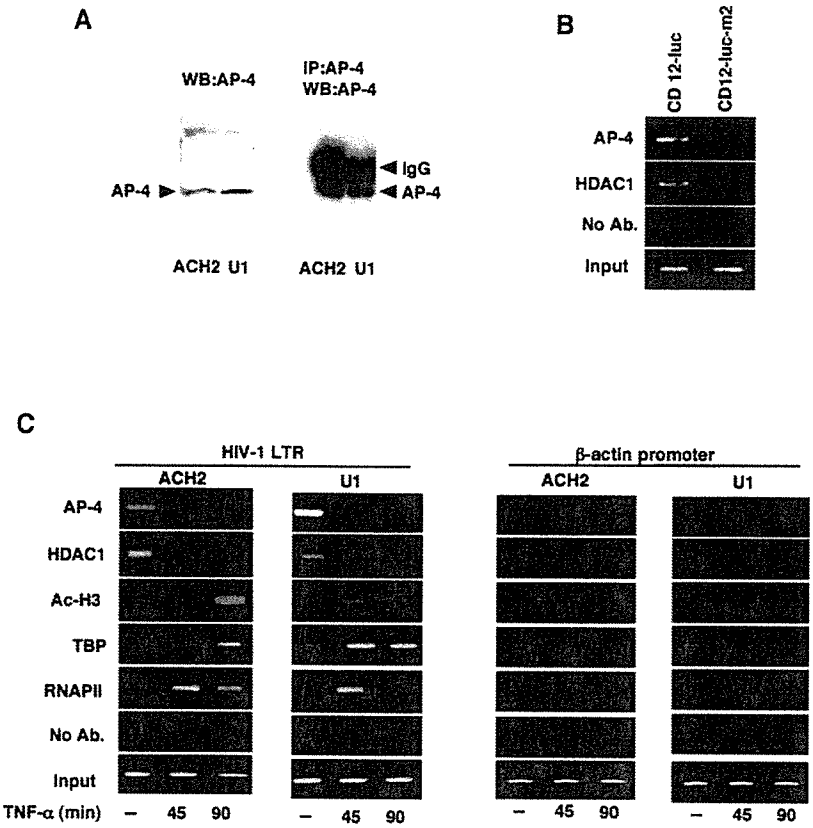

FIGURE 8. **ChIP assays detecting AP-4 and HDAC1 on HIV-1 LTR.** *A*, immunoreactivity of anti-AP-4 antibody. To assess the immunoreactivity of anti-AP-4 antibody for immunoblotting (*left panel*) and immunoprecipitation (*right panel*), ACH2 and U1 cell lysates were reacted with the rabbit anti-AP-4 antibody. *Arrowheads* indicate the locations endogenous AP-4. Position of IgG was also indicated (*right*). The AP-4 immune complex was separated on SDS-PAGE followed by immunoblot with anti-AP-4 antibody. *B*, AP-4 recruits HDAC1 to the HIV-1 LTR. 293 cells were transfected with CD12-luc or CD12-luc-m2, incubated for 48 h, and ChIP assay was performed. Cross-linked chromatin fragments were prepared, and the association of AP-4 and HDAC1 was analyzed by ChIP assay using antibodies to AP-4 and HDAC1. The recovered DNA was amplified by PCR with promoter-specific primers (spanning from −109 to +79 of HIV-1 LTR) and analyzed on a 2% agarose gel. Input DNA represents total input chromatin (1%) while immunoprecipitation with no antibody (*No Ab.*) serves as negative control. *C*, dynamic association/dissociation of AP-4, HDAC1, acetylated histone H3, TBP, and RNAP II. ChIP assays were performed with latently infected cell lines. ACH2 and U1 cells were either untreated or treated with TNF-α (3 ng/ml) for indicated times and subjected to ChIP assays. Cross-linked chromatin fragments were prepared, and the association of AP-4, TBP, RNAPII, HDAC1, Ac-H3, and HIV-1 LTR DNA (−109/+79) was analyzed by ChIP assay as described in *B*. The β-actin promoter DNA (−980/−915) was similarly analyzed as a control. The experiments were repeated performed with reproducible results, and the representative results are shown.

duction (Fig. 9*C*). These results indicate that AP-4 can inhibit HIV-1 production and that the DNA binding activity of AP-4 is crucial for its inhibitory action.

In Fig. 9*D*, we examined the effect of depleting endogenous AP-4 on HIV-1 production using siRNA for AP-4. AP-4 depletion resulted in significant increase in the basal HIV-1 production (3.2-fold as compared with control siRNA (GFP)). Similarly, TNF-α-stimulated HIV-1 production was elevated by the treatment with AP-4 siRNA (5.7-fold as compared with control siRNA). We also observed the elevation of viral protein synthesis in the transfected cells by AP-4 depletion (Fig. 9*D*, *right panel*).

Finally, we examined the effect of AP-4 on HIV-1 replication in Jurkat CD4+T cells. To examine the effect of AP-4 binding site within HIV-1 LTR, we created a mutant pNL4-3 lacking the AP-4 binding and quantified the amounts of HIV-1 virions in the culture supernatant of Jurkat cells transfected with either the wild-type or the mutant pNL4-3. As shown in Fig. 9*E*, the amounts of HIV-1 production were not significantly changed over time between the wild-type and the mutant HIV-1 clones. In Fig. 9*F*, the repressive effect of AP-4 on HIV-1 production was examined with or without TNF-α stimulation. When the mutant pNL4-3, containing mutation in the AP-4 binding, was cotransfected with AP-4, the inhibitory effect of AP-4 was abolished irrespective of the TNF-α stimulation (Fig. 9*F*, *right panel*).

DISCUSSION

The extent of HIV-1 replication is controlled at the step of transcription and the level of transcription is governed by the coordinated actions of viral and cellular transcription factors acting on LTR both in positive and negative fashions (1, 2, 9). In contrast to the abundant literatures reporting actions of positive transcription factors, mostly NF-κB (1, 2), little is known about the action of negative transcription regulators. Here, we demonstrate evidences demonstrating that AP-4 is a negative regulator of HIV-1 transcription and its production. Although a previous study reported that AP-4 blocks TBP binding to TATA box *in vitro* (10) and suggested that AP-4 negatively regulates HIV-1 transcription, no evidence has been presented supporting the repressive action of AP-4 on HIV-1 transcription and viral production. In this study, we were able to demonstrate that AP-4 acts as a negative transcription factor for HIV-1 gene expression by recruitment of HDAC1, as well as by preventing the TBP (TFIID) binding to the TATA box, and that AP-4 is actively involved in the transcriptional silencing of HIV-1 gene expression in latently infected cell lines.

The AP-4 binding site in the HIV-1 LTR is located immediately downstream of TATA box (2, 10, 13). Among various HIV/SIV isolates AP-4 sites are conserved among HIV-1 subtypes A, B, C, D, and CRF02-AG, HIV-2, and SIVcpz-gab. However, although most (92%) of the clinical HIV-1 isolates had functional AP-4 binding site (11), no clear cor-

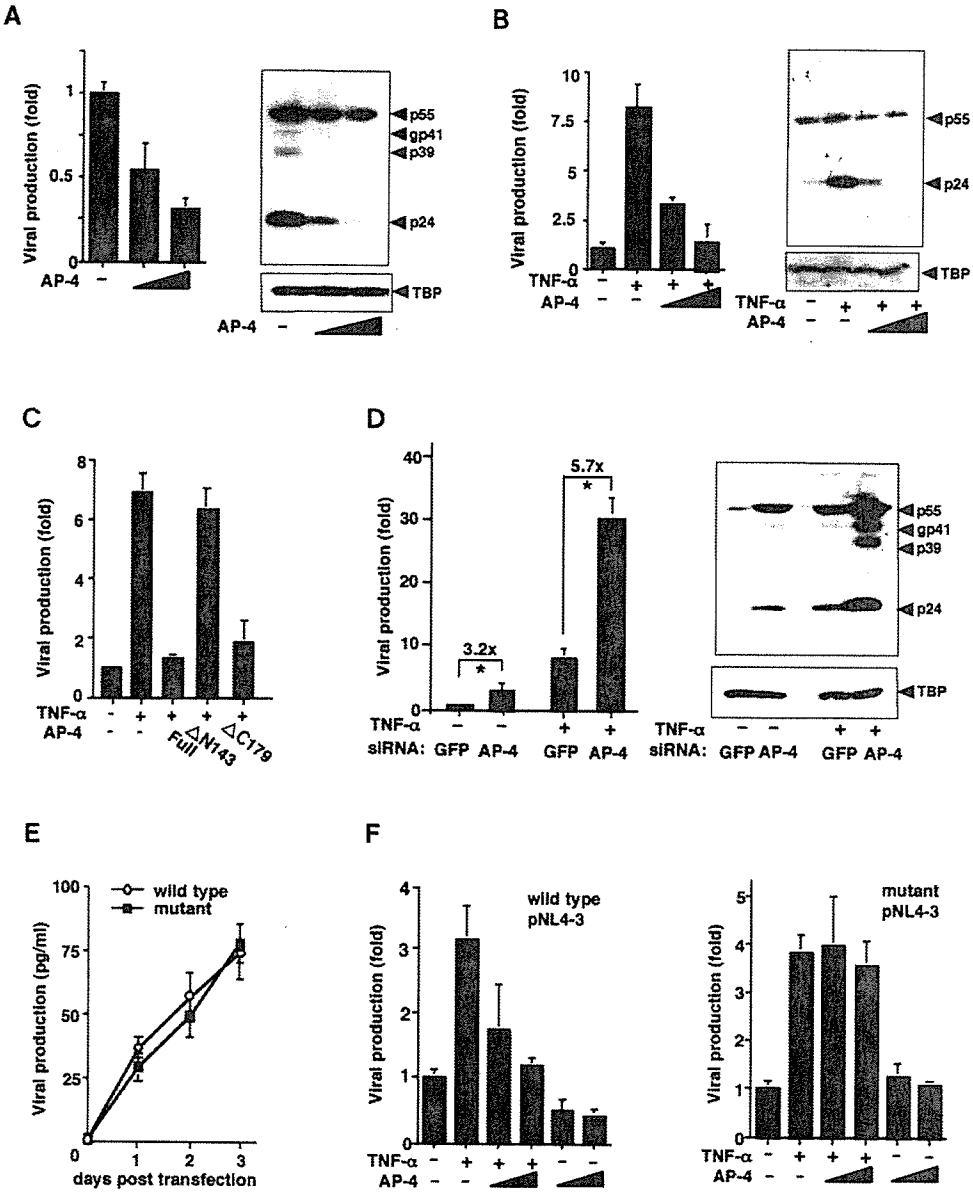

FIGURE 9. **Repression of HIV-1 production by AP-4.** *A* and *B*, HIV-1 production and AP-4-mediated repression in transfected cells. 293 cells were transfected with pNL4-3 and pFLAG-AP-4. After 36 h of transfection, cells were either untreated (*A*) or treated (*B*) with TNF-α (3 ng/ml) and incubated for additional 24 h. The culture supernatants and cell lysates were collected and subjected to the determination of p24 antigen level by ELISA and detection of virus proteins by immunoblot with AIDS patient serum, respectively. *C*, effects of AP-4 mutants on HIV-1 production. 293 cells were transfected with pNL4-3 together with expression vectors for wild-type or mutant AP-4. After 24 h of transfection, cells were untreated or treated with TNF-α (3 ng/ml) and incubated for additional 24 h. The p24 antigen level in the culture supernatant was determined as in *A*. *D*, effects of AP-4 knockdown on HIV-1 production. 293 cells were transfected with pNL4-3, and AP-4 siRNA-1 (Fig. 4) or control siRNA (GFP). After 36 h of transfection, cells were untreated or treated with 3 ng/ml of TNF-α and incubated for an additional 24 h. The culture supernatants and cell lysates were collected and analyzed the p24 antigen level and viral protein expression, respectively, as in *B*. *, $p < 0.01$. *E*, HIV-1 production of the pNL4-3 mutant lacking the AP-4 sites within both LTR. Either the wild-type or the mutant pNL4-3 was transfected into Jurkat CD4+T cells, and the amounts of HIV-1 virion production were quantified. The culture supernatants were collected after 1, 2, and 3 days post-transfection, and the p24 antigen levels were measured as in *A*. *F*, repression of HIV-1 production by AP-4 in Jurkat CD4+T cells. Jurkat cells were transfected either with the wild-type (*left panel*) or the mutant (*right panel*) pNL4-3 lacking the AP-4 sites in both 5′- and 3′-LTR, together with various amounts of pFLAG-AP-4 (0.2 and 0.8 μg per transfection). After 36 h of transfection, cells were either untreated or treated with TNF-α (3 ng/ml) and incubated for an additional 24 h. The culture supernatants were prepared, and the p24 antigen levels were measured. Experiments were repeated at least three times, and reproducible results were obtained. The representative data are shown.

relation with the clinical stage was observed. Thus, the biological significance of the presence of AP-4 site in HIV-1 LTR in determining the virulence awaits further *in vivo* investigations.

There are a number of transcription factors acting as repressors. However, the mechanisms of their actions are not uniform and multiple modes of action are reported including: 1) inhibition of transcriptional activators upon its DNA binding or interaction with co-activators (36–38), 2) prevention of the binding of general transcription factors such as TBP (TFIID) and TFIIB to the promoter (39–43), 3) direct repression of promoter activity by recruiting co-repressors (8, 28, 30), 4) alteration of chromatin structure (19, 40), and 5) inhibition of transcriptional elongation (44, 45). AP-4 appears to exert transcriptional repression of HIV-1 promoter through bimodal mechanisms: 1) masking the HIV-1 TATA element from TBP binding and 2) recruiting HDAC1. In this context, it is noted that HIV-1 does not appear to use the TATA box but instead use the CATA box motif located two nucleotides upstream of the conventional TATA box (46). It is possible that AP-4 may preclude the usage of TATA box by physical masking. However, since we observed that AP-4 blocked the TBP binding to CATATA box in EMSA (Fig. 1*D*), AP-4 may also block the CATA box.

Like other members of bHLH family to which AP-4 belongs, the AP-4 HLH motif and the adjacent basic domain are necessary to confer site-specific DNA binding (20). Unlike other HLH proteins, AP-4 also contains two additional protein dimerization motifs consisting of leucine repeat elements LR1 and LR2, through which AP-4 forms a homodimer (20). We found that both bHLH motif and two LR elements were necessary to exert transcriptional repression, presumably by masking the TATA box. However, unlike HIV-1 LTR, most other promoters where AP-4 has negative role have AP-4 sites at distant locations from the TATA box and even some promoters are TATA-less (22–24) and the mechanism by which AP-4 represses transcription other than masking the TATA box has not been elucidated. In addition, we found that AP-4 could exert transcriptional repression of HIV-1 even when the AP-4 site was located distant from the TATA box. Subsequent experiments have revealed that AP-4 could recruit HDAC1 to the promoter and that the AP-4-mediated repression could be restored by the treatment with a histone deacetylase inhibitor TSA. These bimodal actions of AP-4 make this factor a strong negative regulator for HIV-1 transcription. However, further studies are needed such as to determine where AP-4 binds to HDAC and to clarify whether AP-4 binds other transcriptional reg-

ulators. Interestingly, AP-4 was reported to be a transcriptional activator of transforming growth factor β (47), immunoglobulin κ chain (48), and SV40 (late promoter) (21) where AP-4 binding sites were found in the enhancer elements of these genes although no direct evidence is thus far available to show that AP-4 actually act as a transcriptional activator for these genes. Mermod *et al.* (21) showed that AP-4 acts in concert with AP-1, binding to the adjacent site of AP-4, in stimulating SV40 transcription *in vitro*. Thus, it is possible that the effect of AP-4 binding on transcription may be modified by other transcription factors recruited to the promoter and depend on the promoter context through combinatorial interaction with other transcription factors.

Our finding that AP-4 is constitutively present on the silent HIV-1 promoter in latently infected cells may have significant biological implications. We also found that TNF-α stimulation abrogated the AP-4-mediated repression of HIV-1 promoter. It is conceivable that nuclear translocation of NF-κB and its binding to the HIV-1 promoter might induce local chromatin remodeling, thus eliminating AP-4 and its repressor complex. It appears that the interplay among various transcription factors on the HIV-1 promoter determines the transcriptional competence of the latent HIV-1 provirus.

The ability of HIV-1 to establish a latent infection is considered crucial for the pathogenesis of AIDS (49, 50). Whereas HIV-1 entry into activated $CD4^+$ lymphocytes leads to a productive infection, the virus remains latent in resting $CD4^+$ lymphocytes (51). For many HIV-infected patients, although current anti-HIV treatment can reduce viral loads to undetectable levels, infected cells persist in a long term and harbor integrated proviruses capable of reseeding virus production after cessation of therapy. Our observation of AP-4 in the negative regulation of HIV-1 gene expression could give us a clue to understand how the latency is maintained at least in cells. Moreover, it is proposed that breakdown of viral latency during the early clinical stage where potent anti-viral cytotoxic T lymphocyte is still present is considered to benefit the outcome of HIV-1 infection by eliminating the otherwise long lasting chronically and latently infected cells (52, 53). Interestingly, Lehrman *et al.* (54) have recently reported a clinical study that combination therapy of conventional anti-HIV therapy with an HDAC inhibitor, valproic acid, could successfully accelerate the clearance of HIV-1 from resting CD4+T cells. It is plausible that HDAC inhibitors have clinical benefit in preventing the clinical development of AIDS. Further studies are needed to clarify the role of AP-4 and other repressor proteins in the maintenance of HIV-1 latency *in vivo* and to determine the clinical benefit of HDAC inhibitors.

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ORIGINAL ARTICLE

Induction of cell death in adult T-cell leukemia cells by a novel IκB kinase inhibitor

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NF-κB is constitutively activated in adult T-cell leukemia (ATL) and is considered responsible for cell growth and prevention of cell death. In this study, we demonstrate that NF-κB is constitutively activated in various HTLV-1-infected T-cell lines and ATL-derived cell lines irrespectively of Tax expression as evidenced by the phosphorylation of IκBα and p65 subunit of NF-κB, activation of NF-κB DNA binding, and upregulation of various target genes including *bcl-x_L*, *bcl-2*, *XIAP*, *c-IAP1*, *survivin*, *cyclinD1*, *ICAM-1* and *VCAM-1*. The effects of a novel IκB kinase (IKK) inhibitor, 2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-yl nicotinonitrile (ACHP), were examined on cell growth of these cell lines and fresh ATL leukemic cells. We found that ACHP could inhibit the phosphorylation of IκBα and p65, as well as NF-κB DNA-binding, associated with downregulation of the NF-κB target genes and induce cell growth arrest and apoptosis in these cells. When Tax-active and Tax-inactive cell lines were compared, ACHP could preferentially inhibit cell growth of Tax-active cells. Moreover, ACHP exhibited strong apoptosis-inducing activity in fresh ATL cells. These findings indicate that ACHP and its derivatives are effective in inducing ATL cell death and thus feasible candidates for the treatment of ATL.
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Keywords: ATL; NF-κB; IKK; chemotherapy; apoptosis

Introduction

Adult T-cell leukemia (ATL) is an aggressive lymphoproliferative disorder closely associated with the human T-cell leukemia virus type 1 (HTLV-1).[1,2] ATL arises after a long latent period of over 50 years and is considered to involve multi-step mechanism of tumorigenesis.[3] ATL is characterized by diffuse lymphadenopathy, hepatosplenomegaly, and infiltration of malignant cells into skin and other organs.[4] Acute type of ATL is an aggressive form of T-cell leukemia with a median survival of only 6 months and a projected 4-year survival of about 5%. Conventional chemotherapies appeared to be ineffective in prolonging the life of patients with ATL, mostly due to the frequent acquisition of drug resistance and adverse effects. Although novel clinical trials using interferon and arsenic trioxide or zidovudine exhibited better therapeutic responses, their efficacies were limited and only small percentages of patients achieved long-lasting remission.[5] Therefore, novel treatment modalities are desperately needed.

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Recent studies have elucidated crucial roles of nuclear factor-κB (NF-κB) in tumor development and progression.[6,7] NF-κB is a transcription factor that regulates expression of various genes involved in cell cycle regulation and inhibition of apoptosis, such as *cyclinD1*, *bcl-2*, *bcl-x_L*, *XIAP*, *c-IAPs* and *survivin* as well as genes involved in inflammatory and immune responses.[8] In addition, intracellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1), which are not only important for cell adhesion but also for T-cell proliferation, are also under the control of NF-κB.[9] NF-κB is a hetero- or homodimer consisting of Rel family proteins, p65 (RelA), RelB, c-Rel, p105/p50 and p100/p52.[10] The p65/p50 heterodimer, a major form of NF-κB, is normally present in the cytoplasm in association with its inhibitor, IκBα. Stimulation by the inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin-1β results in the activation of IκB kinase (IKK) complex through mitogen-activated protein kinase /extracellular signal-regulated kinase kinase 1,3 or NF-κB inducing kinase (NIK). IKKβ serves as catalytic subunit that phosphorylates IκBα on two serine residues (Ser32/S36) (called 'canonical NF-κB activation pathway').[11] On the other hand, IKKα is activated through NIK by alternative stimuli such as lymphotoxin β (LTβ), B-cell activating factor and CD40 ligand, and mediates the processing of p100 to p52 ('noncanonical NF-κB activation pathway'). In addition, we have previously reported that phosphorylation of p65 at Ser 536 is crucial for NF-κB transcriptional activity and is mediated by IKKα upon LTβ signaling.[12] Importantly, such non-canonical NF-κB activation cascade mediated by NIK-IKKα does not necessarily associate with phosphorylation of IκBα followed by its degradation. Thus, inhibitors for IκBα degradation may not be sufficient in inhibiting the NF-κB activity.

In ATL cells and HTLV-1-infected T-cells, a number of reports demonstrated the constitutive activation of NF-κB and its involvement in tumorigenesis.[13–15] These carcinogenic actions of HTLV-1 have been ascribed to Tax, a transactivator protein encoded by the virus, which is responsible for the activation of NF-κB.[16–18] For instance, Suzuki *et al.*[16] reported that Tax directly binds to and activates NF-κB. Tax is also known to activate NF-κB by stimulating IKK complex by interacting with IKKs.[17] Furthermore, Xiao *et al.* demonstrated that Tax promotes the proteolytic processing of p100.[18] In fresh ATL cells, however, NF-κB was shown to be constitutively activated although Tax is not expressed.[14,15] As p52 expression is elevated in these cells, the involvement of the noncanonical NF-κB activation pathway is suggested in ATL cells. Thus, NF-κB plays a major role in ATL leukemogenesis and is considered to be a feasible target in the chemotherapy of ATL.

In this study, we confirmed the constitutive activation of NF-κB in HTLV-1-infected T-cell and ATL cell lines and examined the effect of a novel IKK inhibitor, 2-amino-6-(2-(cyclopropylmethoxy)-6-hydroxyphenyl)-4-piperidin-4-yl nicotinonitrile

(ACHP), on the growth and survival of HTLV-1-infected T-cell lines, ATL cell lines and the cells derived from ATL patients. ACHP exhibited a high selectivity for IKKβ and IKKα over other kinases, and have an unique specificity on IKK subtypes (the 50% of inhibitory concentration (IC_{50}) values for IKKβ and IKKα are 8.5 and 250 nM, respectively; IC_{50} for IKKγ, Syk and MKK4 are over 20 μM, measured by *in vitro* kinase assays).[19,20] Here, we demonstrate that ACHP could efficiently induce cell growth arrest and apoptotic cell death by blocking NF-κB in ATL cells.

Materials and methods

Cells and reagents

HTLV-1-infected T-cell lines, ATL-35T,[21] 81–66/45,[22] MJ,[23] and MT-2[23] cells, human ATL cell lines established from ATL patients, ATL-102,[24] ED-40515(−)[25] and TL-Om1[14] cells, and a HTLV-1-negative T-cell leukemia cell line Jurkat were used in this study. ED-40515 (−) and TL-Om1 cells were kind gifts from Drs M Maeda and M Matsuoka (Kyoto University, Japan). Fresh leukemia cells derived from acute-type ATL patients were obtained with informed consent from each patient at Nagoya City University Hospital and Imamura Bun-in Hospital after approval by the institutional ethical committee. All samples from ATL patients contained more than 80% leukemic cells. Peripheral blood mononuclear cells were derived from four healthy donors upon informed consent. All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 incubator. The novel IKK inhibitor, ACHP,[19,20] was a kind gift from Drs T Murata and K B Bacon of Bayer Yakuhin Ltd (Kyoto, Japan).

Immunoblot analysis and antibodies

Immunoblot analyses were performed as previously described.[20] Briefly, 1.0×10^6 cells were treated with or without ACHP. The cells were lysed in 50 μl of lysis buffer (20 mM HEPES-KOH (pH 7.9), 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF, 1 mM PMSF, 0.2% Triton X, protease inhibitor cocktail (Roche, Indianapolis, IN, USA)) and the supernatant was collected. Equal amounts of the proteins were electrophoresed on 10% SDS–PAGE and transferred onto a nitrocellulose membrane. The membranes were incubated with TBS-T (10 mM Tris-HCl (pH 8.0), 15 mM NaCl, 0.1% Tween) with 5% nonfat milk containing 1:1000 diluted primary antibodies against either phospho-IκBα (Ser32), phospho-p65 (Ser536), phospho-p65 (Ser276), phospho-Akt (Ser473), Akt, phospho-IKKα (Ser181), phospho-p38 MAPK (Thr180/Tyr182), phospho-ATF-2 (Thr71) and phospho-JNK (Thr183/Tyr185) (Cell Signaling Technology, Beverly, MA, USA), p65, p52/p100, IκB-α or α-tubulin (Santa Cruz, Santa Cruz, CA, USA). After incubation, the membranes were rinsed and further incubated with HRP-conjugated secondary antibodies (Amersham Biosciences, Buckinghamshire, UK) in TBS-T with 5% nonfat milk. Each protein was detected by SuperSignal (PIERCE, Rockford, IL, USA).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously.[20] Nuclear extracts were prepared from 1.0×10^6 cells treated with or without ACHP. EMSA was performed using the nuclear extracts and the oligonucleotide probe containing NF-κB sequence in the promoter of *HIV1-LTR*. The sequence was as follows: 5′-TTT CTA GGG ACT TTC CGC CTG GGG ACT TTC CAG-3′. The DNA probe was 5′end-labeled using T4 polynucleotide kinase and [γ-^{32}P] ATP (Amersham Biosciences). DNA binding reactions were performed at 30°C for 15 min with labeled DNA probe and 15–30 μg protein of nuclear extract in 20 μl binding buffer (22 mM HEPES-KOH (pH7.9), 80 mM KCl, 5% glyceol, 0.1% NP-40, 1 mM dithiothreitol, 2 μg poly dI-dC, 2 g tRNA and protease inhibitor). The samples were subjected to electrophoresis on 5% nondenaturing polyacrylamide gel with 0.5 × Tris-borate-EDTA buffer at 4°C, followed by autoradiography. For competition and supershift analyses, nuclear extracts were preincubated with unlabeled probe DNA and antibodies (4 μg) against p65, RelB, c-Rel, p52 or p50 (Santa Cruz), respectively, for 30 min at 30°C.

Reverse transcription-PCR

Reverse transcription–PCR (RT-PCR) was performed as described previously.[20] Total RNA was prepared from 1.0×10^6 cells. After incubation with DNase I (Invitrogen, Carlsbad, CA, USA), RNA was reverse transcribed using SuperScript System (Invitrogen). Each sample was subjected to PCR amplification, and the products were analyzed by 1% agarose gel electrophoresis. The oligonucleotide primers for *bcl-2*, *cyclinD1*, *XIAP*, *cIAP-1* and *β-actin*, were used as described previously.[20] The primers for *bcl-x_L* and *survivin* were as follows: *bcl-x_L* sense, 5′-CAGAGCAACCGGGAGCTGGT-3′, antisense, 5′-GAT CCA AGG CTC TAG GTG GTC-3′; *survivin* sense, 5′-GGC ATG GGT GCC CCG ACG TTG-3′, antisense, 5′-CAG AGG CCT CAA TCC ATG GCA-3′.

Growth inhibition assay

Approximately 1.5×10^4 cells were cultured in 96-well plate in triplicates at 37°C. Growth inhibitory effect of ACHP was determined using MTT assay as previously described.[20] Optical densities (OD) at 570 and 630 nm were measured with multiplate reader. Cell viability (%) was calculated as follows: (OD_{630}–OD_{570} of the samples/OD_{630}–OD_{570} of the control) × 100 (%).

Flowcytometric analysis

The flowcytometric analyses were performed using FACSCAN (BD Bioscience, San Jose, CA, USA) and CellQuest program as previously described.[20] For cell cycle analysis, cells were incubated with propiodium iodide (PI) (Sigma). For apoptosis analysis, cells were incubated with fluorescein isothiocynate (FITC)-conjugated annexinV (MBL, Nagoya, Japan). For ICAM-1 and VCAM-1 expression analysis, cells were incubated with phycoerythrin (PE)-conjugated mouse antibody against human ICAM-1 or VCAM-1, or isotype matched control IgG (BD Biosciences).

Results

Constitutive NF-κB activation in HTLV-1-infected T-cell lines and ATL cell lines

In order to analyze the NF-κB signaling in HTLV-1-infected T-cell lines and ATL cell lines, we examined the phosphorylation status of IκBα and p65. ATL-35T, 81–66/45, MJ, MT-2 and ATL-102 cells are known to express Tax ('Tax-active'), whereas ED-40515(−) and TL-Om1 cells are known not to express Tax ('Tax-inactive').[14,21–25] As shown in Figure 1a, the phosphorylation of IκBα at Ser32 and p65 at Ser536 were detected in all cell lines except Jurkat, a control T-cell line, in the absence of any stimuli. We also detected prominent band of p100/p52 in five

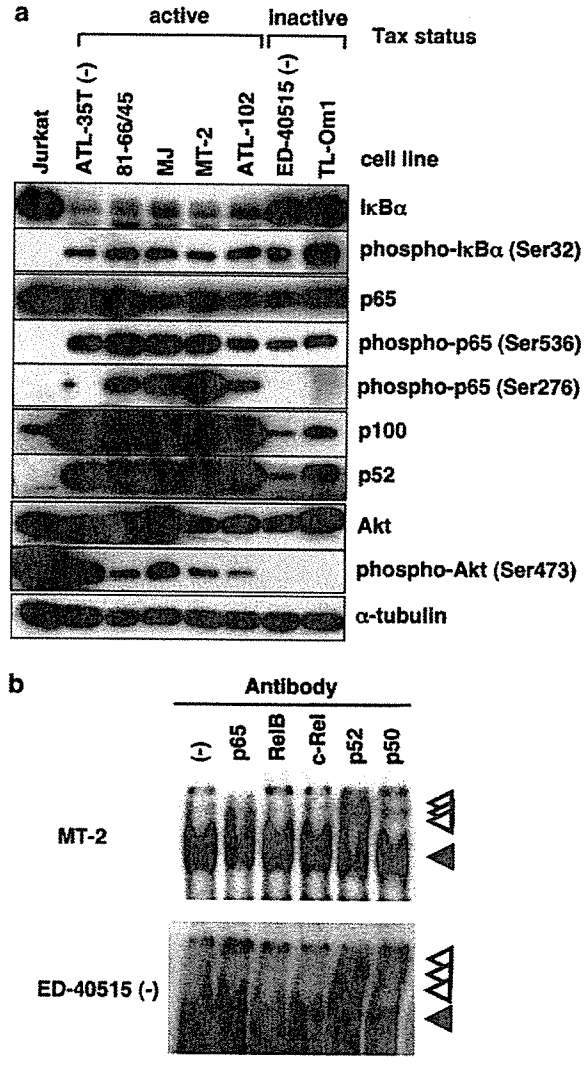

Figure 1 Constitutive NF-κB activation in HTLV-1-infected T-cell lines and ATL-derived cell lines. (**a**) Constitutive phosphorylation of IκBα, p65 and Akt. The whole cell-extracts obtained from the HTLV-1-infected T-cell lines (ATL-35T(−), 81–66/45, MJ, MT-2) and 3 ATL-derived cell lines (ATL-102, ED-40515(−), TL-Om1). Immunoblotting analyses were performed with antibodies against IκBα, phospho-IκBα, p65, phospho-p65, phospho-p65, p100/p52, Akt, phospho-Akt and α-tubulin (internal control). (**b**) Constitutive activation of NF-κB. The nuclear extracts were prepared from MT-2 and ED-40515(−) cell lines and EMSA was performed with specific DNA probe containing the NF-κB sequence. The results of supershift analysis using antibodies against each NF-κB subunits are shown. Closed and open arrowheads indicate positions of the specific DNA-NF-κB complex and the supershifted complex, respectively.

Tax-active cell lines, but not in Tax-inactive and control cell lines. The phosphorylation of p65 at Ser 276, which is not mediated by IKK,[26] was not correlated with IκBα phosphorylation. Constitutive phosphorylation of Akt at Ser473 was observed in five Tax-active cell lines and Jurkat.

We then performed EMSA using specific probes containing NF-κB binding sequence in the HIV-1 LTR promoter. The results revealed that NF-κB was constitutively activated in these HTLV-1-infected T-cell lines and ATL-derived cell lines, but not in Jurkat cells (Figures 1b and 2d). Representative results of MT-2 (Tax-active) and ED-40515 (−) (Tax-inactive) cells are shown in Figure 1b. Figure 1b also shows the result of supershift analysis using competitive antibody against each NF-κB subunit. In both cells, constitutive DNA binding of NF-κB was detected without any stimuli, and supershift bands were observed by addition of antibodies against p65, p52 or p50, indicating that activated NF-κB consisted of p65, p52 and p50 but not RelB or c-Rel.

Inhibitory effects of ACHP on NF-κB activation

We then examined the inhibitory effect of ACHP, a specific inhibitor for IKKβ and IKKα, on the phosphorylation of IκBα and p65 in these cell lines. Representative results are shown in Figure 2a with MT-2 and ED-40515(−) cells. ACHP efficiently inhibited phosphorylation of IκBα and p65 (IC_{50} values in MT-2 cells were 0.4 and 0.2 μM, respectively. IC_{50} values in ED-40515 (−) cells were 10.2 and 29.5 μM, respectively. See the Supplementary Information). Inhibitory effect of ACHP was observed as early as 5 min after the treatment (Figure 2b). It is noted that ACHP-treated MT-2 cells appeared to have an increasing net amount of IκBα concomitantly with the inhibition of IκBα phosphorylation. ACHP also exhibited similar effects with other Tax-active cell lines (data not shown). On the other hand, in ED-40515(−) cells, the amount of IκBα remained at the same level and the inhibitory dose was higher than MT-2 cells. In Figure 2c, the inhibitory effect of ACHP on other kinases involved in NF-κB pathways and other major signal transduction pathways are shown. ACHP showed no effect on phosphorylation of IKKα at Ser 181, processing of p100/p52, or phosphorylation of p65 at Ser 276. Moreover, ACHP did not inhibit the phosphorylation of p38 MAPK, ATF-2 and JNK. Unexpectedly, ACHP inhibited the phosphorylation of Akt (IC_{50} values were 4.5 μM. See the Supplementary Information), which is presumably responsible for the cytotoxic effect of ACHP on Jurkat cell survival. In Figure 2d, the inhibitory effect of ACHP on the DNA-binding activity of NF-κB was examined in MT-2, ED-40515(−) and Jurkat cells, using specific probes containing NF-κB binding sequence in the HIV-1 LTR promoter. In MT-2 cells, the NF-κB DNA binding was decreased by ACHP at greater than 1 μM. On the other hand, in ED-40515(−) cells, where the NF-κB DNA binding was weaker than MT-2, the significant inhibitory effect of ACHP was not observed until 50 μM.

Inhibitory effect of ACHP on expression of NF-κB target genes

In ATL, constitutive transcription of antiapoptotic genes such as *bcl-x_L*, *XIAP*, *c-IAPs* and *survivin* has been reported and ascribed to the resistance against anticancer agents.[27,28] NF-κB is known to be involved in the proliferation of HTLV-1-infected cell lines and fresh ATL cells by upregulating growth-promoting genes such as *cyclin D1*.[29] In addition, NF-κB stimulates the expression of *ICAM-1* and *VCAM-1* genes, which mediate T-cell activation and proliferation.[9,30]

As shown in Figure 3a, a distinct inhibition of *cyclin D1* gene expression was observed in MT-2 and ED-40515 (−) cells. Inhibition of *bcl-x_L*, *bcl-2*, *XIAP*, *c-IAP1* and *survivin* gene expressions was observed at high concentration of ACHP. These findings were also reproducibly observed using quantitative real-time RT-PCR analysis (data not shown), although we do not currently know the reason of the different susceptibilities of individual genes to ACHP. In Jurkat cells, inhibition of these gene expressions was weaker than ATL cell lines even at high concentration of ACHP. Figure 3b and c demonstrate that ICAM-1 and VCAM-1are highly expressed in MT-2 and ATL-102 cells, and that significant inhibitory effect was evident even at 1 μM ACHP. Whereas ACHP downregulated the expression of VCAM-1

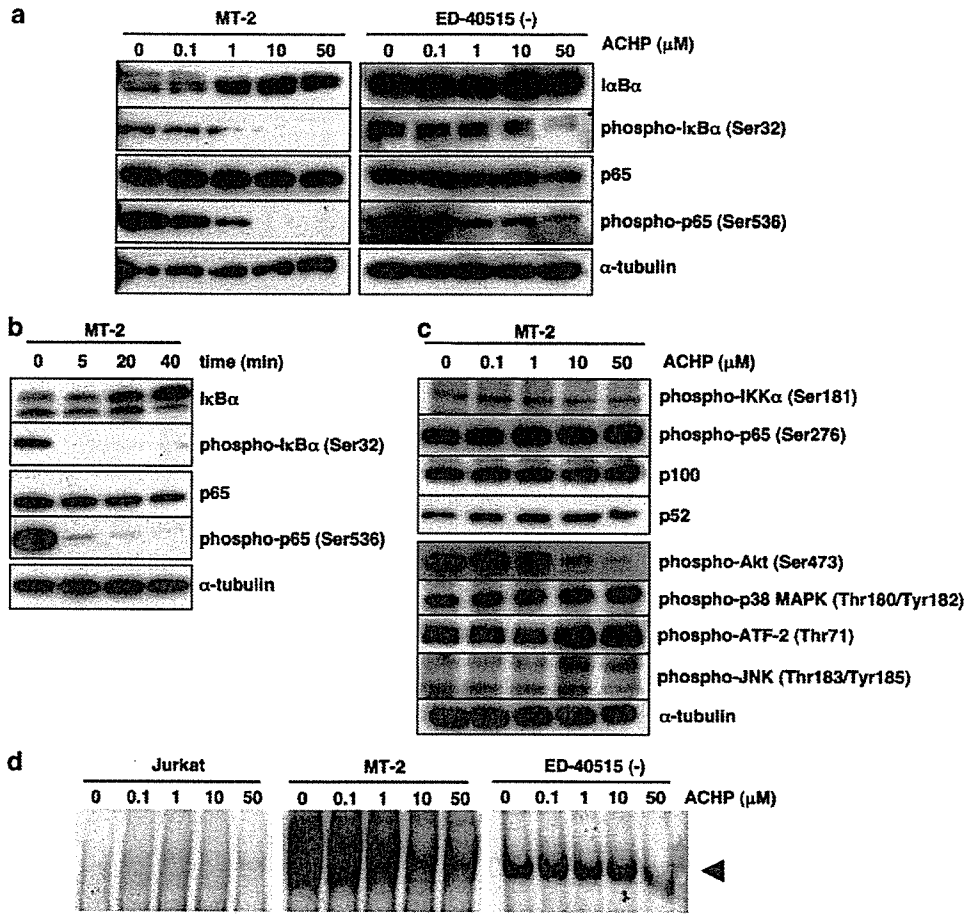

Figure 2 Inhibitory effects of ACHP. (**a**) Dose-dependent inhibition of IκBα and p65 phosphorylation. MT-2 and ED-40515(−) cells were treated with ACHP (0–50 μM) for 20 min and were subjected to immunoblotting analyses with the indicated antibodies. (**b**) Time-course of inhibition of IκBα and p65 phosphorylation. MT-2 cells were treated with ACHP (10 μM) and whole-cell extracts were subjected to immunoblot analysis. (**c**) Specificity of ACHP on IKK and other kinases. MT-2 cells were treated with ACHP (0–50 μM) for 20 min and were subjected to immunoblot analysis with the indicated antibodies. (**d**) Inhibition of NF-κB DNA binding. Cells were treated with ACHP (0–50 μM) for 4 h. Equal amounts (15 μg protein) of nuclear extracts were analyzed for NF-κB binding activity by EMSA using specific probe containing the NF-κB. Closed arrowhead indicates the location of the DNA-NF-κB complex.

in ATL-102 and MT-2 cells, no significant inhibition was observed in ED-40515 (−) cells. ICAM-1 was not expressed in ED-40515 (−) cells. When expression of CD25, as a negative control, was examined, ACHP treatment did not change the level of CD25 (data not shown).

Suppression of cell cycle progression and induction of apoptosis by ACHP

As shown in Figure 4a, ACHP reduced the fraction of cells at S phase in MT-2 and ED-40515 (−) cells, whereas no effect was observed in control Jurkat cells. These findings indicate that ACHP inhibits the growth of HTLV-1-infected T-cell lines in which NF-κB is constitutively activated. In Figure 4b, the number of cells undergoing apoptosis (annexinV-positive) was measured. In all HTLV-1-infected T-cell cell lines, apoptosis induction was remarkably observed at 50 μM, after 8 h treatment with ACHP. In addition, ACHP induced the cleavage of PARP in MT-2 and ED-40515 (−) cells (data not shown). We then examined the effects of ACHP on the growth of seven HTLV-1-infected T-cell lines and a control noninfected T-cell line (Jurkat). As shown in Figure 4c, ACHP inhibited the growth of these cells in a dose-dependent manner. Tax-active cell lines were more susceptible to ACHP than Tax-inactive cell lines and Jurkat (IC_{50} values in Tax-active cell lines, Tax-inactive cell lines or Jurkat were 3.1 ± 1.3 μM, 10.7 ± 1.7 μM and 23.6 μM, respectively), suggesting that the growth of Tax-active cells depends on NF-κB more than Tax-inactive cells. These observations are consistent with augmented NF-κB DNA binding and accelerated turn over of IκBα in Tax-active cells (Figures 1 and 2).

Growth inhibition and apoptosis induction of fresh ATL cells by ACHP

We then evaluated the effects of ACHP on the growth of fresh ATL cells obtained from four independent acute-type ATL patients. Peripheral blood mononuclear cells (PBMCs) contained greater than 90% leukemic cells. Control PBMCs were similarly obtained from four healthy individuals. As shown in Figure 5a, ACHP inhibited growth of fresh ATL cells. Fresh ATL leukemic cells were more susceptible to the ACHP-induced cell growth inhibition than control PBMCs from healthy individuals with IC_{50} values of 8.6 ± 1.1 and 55.7 ± 7.5 μM, respectively ($p < 0.01$). Compared with the data in Figure 4c with T-cell lines, the susceptibility of fresh ATL cells to ACHP was between Tax-active and inactive cells. However, healthy mononuclear cells were far more resistant to ACHP than Tax-inactive cells. These findings suggested that fresh ATL cells depend on NF-κB.

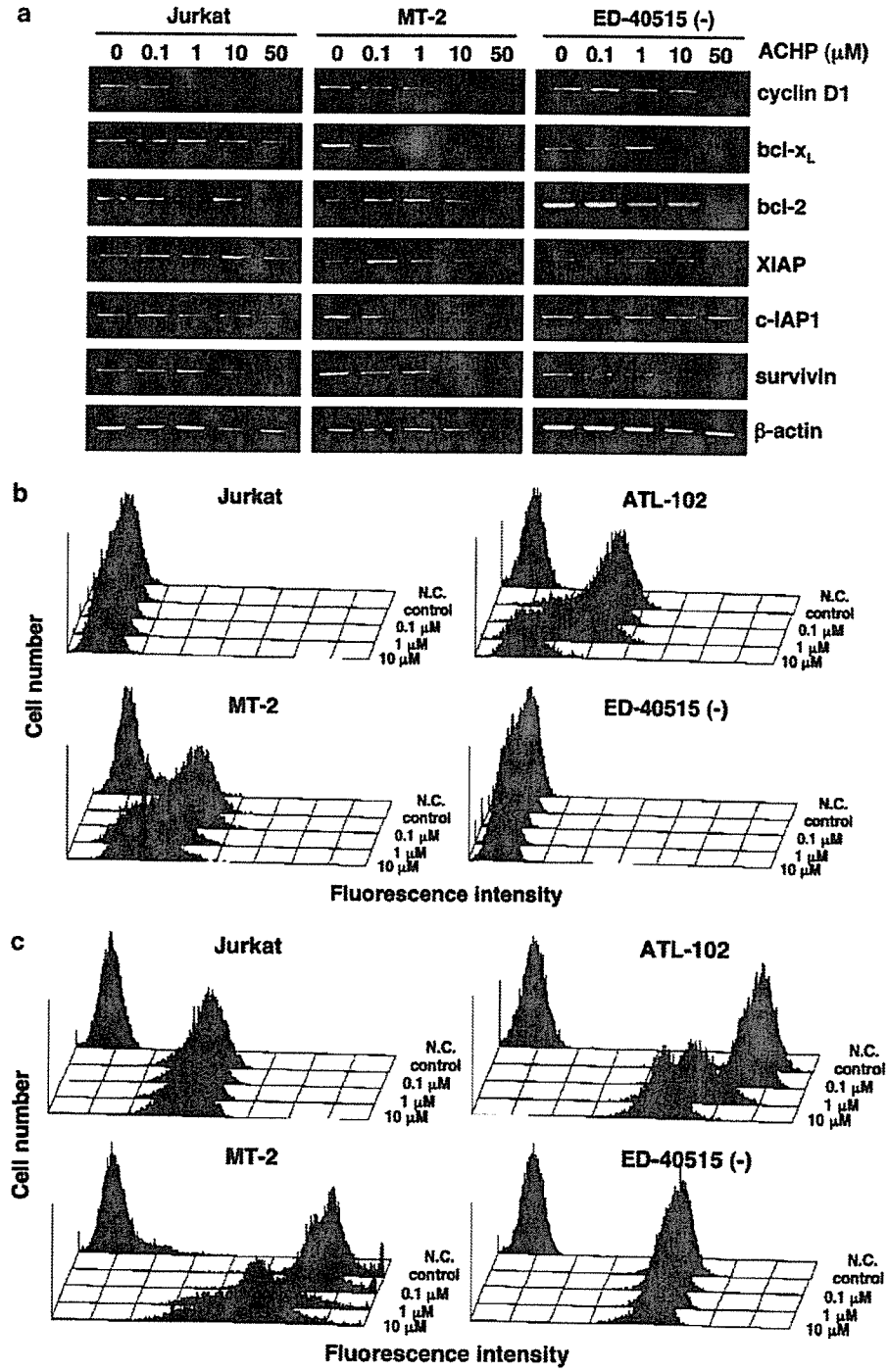

Figure 3 Inhibition of the NF-κB-dependent gene expression by ACHP. (**a**) Inhibition of gene expression by ACHP. The mRNA levels of various NF-κB target genes were examined by RT-PCR. Cells were treated with ACHP (0–50 μM) for 4 h, and total RNA were prepared and subjected to the determination of mRNA levels of *cyclin D1*, *bcl-xL*, *bcl-2*, *XIAP*, *c-IAP1*, *survivin* and *β-actin*. Each sample was subjected to PCR amplification for 35 cycles (*cyclin D1* in Jurkat cells) or 30 cycles (other genes). (**b**) and (**c**) Downregulation of ICAM-1 and VCAM-1 expression by ACHP. Surface expression of ICAM-1 (**b**) and VCAM-1 (**c**) was examined with Jurkat, ATL-102, MT-2 and ED-40515(−) cells in the absence (control) or presence of ACHP (0.1–10 μM) for 48 h by flowcytometry using specific antibodies. NC, negative control stained with isotype-matched IgG.

We then examined the levels of IκBα and p65 phosphorylation, as well as the NF-κB DNA binding, of fresh ATL cells and their susceptibility to ACHP-mediated apoptosis. As shown in Figure 5b, constitutive phosphorylation of p65 at Ser 536 and NF-κB DNA binding were observed and inhibited by ACHP (IC_{50} value: 0.6 μM), however, phosphorylation of IκBα at Ser 32 was not detected in fresh ATL cells. Identical results of protein phosphorylation were observed with other fresh ATL cells, whereas no constitutive phosphorylation of p65 and IκBα was observed with control PBMCs (data not shown). The flowcytometric analysis of fresh ATL cells (Figure 5c) revealed the appearance of prominent sub G_0/G_1 population suggesting the presence of cells undergoing apoptosis upon treatment with ACHP. Consistently, annexinV-positive cells were detected even

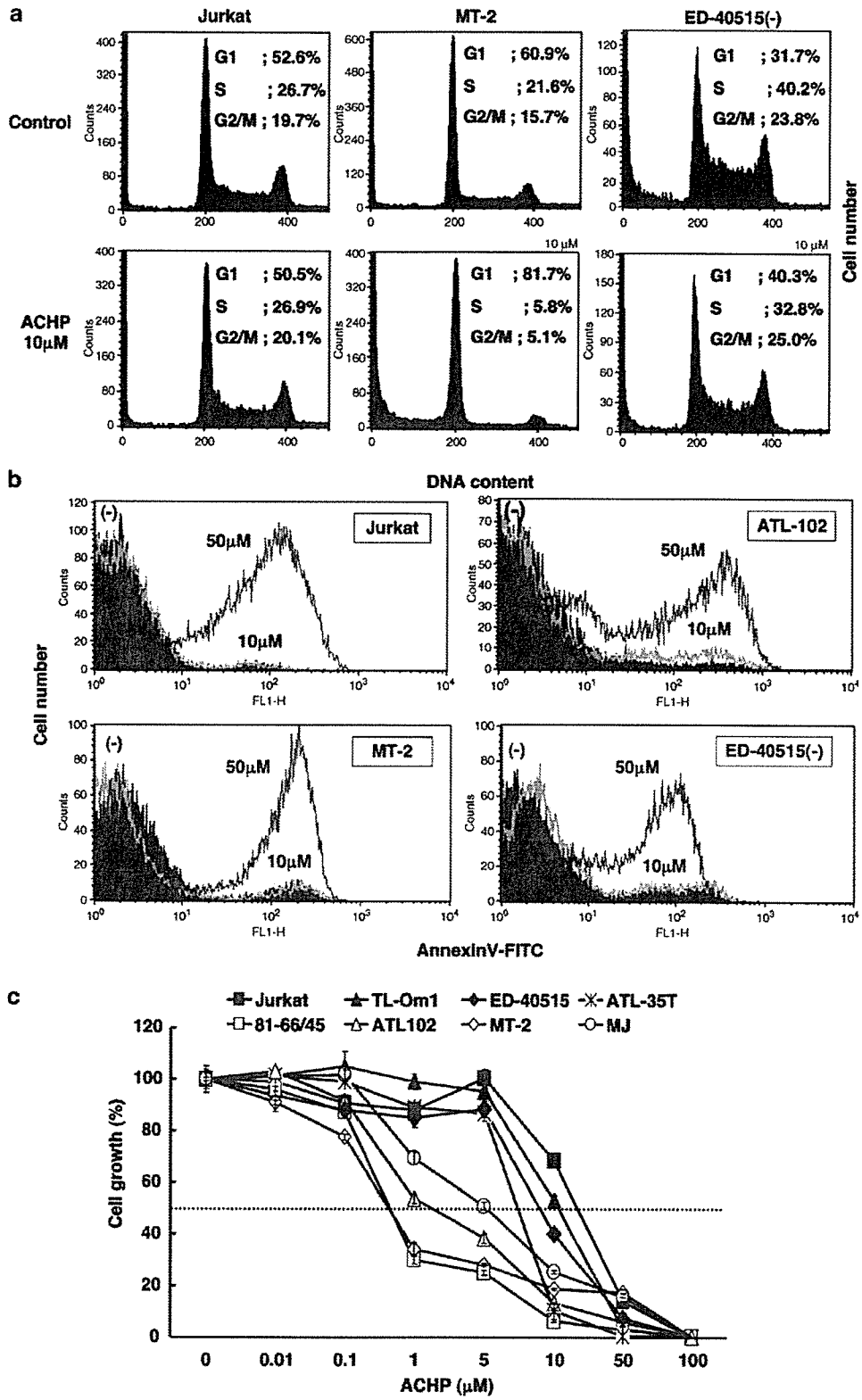

Figure 4 Growth inhibition, cell cycle arrest and induction of apoptosis by ACHP. (**a**) Inhibition of cell cycle progression. Cells were treated with or without ACHP (10 μM) for 24 h, stained with PI and subjected to the flowcytometric analysis. The fractions of each cell-cycle phase (%) are analyzed by CellQuest analysis program. Each experiment was repeated at least three times with reproducible results. The representative data are shown. (**b**) Induction of apoptosis. Cells were treated with ACHP (0, 10, 50 μM) for 8 h, stained with FITC-conjugated annexin V, and analyzed by flowcytometry. Closed areas indicate the intensities of nontreated control cells denoted as '(−)'. (**c**) Cell growth inhibition by ACHP. Cells were treated with ACHP for 3 days, before the cytotoxicity was evaluated by MTT assay. The results are indicated as percentage compared to the untreated control. These experiments were performed in triplicates and the mean values ± s.d. are shown.

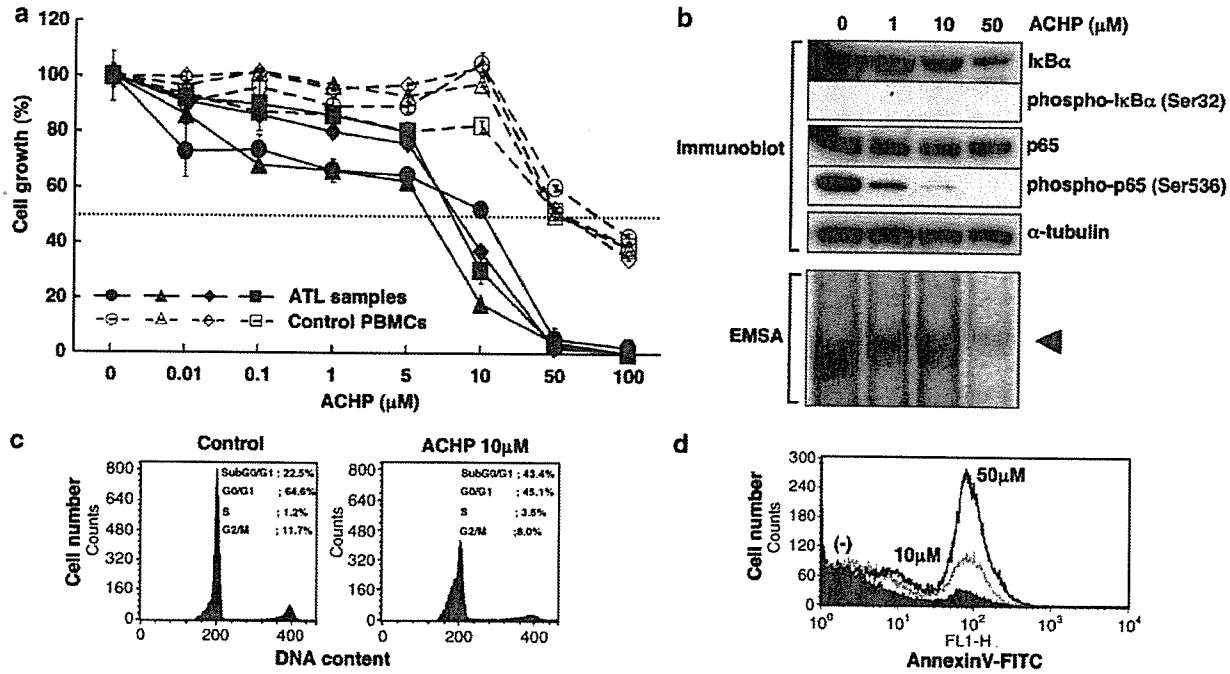

Figure 5 Growth inhibition and apoptosis induction of fresh ATL cells by ACHP. (**a**) Effects of ACHP on the cell growth of fresh ATL cells and control PBMCs. Four fresh ATL samples derived from acute-type ATL patients and four PBMCs derived from healthy donors were cultured in the absence of mitogen and treated with ACHP) for 3 days. The results are indicated as percentage compared to the untreated control. These experiments were performed in triplicates and the mean values ± s.d. are shown. (**b**) Dose-dependent inhibition of p65 phosphorylation and NF-κB DNA binding in fresh ATL cells. Fresh ATL cells were treated with ACHP for 6 h. Whole-cell extracts were subjected to immunoblotting analysis with the indicated antibodies, and nuclear extracts were subjected to EMSA. Closed arrowhead indicates the location of the DNA-NF-κB complex. (**c**) Cell cycle distribution analysis of fresh ATL cells upon treatment with ACHP. Fresh ATL cells were treated with or without ACHP (10 μM) for 24 h, stained with PI and subjected to the flowcytometric analysis. The fractions of each cell-cycle phase (%) are shown. Note the rapid appearance of subG$_0$/G$_1$ cells upon ACHP treatment. (**d**) Induction of apoptosis by ACHP. Fresh ATL cells were treated with ACHP (10 or 50 μM) for 8 h, stained with FITC-conjugated annexin V, and analyzed by flowcytometry. Closed areas indicate the intensities of nontreated control cells denoted as '(−)'.

with 10 μM of ACHP (Figure 5d). Thus, ACHP was effective in blocking the growth and inducing the apoptosis of ATL cells as well as HTLV-1-infected T-cell lines.

Discussion

In this study, we demonstrated that a novel IKK inhibitor, ACHP, efficiently inhibited NF-κB that is constitutively activated in ATL cell lines and fresh ATL cells. We also observed that it could block their growth by inducing apoptosis and cell cycle arrest. The constitutive activation of NF-κB has been reported in other neoplasms such as colorectal cancer, hepatocellular carcinoma, multiple myeloma and various forms of malignant lymphoma.[6,7,20] In these neoplasms, NF-κB was shown to be involved in tumorigenesis and disease progression by upregulating anti-apoptotic proteins, cell cycle regulators, and cell adhesion molecules. In fact, inhibitors of NF-κB such as Bay11-7082[13] showed antitumor effects. In addition, we have previously reported the growth inhibitory effect of ACHP on multiple myeloma cell lines and the synergism with conventional chemotherapeutic agents.[20]

We observed the growth inhibitory effect of ACHP especially in Tax-active HTLV-1-infected T-cell lines. In MT-2 cells, for example, the NF-κB DNA binding was prominent and was efficiently inhibited by ACHP (Figure 2d). On the other hand, in Tax-inactive cell lines such as ED-40515(−), phosphorylation of IκBα/p65 and NF-κB DNA binding was not inhibited until higher concentration (greater than 10 μM) of ACHP was used (Figure 2a and d). It was noted that the effective ACHP concentrations in blocking the phosphorylation and NF-κB DNA binding in these cell lines correlated with the extents of growth inhibition by ACHP (Figure 4c). Similar observations were reported by Mori *et al.*[13] where Tax-active HTLV-1-infected T-cell lines were more susceptible to the growth inhibitory effect of Bay 11-7082, another inhibitor of IKK, than Tax-inactive ATL cell lines.

The functional interaction between Tax and NF-κB activation pathway has been well investigated in previous studies by others.[16–18] In addition, constitutive activation of NF-κB is also reported in Tax-independent ATL cells[14,15] although the mechanism is not established yet. In these cells, it is postulated that the overexpression of TNFα or LTβ, which is often found in ATL patients,[22,31] could substitute the effects of Tax. We have demonstrated that LTβ receptor-mediated signaling specifically involves IKKα and induces p65 phosphorylation at Ser 536.[12] The constitutive phosphorylation of p65 at Ser 536 was evident in HTLV-1-infected T-cell lines irrespectively of the expression of functional Tax (Figure 1). In support of this possibility, Hironaka *et al.*[15] reported that dominant-negative IKKα mutant, but not dominant-negative IKKβ mutant, could inhibit the NF-κB activity in ATL-derived cell lines. Interestingly, we observed the NF-κB DNA binding associated with the constitutive p65 Ser 536 phosphorylation but not with the IκBα phosphorylation in fresh ATL samples (Figure 5b). In these cells, even lower concentrations of ACHP could induce apoptosis and cell growth arrest as compared with ATL cell lines (Figures 5c and d). These findings collectively suggest that the IKKα-mediated NF-κB activation pathway may play a crucial role in ATL.

In this study, we observed that various NF-κB target genes showed different susceptibility to the inhibitory effects of ACHP (Figure 3). For example, whereas expression of ICAM-1 and VCAM-1 was inhibited by the low dose of ACHP (Figure 3b and c), expression of anti-apoptotic genes, such as *bcl-XL, bcl-2, XIAP, c-IAP2* and *survivin*, required higher doses of ACHP (Figure 3a). It is possible that various NF-κB target genes are regulated by distinct NF-κB activation pathways. In this context, we found that IκBα phosphorylation, primarily catalyzed by IKKβ, was inhibited by low dose ACHP in HTLV-1-infected T cell lines (Figure 2). Thus, it appears that the ACHP-mediated inhibition of NF-κB target genes favors the canonical activation pathway. In addition, we found that ACHP could also induce apoptosis in Jurkat cells unexpectedly (Figure 2c) because no constitutive NF-κB activation was detected in these cells (Figures 1 and 2). In Jurkat cells the genetic defect of PTEN phosphatase is considered responsible for the cellular transformation.[32] The genetic defect of PTEN causes the constitutive Akt phosphorylation mediated by phosphatidylinositol-3 phosphate kinase (PI3K) and its inhibition by ACHP may have caused cell death in Jurkat. The constitutive Akt phosphorylation is also observed in the Tax-active cell lines (Figure 1a). Thus, it is possible that net effects of ACHP in inhibiting the growth of ATL cell lines may be through IKK-NF-κB and PI3K-Akt kinase pathways.

In conclusion, our results indicate the therapeutic efficacy of ACHP and its derivatives in the treatment of ATL by blocking the signal transduction pathway leading to constitutive activation of NF-κB, as well as Akt phosphorylation. Additionally, we observed the apparent differences in the NF-κB activation pathways involved in Tax-active, Tax-inactive, and most notably, fresh ATL cells and highlighted the role of IKKα in ATL leukemogenesis. Thus, ACHP and its derivatives could be feasible components of the novel anti-ATL chemotherapeutic regimen by sensitizing leukemic cells to the conventional cytotoxic agents.

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Inhibition of Human Immunodeficiency Virus Type 1 Replication in Latently Infected Cells by a Novel IκB Kinase Inhibitor

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In human immunodeficiency virus type 1 (HIV-1) latently infected cells, NF-κB plays a major role in the transcriptional induction of HIV-1 replication. Hence, downregulation of NF-κB activation has long been sought for effective anti-HIV therapy. Tumor necrosis factor alpha (TNF-α) stimulates IκB kinase (IKK) complex, a critical regulator in the NF-κB signaling pathway. A novel IKK inhibitor, ACHP {2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-yl-nicotinonitrile}, was developed and evaluated as a potent and specific inhibitor for IKK-α and IKK-β. In this study, we examined the ability of this compound to inhibit HIV-1 replication in OM10.1 cells latently infected with HIV. When these cells were pretreated with ACHP, TNF-α-induced HIV-1 replication was dramatically inhibited, as measured by the HIV p24 antigen levels in the culture supernatants. Its 50% effective concentration was approximately 0.56 μM, whereas its 50% cytotoxic concentration was about 15 μM. Western blot analysis revealed inhibition of IκBα phosphorylation, IκBα degradation, p65 nuclear translocation, and p65 phosphorylation. ACHP was also found to suppress HIV-1 long terminal repeat (LTR)-driven gene expression through the inhibition of NF-κB activation. Furthermore, ACHP inhibited TNF-α-induced NF-κB (p65) recruitment to the HIV-1 LTR, as assessed by chromatin immunoprecipitation assay. These findings suggest that ACHP acts as a potent suppressor of TNF-α-induced HIV replication in latently infected cells and that this inhibition is mediated through suppression of IKK activity.

Although the recent progress in combination therapy against viral reverse transcriptase and protease has achieved considerable reduction of the viral load in human immunodeficiency virus type 1 (HIV-1)-infected individuals and significant improvement in survival, chemotherapy could not be terminated unless chronically infected cell populations, such as resting memory T cells and monocytes/macrophages, could be eradicated (15, 51, 53). Thus, it is crucial to inhibit HIV-1 replication in the latently infected cells. Molecular analyses of HIV-1 replication have revealed a concerted complexity that regulates the viral life cycle (52). Among the various steps of the viral life cycle, the step of transcription from HIV-1 provirus is conceived to be crucial for viral replication, since amplification of the viral genetic information is attainable only through transcription. It is through this step that HIV acquires genetic variation, thus enabling the emergence of HIV quasispecies containing clones resistant to host immune responses and anti-HIV drugs. In addition to the virus-encoded transcriptional transactivator Tat, several cellular factors are known to regulate HIV-1 transcription (29, 52). Among these host factors, nuclear factor κB (NF-κB) is known to play a major role in regulated HIV-1 gene expression (44, 48, 52).

NF-κB is an inducible cellular transcription factor that regulates a wide variety of cellular and viral gene expression, including that of HIV (6, 7, 22, 44, 48, 50, 65). Recently, two major signaling pathways leading to receptor-mediated NF-κB activation have been classified: the canonical and noncanonical (alternative) pathways. In the canonical pathway, a diverse range of stimuli, such as tumor necrosis factor alpha (TNF-α), viral and bacterial pathogens, and stress-inducing agents (24), stimulate the signal transduction pathways that lead to the activation of NF-κB. In cells, NF-κB, a hetero- or homodimer consisting of the Rel family proteins p65 (RelA), RelB, c-Rel, p50/p105, and p52/p100, resides in the cytoplasm and is complexed with an inhibitory molecule, IκB (6). Stimulation activates the IκB kinase (IKK) complex composed of two catalytic subunits, IKK-α and IKK-β, and a regulatory subunit, IKK-γ (22). IKK rapidly phosphorylates IκBα on its two NH_2-terminal serine residues (Ser32 and Ser36) (21, 41, 71). Phosphorylation targets IκBα for its ubiquitination and degradation by the β-transducin repeat-containing protein ubiquitin ligase and 26S proteasome, respectively, thus allowing free NF-κB to translocate to the nucleus to activate gene expression (22). In this event, IKK-β and IKK-γ mainly regulate IκB degradation, while IKK-α is dispensable (22), although its nuclear function remains essential for the transcriptional activity of NF-κB (1, 70). The noncanonical pathway, however, is strictly dependent on the NF-κB-inducing kinase (NIK)-mediated activation of IKK-α, which phosphorylates p100, causing its inducible processing into p52 (60, 68). This IKK-β/IKK-γ-independent pathway is induced in response to stimuli, such as lymphotoxin B (18), B-cell-activating factor (16), and CD40 ligand (17). Moreover, recent reports by us and others have shown that IKK-α also phosphorylates p65 at Ser536, which is pivotal for the transcriptional competence of NF-κB when it is bound to the promoter sequence of target genes in the nucleus (27, 28, 56).

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The role of NF-κB in activating HIV transcription has been extensively studied. HIV-1 replication is positively regulated by several cytokines, or T-cell activators, most of which act either completely or partially via NF-κB (4, 8, 38). NF-κB has been shown to regulate viral transcription via the two NF-κB sites located in the HIV-1 long terminal repeat (LTR) enhancer region (44) and is further enhanced through synergism with Sp1 (34, 49). In HIV-1 latently infected cells, activation of NF-κB could trigger the transcription of viral genes, including the transactivator Tat, resulting in an explosive increase in HIV replication (29, 47, 52). Treatment with compounds that block NF-κB activation inhibits HIV-1 gene expression and viral replication (3, 20, 33, 45, 59, 63, 64). Hence, downregulation of NF-κB activity by suppressing NF-κB or the signaling proteins involved in the NF-κB activation pathway, such as the IKKs (23, 30), is considered a feasible target for future anti-HIV therapy.

To control HIV-1 expression from latently infected cells, we examined the effect of a novel IKK inhibitor, 2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-ylnicotinonitrile (ACHP). ACHP was found on a massive screening to have specific inhibitory action on IKK-β and IKK-α (42, 43). The 50% inhibitory concentrations for IKK-β and IKK-α are 8.5 and 250 nmol/liter, respectively, measured by in vitro kinase assays, and those for other kinases, such as IKK-γ, Syk, and mitogen-activated protein kinase kinase kinase 4, were greater than 20 μmol/liter (42). ACHP also showed good aqueous solubility and cell permeability, thus demonstrating high bioavailability in mice and rats (43).

In this study, we demonstrate the inhibitory action of ACHP on IκBα phosphorylation and its degradation, as well as the nuclear translocation and phosphorylation of p65, resulting in the reduction of HIV production in HIV-1 latently infected cells. Furthermore, NF-κB (p65) binding to the HIV-1 LTR was also abolished by this compound. From these findings, this compound and its derivatives appear to be feasible candidates for novel anti-HIV therapy.

MATERIALS AND METHODS

Reagents. An IKK inhibitor, ACHP, was a kind gift from T. Murata of Bayer Yakuhin Inc. (Kyoto, Japan). Human recombinant TNF-α was purchased from Roche and used at 1 ng/ml for NF-κB stimulation. Antibodies for IκBα, p65 (RelA), and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while antibodies for phospho-IκBα (Ser32) and phospho-p65 (Ser536) were purchased from Cell Signaling Technology (Beverly, MA). The pooled sera from HIV-1-infected individuals were kindly given by T. Hamano (National Institute of Infectious Diseases, Tokyo, Japan). Horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences (Little Chalfont, United Kingdom) (rabbit and mouse) and from DAKO (DAKO A/S, Denmark) (goat).

Cell lines. OM10.1 cells (13), a human macrophage/monocytic cell line latently infected with HIV-1, and MOLT4/III_B cells (3), a T-cell line chronically infected with HIV-1 (III_B strain), were used in the antiviral assays. The OM10.1 cells and MOLT4/III_B cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37°C in a 5% CO_2 incubator. To maintain the latency in OM10.1 cells, 20 μM of AZT was added to culture media and was removed prior to experiments (3, 58, 59, 64).

Antiviral assay. The antiviral activity of ACHP was evaluated based on the extent of inhibition of p24 antigen production in OM10.1 cells as previously described (59, 64). Cells (2×10^5/ml) were incubated with or without ACHP for 1 h and then stimulated with TNF-α (1 ng/ml) for 24 h at 37°C. A time course experiment (until 72 h) was also conducted. On the other hand, MOLT4/III_B cells (2×10^5/ml) were cultured in the absence or presence of ACHP with or without TNF-α stimulation. The culture supernatants were then collected and assayed for viral p24 antigen. Experiments were carried out in triplicate and repeated at least twice. The cytotoxicity of the test compound was also determined by the WST-1 method (Roche) (58).

Quantitation of HIV-1 replication. Viral p24 antigen levels in the cell supernatants of OM10.1 and MOLT4/III_B cells were determined using the commercial Retrotek HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (Cellular Products, Buffalo, NY) according to the manufacturer's instructions. Assays were performed in triplicate and repeated at least twice.

Transient luciferase assay. 293 cells (1×10^5/well) were transfected with reporter plasmids using FUGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's instructions. For each transfection, 0.3 μg of reporter plasmid, CD12wt-Luc containing wild-type HIV-1 LTR (47) or CD12mut-Luc containing mutated NF-κB binding sites (59), and 0.1 μg of the internal control plasmid, phRL-TK, expressing *Renilla* luciferase, were used (57). Twenty-four hours after transfection, the cells were treated with ACHP for 30 min and stimulated with TNF-α (5 ng/ml) for 4 h. The transfected cells were then harvested, and the extracts were subjected to luciferase assay using the Luciferase Assay System (Promega). The luciferase activity was normalized with *Renilla* luciferase activity as an internal control to assess the transfection efficiency. The data are presented as the increase in luciferase activities (means ± standard deviations) relative to the control from triplicate transfections.

Preparation of whole-cell and nuclear extracts. OM10.1 cells (1×10^6/ml) were treated with or without ACHP for 1 h and stimulated with or without TNF-α (1 ng/ml) at various times. The cells were then washed with cold phosphate-buffered saline and resuspended in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride [PMSF], 1% Triton X-100, and protease inhibitors [Roche Diagnostics GmbH, Mannheim, Germany]), incubated on ice for 10 min, and centrifuged at 15,000 rpm for 15 min. The supernatant was then collected (whole-cell extract) and stored at −80°C until it was used. In order to prepare the nuclear extract, sedimented cells were resuspended in cytoplasmic lysis buffer (Chemicon International, Tenecula, CA) and incubated for 15 min on ice. The cells were vortexed and then centrifuged at 15,000 rpm for 10 min, and the supernatant was removed. The cell pellets were washed twice with cytoplasmic buffer to remove any trace of proteins from the cytoplasmic extracts, resuspended in 20 μl of nuclear lysis buffer (Chemicon International, Tenecula, CA), and incubated on ice for 15 min. The cell suspensions were then sonicated for 10 s and centrifuged at 15,000 rpm for 10 min, and the supernatant fractions were stored at −80°C until they were used. The protein content was measured by a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA).

Western blotting. Equal amounts of the proteins (14 μg) were resolved on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred onto a polyvinylidene fluoride membrane (Millipore Corporation, MA), and reacted with specific antibodies for various proteins. Expression of HIV-1 viral proteins was examined by reaction with pooled sera from HIV-1-infected individuals. Detection of immunoreactive bands was visualized by chemiluminescence using Super-Signal (Pierce, Rockford, IL).

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were performed with a previously described protocol with some modifications (25). In brief, cells either with or without ACHP treatment and with or without TNF-α stimulation were cross-linked by adding formaldehyde to the medium (1% final concentration) and incubated at 37°C for 10 min. The cells were then washed with cold phosphate-buffered saline containing protease inhibitors and PMSF and lysed in SDS-lysis buffer (1% SDS, 50 mM Tris-HCl [pH 8.0], 16.7 mM NaCl, PMSF, and protease inhibitors), and the chromatin was sheared by sonication 20 times for 30 s each time at one-third of the maximum power with 1 min of cooling on ice between each pair of pulses (Bioruptor; COSMO Bio, Tokyo, Japan). The lysates were then collected after centrifugation at 15,000 rpm for 15 min, followed by the addition of specific antibodies, and the mixture was rotated at room temperature for 2 h and further incubated for another hour at 4°C. DNA samples were then precipitated with salmon sperm DNA and protein G-agarose beads (Upstate Biotechnology, Lake Placid, NY), and cross-linking of the immunoprecipitates and input DNAs was reversed by incubation at 65°C for 6 h. The DNAs were then purified using Qiaquick spin columns (QIAGEN), and PCR was performed with a HotStar*Taq* Master Mix kit (QIAGEN). The PCR primers used for amplifying promoters containing the NF-κB binding sites included HIV-1 LTR promoter (−176 to +61), 5′ CGA GAG CTG CAT CCG GAG TA-3′ and 5′ AGC TTT ATT GAG GCT TAA GC 3′ (37); human IκBα promoter (−316 to −15), 5′-GAC GAC CCC AAT TCA AAT CG 3′ and 5′ TCA GGC TCG GGG AAT TTC C-3′ (70); and human β-actin promoter (−980 to −915), 5′-TGC ACT GTG CGG CGA AGC-3′ and 5′-TCG AGC CAT AAA AGG CAA-3′ (70).

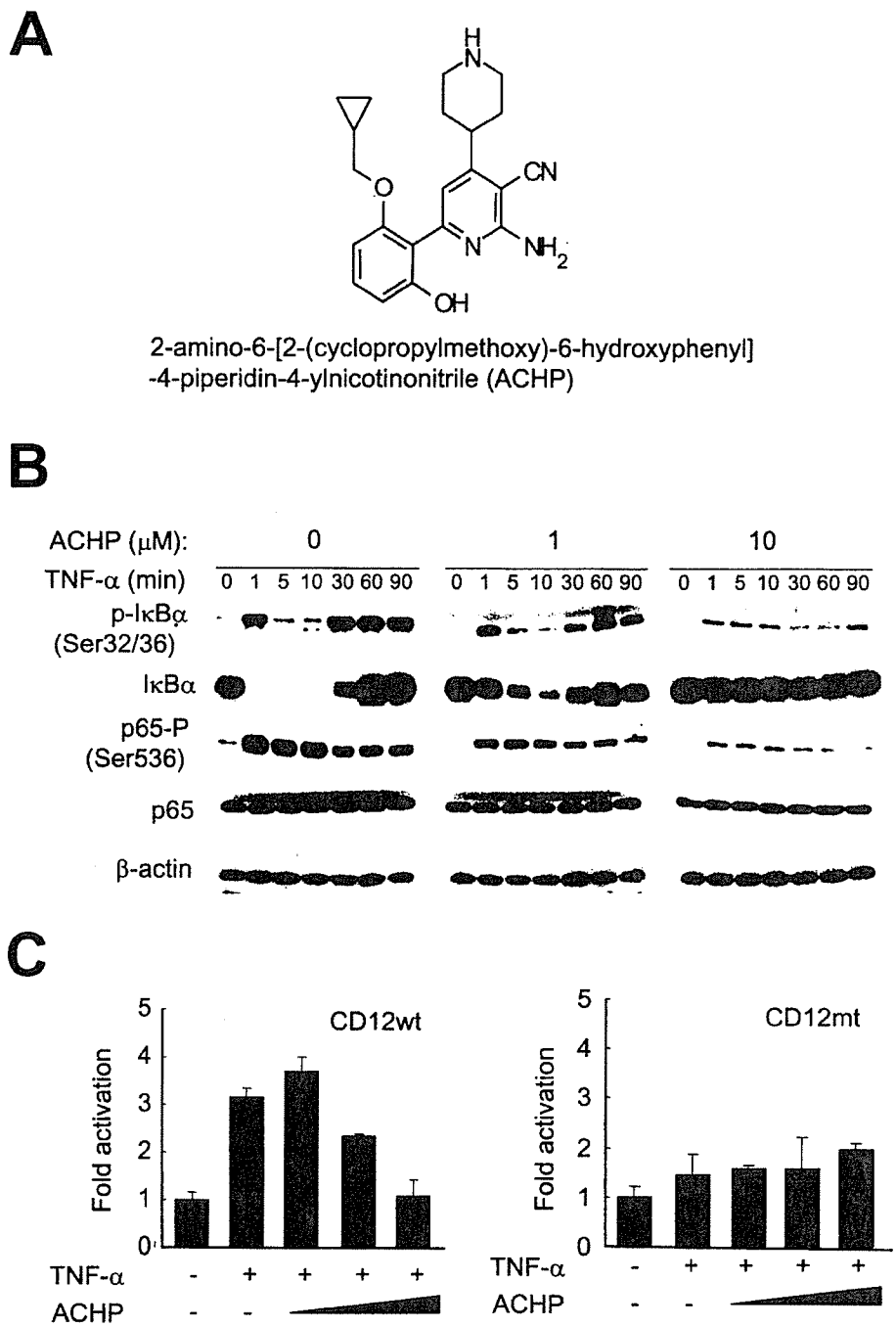

FIG. 1. Effect of ACHP on IκBα phosphorylation and degradation and p65 phosphorylation induced by TNF-α. (A) Chemical structure of ACHP. (B) Inhibition of phosphorylation of IκBα and p65 by ACHP. OM10.1 cells (1×10^6/ml) were pretreated with or without ACHP for an hour and then stimulated with TNF-α (1 ng/ml) for the indicated time periods. Whole-cell extracts were fractionated on 10% SDS-polyacrylamide gel electrophoresis and subjected to immunoblot analysis with the specified antibodies. Immunoblotting of β-actin indicated that equal amounts of protein were applied in each lane. (C) Inhibitory effect of ACHP on HIV-1 LTR-driven gene expression in 293 cells. A luciferase reporter plasmid containing wild-type NF-κB binding sequence (CD12wt, left) or its mutant (CD12mt, right) was transfected into 293 cells under the control of the HIV-1 LTR. After transfection, the cells were incubated in the absence or presence of ACHP for 30 min, stimulated with TNF-α (5 ng/ml) for 4 h, and harvested for luciferase assay, as described in Materials and Methods. The luciferase activity is indicated as increase relative to the untreated control (lane 1). The data are mean values plus standard deviations of triplicate experiments.

RESULTS

ACHP inhibited TNF-α-induced IκBα phosphorylation and degradation and p65 phosphorylation. Activated IKKs target the IκBα inhibitor of NF-κB for phosphorylation on Ser32 and Ser36, leading to the rapid ubiquitylation and degradation of the inhibitor by the 26S proteasome (44). To assess whether ACHP (Fig. 1A) modulates this cascade, OM10.1 cells, a macrophage/monocyte cell model widely used in studying thera-

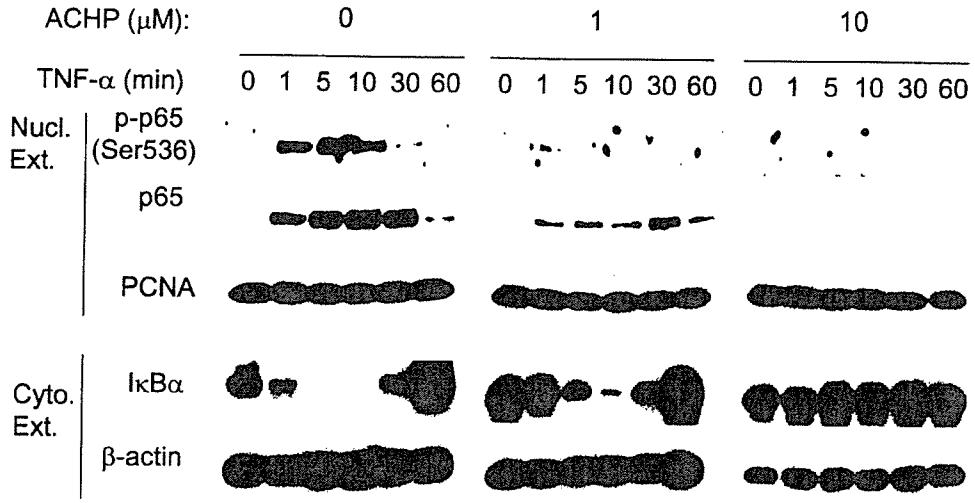

FIG. 2. Effect of ACHP on TNF-α-induced p65 translocation and phosphorylation in the nucleus. OM10.1 cells (1×10^6/ml) were pretreated with or without ACHP for an hour and then stimulated with TNF-α (1 ng/ml) for the indicated time periods. Nuclear extracts (Nucl. Ext.) were fractionated by 10% SDS-polyacrylamide gel electrophoresis and immunoblotted with the anti-phospho-p65 (Ser536) antibody (top). The membrane was reprobed with anti-p65 (upper middle) and anti-PCNA (lower middle) antibodies. Cytoplasmic extracts (Cyto. Ext.) were immunoblotted with the anti-IκBα and β-actin antibodies (bottom). The purity of the nuclear and cytoplasmic extracts was confirmed with antibodies specific for PCNA, a known nuclear protein (lower middle) and β-actin, a cytoplasmic protein (bottom), respectively.

peutic interventions (3, 13, 58, 64), were stimulated with TNF-α for the indicated times in the absence or presence of ACHP and analyzed for IκBα and NF-κB (p65) expression and for their phosphorylation status by Western blot analysis.

As shown in Fig. 1B, immunoblotting analysis showed that IκBα was readily phosphorylated at Ser32 and Ser36 as early as 1 min after TNF-α stimulation. Following its degradation, a massive increase was observed, indicating the activation of the IκBα gene by NF-κB (Fig. 1B, first gel, first row) (12, 14, 37, 61). However, treatment with ACHP reduced IκBα phosphorylation in a dose-dependent manner (second and third gels, first row). ACHP (10 μM) maintained the phosphorylation approximately at basal levels throughout the time course experiment (third gel, first row). Because IκBα normally undergoes degradation following phosphorylation, we proceeded to ascertain whether ACHP blocked its degradation. Remarkably, IκBα expression levels were apparently sustained at basal levels, and no degradation was observed when cells were treated with 10 μM ACHP, confirming that the preceding step, IκBα phosphorylation, was inhibited (third gel, second row).

To further explore the status of NF-κB activation in these cells, we also investigated the phosphorylation levels of the p65 subunit of NF-κB. Figure 1B (third row of each gel) shows constitutive phosphorylation of p65 at Ser536, which was markedly induced a minute after TNF-α treatment and gradually decreased in a time-dependent manner. In contrast, pretreatment with ACHP reduced this TNF-α-stimulated phosphorylation of p65 (third gel, third row), whereas the total protein level of p65, like that of IκBα, was maintained at basal levels in the absence or presence of TNF-α (fourth row of each gel). β-Actin levels were unchanged (bottom panels), indicating equal loading of proteins in the gel. These results indicate that ACHP promoted a substantial defect in the TNF-α-induction of NF-κB activation.

We then examined the effect of ACHP on HIV-1 gene expression using a transient luciferase assay. As shown in Fig. 1C, TNF-α stimulated the gene expression from CD12wt containing the wild type HIV-1 LTR (47) by approximately threefold. When cells were pretreated with ACHP, the gene expression was inhibited in a dose-dependent manner (Fig. 1C, left). However, when the NF-κB sites were mutated (CD12mt), no such activation by TNF-α or inhibitory effects of ACHP were observed ((Fig. 1C), right).

ACHP repressed TNF-α-induced nuclear translocation and phosphorylation of p65. Because IκBα phosphorylation and its degradation were inhibited by treatment with ACHP, and since both are prerequisite steps for the release and transport of NF-κB to the nucleus, we also examined whether ACHP modulated this event. Following TNF-α stimulation at 1, 5, 10, 30, and 60 min, in either the absence or the presence of ACHP, nuclear extracts from OM10.1 cells were isolated and NF-κB (p65) was examined by Western blotting analysis. TNF-α treatment resulted in a rapid nuclear accumulation of p65 as early as 1 min, followed by increase until 30 min, and then drastically decreased at 60 min (Fig. 2, "Nucl. Ext.," first gel, second row). Concurrently, IκBα appeared, indicating its nuclear-export function (data not shown). Consistent with previous studies using OM10.1 cells (3, 58, 64), a low level of NF-κB activation, i.e., nuclear localization of p65, was observed in unstimulated cells, which was notably reduced by the presence of ACHP alone (Fig. 2, lanes at 0 min, second row). Whereas TNF-α induced the accumulation of p65 in the nucleus, ACHP almost totally abolished this effect (Fig. 2, "Nucl. Ext.," third gel, second row), suggesting that the nuclear transport of NF-κB was inhibited. Immunoblots of the cytoplasmic extracts also revealed that 10 μM ACHP maintained IκBα protein levels at basal levels, reaffirming the persistence of latent NF-κB–IκB complexes (Fig. 2, "Cyto. Ext.," first row of each gel). In addition to nuclear translocation, p65 phosphorylation is also essential for its maximum transcriptional activity (28, 66, 72).

Concomitant with the appearance of p65 in the nucleus, rapid phosphorylation of p65 at Ser536 was detected (Fig. 2, "Nucl. Ext.," first row). This phosphorylation, however, occurred only transiently, followed by a marked reduction after 10 min of TNF-α stimulation, suggesting dephosphorylation of p65. In contrast, no phosphorylation was observed in the presence of 10 μM ACHP, implying that ACHP efficiently blocked the phosphorylation of p65 in the nucleus (Fig. 2, "Nucl. Ext.," third gel, first row). These observations further strengthen the view that ACHP impairs the IKK activity that enables p65 to translocate to the nucleus and phosphorylates p65.

Inhibition of TNF-α-induced HIV-1 production by ACHP from OM10.1 cells. To examine the effect of ACHP on TNF-α-induced HIV replication in latently infected OM10.1 cells, the cells were treated with various concentrations of TNF-α in the absence or presence of ACHP. After 24 h of incubation, the culture supernatants were collected and assayed for HIV-1 p24 antigen levels. In agreement with previous studies (3, 58, 64), HIV-1 production was dose-dependently induced by TNF-α (Fig. 3A). However, in the presence of ACHP, a dose-dependent inhibition of virus production was observed. A similar effect was also observed in the time course experiment (Fig. 3B). This strongly suggests that ACHP compelled a defect in the TNF-α-induced NF-κB activation in HIV. No significant induction was observed with ACHP alone. An effective concentration for 50% reduction of HIV production was estimated to be approximately 0.56 μM. Meanwhile, the 50% cytotoxic concentration (CC_{50}) was approximately 15 μM, and thus, the estimated therapeutic window of ACHP was approximately 27 (Fig. 3D). Moreover, immunoblotting of whole-cell extracts (Fig. 3C) with the pooled sera from individuals infected with HIV-1 revealed inhibition of the envelope (*env*) gene products gp160 and gp41 and the *gag* gene products p66, Pr55, and p39 in the presence of 10 μM ACHP, thus confirming the effect of ACHP on the viral-production step.

We also explored the effect of ACHP on viral production from MOLT4/III_B cells chronically infected with HIV-1 (Fig. 4). Whereas ACHP efficiently suppressed viral production in latently infected cells, no significant inhibition was observed in chronically infected cells. A slight decrease in HIV production at 10 μM ACHP, although not statistically significant, was presumably due to a nonspecific cytotoxic effect of ACHP (Fig. 4A, left). The presence of constitutive viral production in the absence of TNF-α suggests the involvement of multiple mechanisms in the regulation of HIV-1 replication (24). From the cytotoxicity profile of ACHP, a CC_{50} value of 11 μM was obtained for these cells (Fig. 4A, right). There was no effect of ACHP on the level of HIV production without TNF-α (Fig. 4B). These results also imply that the anti-HIV-1 activity of ACHP in chronic infection is cell type specific, as previously reported by others (46).

ACHP suppressed NF-κB binding to HIV-1 LTR. HIV-1 replication, particularly in latently infected cells, such as OM10.1, is promoted by NF-κB binding to the HIV-1 LTR upon induction with TNF-α (37, 44, 55). To assess whether NF-κB binding to the HIV-1 LTR is inhibited by ACHP, ChIP assays were performed. OM10.1 cells were preincubated with ACHP and stimulated with TNF-α for 10 min, the time at which maximum NF-κB binding was observed in repeated experiments. Following stimulation, the cells were cross-linked

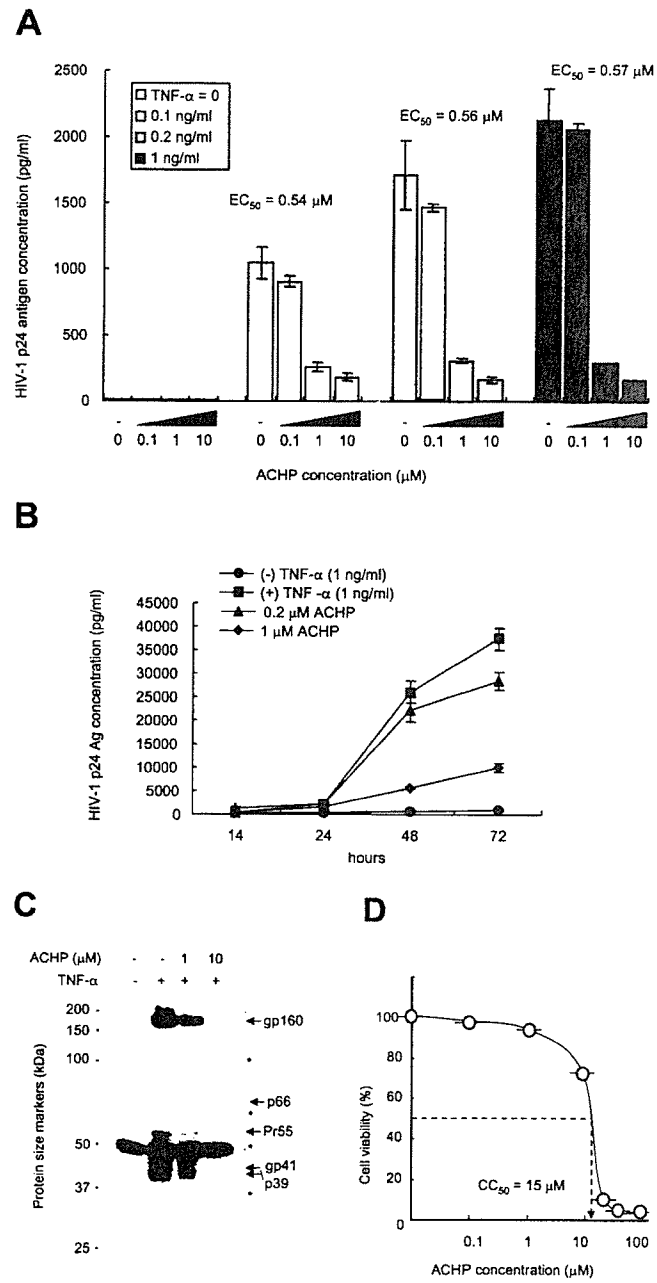

FIG. 3. Effect of ACHP on TNF-α-induced HIV-1 production in latently infected cells. (A) OM10.1 cells (2×10^5/ml) were pretreated with or without ACHP for 1 hour and then stimulated with TNF-α (1 ng/ml) for 24 h. The cell supernatants were then collected and analyzed for HIV-1 p24 antigen levels using a commercial ELISA kit. The data are mean values ± standard deviations of triplicate experiments. EC_{50}, 50% effective concentration. (B) Time course experiment showing the effect of ACHP in OM10.1 cells. Cells (2×10^5/ml) were pretreated in the absence or presence of ACHP for 1 hour and then stimulated with or without TNF-α (1 ng/ml) for 24 h. The cell supernatants were then collected and analyzed for HIV-1 p24 antigen levels using a commercial ELISA kit. The data are mean values ± standard deviations of triplicate experiments. (C) Whole-cell extracts were prepared and immunoblotted with human HIV-1-infected serum for the analysis of HIV-1 viral proteins (whole-cell extracts). The location of each viral product is indicated by an arrow. *, nonspecific bands. (D) Cytotoxicity of ACHP on OM10.1 cells. Cell viability was determined by the WST-1 method, and the CC_{50} value was extrapolated from this measurement.

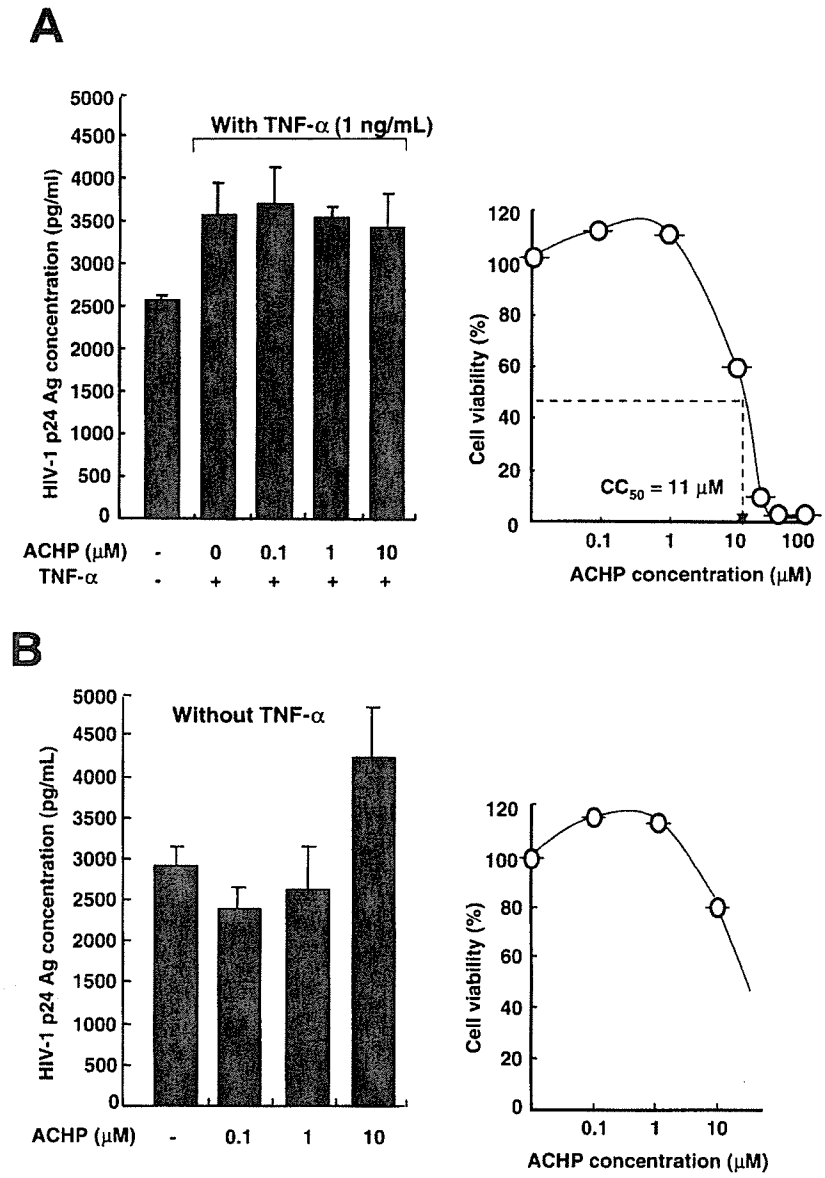

FIG. 4. Effect of ACHP on chronically HIV-1 infected cells. (A and B, left) Effect of ACHP on HIV-1 production. MOLT4/IIIB cells (2×10^5/ml) were pretreated with or without ACHP for 1 hour and then stimulated with or without TNF-α for 24 h. Cell supernatants were then collected and analyzed for HIV-1 p24 antigen levels by ELISA. (A and B, right) Cytotoxicity of ACHP on MOLT4/IIIB cells. Cell viability was determined by the WST-1 assay. The CC_{50} value was extrapolated from this measurement. The data are mean values plus standard deviations of triplicate experiments.

and lysed, and DNA was sheared by sonication. The lysates were immunoprecipitated with anti-p65 antibody and agarose beads, and DNA-protein cross-links were reversed. The p65-immunoprecipitated samples and controls were probed by PCR for HIV-1 LTR sequence containing NF-κB binding sites. In agreement with our Western blotting results, the kinetics of NF-κB binding to the HIV-1 LTR showed a rapid recruitment of NF-κB (p65) to the promoter in response to TNF-α (Fig. 5A, first gel, first row). A remarkable association was observed until 30 min and thereafter decreased to an almost undetectable level, suggesting the cytoplasmic export of p65. The presence of constitutive, almost undetectable NF-κB (p65) binding in the HIV-1 LTR in unstimulated cells again correlated with our Western blot analysis of nuclear p65, thus conforming to previous studies (19, 26). Similarly, NF-κB (p65) binding in the IκBα promoter was also detected, although with a slower onset of association (Fig. 5A, second gel, first row).

In contrast, in the presence of ACHP, both basal and TNF-α-induced p65 recruitment in both promoters was abolished in a dose-dependent manner (Fig. 5B, first and second gels, first row). No amplification was detected with the β-actin promoter (internal control) or in the absence of anti-p65 antibody, confirming the specificity of the DNA immunoprecipitation (Fig.

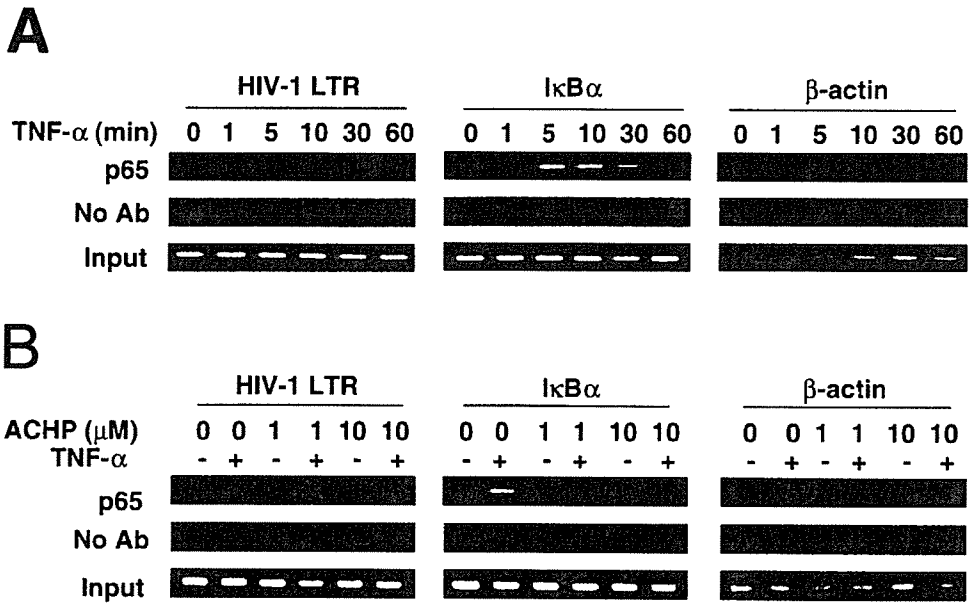

FIG. 5. ChIP assays for NF-κB (p65) binding to promoters. (A) Kinetics of TNF-α-induced NF-κB binding to the HIV-1 LTR. Ab, antibody. (B) ACHP-mediated inhibition of TNF-α-induced NF-κB binding to the HIV-1 LTR. OM10.1 cells (1×10^5/ml) were treated with TNF-α (1 ng/ml) for the indicated time periods, and ChIP assays were performed with anti-p65 antibody or no antibody (negative control). The detection of the immunoprecipitated DNAs in the HIV-1 LTR (first gel), IκBα (second gel), or β-actin (third gel) promoter was analyzed by PCR with promoter-specific primers. Input DNA represents total input chromatin (1%).

5B, second row of each gel). Collectively, these findings suggest that ACHP can efficiently block the binding of NF-κB to the HIV-1 LTR promoter.

DISCUSSION

In this study, we have addressed the question of whether a novel IKK inhibitor, ACHP, can inhibit HIV-1 replication in a macrophage/monocyte cell line latently infected with the virus. We observed that TNF-α-mediated NF-κB signaling could efficiently induce HIV-1 replication, which was subsequently blocked by ACHP. IκBα phosphorylation and degradation, p65 nuclear translocation, and p65 phosphorylation at Ser536 were effectively inhibited by ACHP. Moreover, ACHP suppressed HIV-1 LTR-driven gene expression through the inhibition of the NF-κB activation pathway. We also found, using a ChIP assay, that TNF-α could activate NF-κB (p65)–DNA binding to the HIV-1 LTR in OM10.1 cells and that treatment with ACHP could abolish its binding.

Although NF-κB plays a central role in mediating inducible HIV-1 gene expression (44, 48, 52), the coordinated HIV-1 replication with the cellular activation is partially ascribed to the ability of HIV to assimilate host signaling pathways to activate viral transcription (24, 55). An essential step in the stimulus-induced activation of the canonical NF-κB pathway is the phosphorylation of IκB proteins by the IKK complex (69). In addition, Asin et al. (2) demonstrated that HIV infection itself could induce NF-κB activation through the canonical pathway involving activation, that is, the Ser32 and Ser36 phosphorylation of IκBα by IKK-β, thus highlighting the role of NF-κB in HIV latent infection (11). In fact, transdominant mutants of IκBα that block NF-κB induction inhibited de novo HIV-1 infection in T cells by interfering with viral transcription (32, 54). Furthermore, CD4 engagement with gp120 selectively enhanced IKK activity and mediated the phosphorylation of IκBα, while dominant-negative forms of IKKs inhibited gp120-induced NF-κB activation (11). Moreover, recruitment of NF-κB to the HIV promoter is considered essential for the action of Tat and efficient transcriptional elongation (9, 67). Recent studies using ChIP assays have indicated the importance of IKK-α for induction of NF-κB-mediated gene expression by forming a complex with p65 or CBP in these promoter regions and thus regulating histone H3 phosphorylation, followed by acetylation of CBP (1, 70).

Whereas IKK-β is largely responsible for cytokine-induced IκBα phosphorylation and NF-κB activation (22, 35, 36), IKK-α was initially implicated in more specified biological actions, such as formation of secondary lymphoid tissues (39, 62). However, recent studies have revealed an essential role of IKK-α in the noncanonical/alternative pathway of NF-κB activation, such as lymphotoxin β receptor signaling (28) and NIK-induced p100 processing (16, 17, 18, 22, 60). Moreover, IKK-α has been implicated in the phosphorylation of p65 at Ser536 (7, 28, 66, 72). Of note, the point mutation of Ser536 eventually resulted in the failure of nuclear translocation of NF-κB (40). In addition, since Ser536 is located in the carboxyl-terminal transactivation domain of p65, it is postulated that the phosphorylated NF-κB (p65 at Ser536), once bound in the target DNA, might further recruit basal transcription factors and transcriptional coactivators, thereby increasing the transcriptional competence of NF-κB (p65) (28).

In spite of a robust effect in latently infected cells, ACHP did not show a significant inhibitory effect in chronically infected

cells in which HIV-1 was actively replicating. The reason for the unresponsiveness of these cells to the compound is unclear. A previous study of the effects of an NF-κB inhibitor (4, 46) against chronic HIV infection reported it to be ineffective in suppressing constitutive HIV-1 production, which is consistent with what we observed in this study. Hence, it is also possible that the cellular uptake or intracellular metabolism of ACHP is different in OM10.1 and MOLT4/III_B cells. Another plausible explanation is that the constitutive activation of NF-κB in chronically infected T cells (24, 31, 55) perpetuates the production of other viral proteins, such as Tat and Nef, yielding more important roles for such proteins in activating and regulating the expression of HIV-1 than for NF-κB in these cells. Thus, NF-κB-independent mechanisms appear to be operating in chronic HIV-1 infection.

Although it is evident that ACHP is not potent in actively replicating cells, considering the presence of latent reservoirs that are sources of viral rebounds and its contribution to disease progression (24), a rationale exists for the use of the compound in HIV-1 infection. Apparently, given its mechanism, use of ACHP might cause aberrant regulation of inflammatory cytokines (5, 10, 43). Therefore, further studies are needed to further evaluate its feasibility as a potential drug candidate for novel anti-HIV therapy.

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