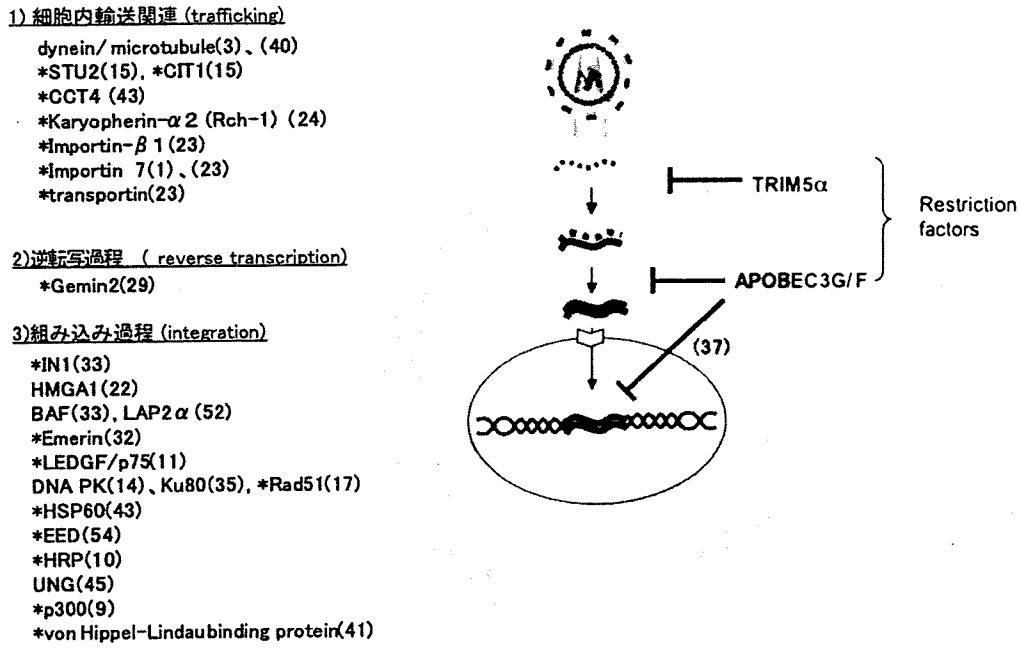

図 2 HIV-1 感染初期過程に関与する宿主因子群
HIV-1 が宿主細胞に吸着/侵入後，ウイルスゲノムが逆転写され染色体 DNA に組み込まれるまでを感染初期過程とよぶ。HIV-1 の感染初期過程において相互作用する宿主因子を細胞内輸送関連，逆転写過程，組み込み過程の各ステップごとに羅列し，括弧内にその関連文献を示す。＊は HIV-1 インテグラーゼとの直接相互作用することが報告された宿主因子を示す。

が存在する。この複合体はウイルスゲノムの状態すなわち逆転写開始以前にあるものと逆転写以降組み込み以前とで大きく 2 つに分けられる[26]。前者は逆転写複合体（reverse transcription complex）とも呼ばれるが，その具体的な構成に関しては未だ十分に解析されていない。一方で，後者の複合体は，含有するウイルス cDNA を外来性のプラスミド DNA に組み込む活性があることからプレインテグレーション複合体（Preintegration complex）と呼ばれている[2]。プレインテグレーション複合体には，逆転写酵素，インテグラーゼに加えてマトリックス蛋白，Vpr 蛋白などがその構成蛋白として報告されている[5]。

MacDonald らは，微小管にトラップされた HIV-1 プレインテグレーション複合体を電子顕微鏡でとらえることに成功し，大きさは直径約 100 nm，長さ 400-700 nm のシリンダー状の形状であることを報告した[40]。核膜孔を単純拡散により通過可能な分子のリミットが直径にして約 28 nm とされていることを考えれば，優にその許容範囲を超えていることになる。したがって，非分裂状態にある細胞に感染を成立させるためには，ウイルスゲノムを効率よく核膜孔を通過させる必要がある。インテグラーゼとの相互作用があると報告された核内輸送に関連する宿主因子としては，これまでに Rch-1[24] や importin7 などの importin ファミリー蛋白[1,23] および核膜の裏打ち構成蛋白のひとつである Emerin が報告されている[32]。しかしながら，これら宿主因子の関与に関しては否定的な論文[48,57] も報告されているので，さらなる探索が必要であろう[1,4]。

逆転写過程：著者らは，酵母 two-hybrid 法により HIV-1 インテグラーゼと結合する新規宿主因子 Gemin2 を同定した[29]。この因子はスプライシングに関与する snRNP 複合体の細胞質内アッセンブリーおよび核内輸送に関与する SMN 複合体の構成因子のひとつとして同定されていた[36] が具体的な役割は U snRNP（U small nuclear ribonuleoproteins）および他のリボヌクレオ複合体のアッセンブリーおよび核内輸送への関与は示唆されているが，未だ不明な点も多い[44]。我々は，Gemin2 が HIV-1 感染後すみやかにインテグラーゼおよびプレインテグレーション複合体と相互作用し，ウイルスゲノムの逆転写反応をサポートするという結果を報告した。さらに，SMN 複合体の構成因子を個々に検討した結果，Gemin2 は既知の SMN 複合体として機能するのではなく，Gemin2 単独もしくは他の未知の宿主因子との複合体として HIV-1 の逆転写過程をサポートしているのではないかと考えている。また，Gemin2 は HIV-1 が吸着侵入直後の逆転写複合体（RTC）の段階ですでに相

互作用するという結果も得ている（未発表）。

一方，インテグラーゼと逆転写酵素の物理的かつ機能的相互作用の報告もなされていたが[30,56]，つい最近，無細胞逆転写アッセイ系において，インテグラーゼ自体にも逆転写促進活性があるという報告がなされた[18]。これらの事実から，逆転写過程におけるインテグラーゼの直接的かつ機能的関与が示されたことになる。

組み込み過程：プロウイルDNAは，宿主細胞の染色体DNAにランダムに組み込まれるのではなく，転写ユニット内に指向性が高いことが知られていた[46]。このHIV-1の組み込み部位の選択に関与する宿主因子として，LEDGF（Lens Epithelium-Derived Growth Factor/p75）が注目されている[13]。LEDGF/p75は，もともとは，転写補助活性化因子であるPC4と相互作用する75kDaの蛋白として[25]，また後にlens epithelial細胞由来のcDNAライブラリーから白内障患者由来の抗体と反応する自己抗原として同定されていた分子である[49]。HIV-1インテグラーゼとの相互作用は，293T細胞で発現させたインテグラーゼと相互作用する分子として報告された[11]。現在までにLEDGF/p75と相互作用が確認されているインテグラーゼはHIV-1，HIV-2，FIVのいずれもレンチウイルス由来であり，γ-レトロウイルスであるMo-MLV，RSVやδ-レトロウイルスであるHTLV-1とは結合しないことが報告されている[7]。こうした生化学的な事実は，各レトロウイルスの組み込み部位の指向性とも相関しており，LEDGF/p75とHIV-1インテグラーゼの構造解析も進んでおり[12]，今後新たなHIV-1阻害剤開発にも貢献が期待されている。

最近，核内のインテグラーゼと結合し，プロテアゾーム経路で分解を促進する宿主因子，VBP1（von Hippel-Lindau binding protein 1）が報告された[41]。この分子はユビキチンライゲース活性をもつcullin-2/VHL複合体と結合することで，インテグラーゼのプロテアゾーム分解を促進するとのことである。VBP1もしくはcullin-2/VHL複合体の構成因子をノックダウンするとウイルス感染後の逆転写過程や組み込みには影響しないが，その後のHIV-1の転写が低下する。しかしながら，tat蛋白によるHIV-1プロモーターの転写活性化には直接関与しないことからインテグラーゼによるウイルスゲノムの組み込みが完了したあとのDNA修復系のリクルートもしくは転写因子のリクルートのためのプロウイルスDNAのリモデリングに関与しているとのことである。組み込み過程以降に関与するインテグラーゼ結合宿主因子として新しい作用点を提示した研究であり，今後の展開が期待される。

その他の組み込みおよびその後の修復に関与する宿主因子も数多く報告されている[51]。また，DNA修復に関与するRAD51[17]やAPOBEC3G/F[37]が，インテグラーゼと相互作用し，組み込み過程阻害に働くという報告もなされた。しかしながら，HIV複製における意義において今後さらなる検討が必要と考えるのでここでは，図中において各因子に関連する論文引用のみにとどめておくこととする。

おわりに

インテグラーゼに結合しHIV複製にもその関与が示唆されている宿主因子を中心に概説した。HIV-1ゲノムの組み込み指向性に関与するLEDGF/p75と組み込み過程以降に機能するVBP1は，両者ともインテグラーゼのセントラルドメイン内の，お互いかなり重複した領域に結合する。一方で，逆転写過程をサポートするGemin2との結合責任ドメインはインテグラーゼのC-末端領域にマップされている。このように，ウイルス感染初期過程の一連の反応のどこかで，逆転写複合体，プレインテグレーション複合体そして組み込み過程後のインテグラーゼにはなんらかの構造変化がともないその結果，宿主因子の受け渡しがあるのかもしれない。インテグラーゼがいかにして多岐にわたる宿主因子と相互作用するのか？　またその機構と意義に関しては今後の重要課題である。

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Reactivation of Latent HIV-1 Infection by the Periodontopathic Bacterium *Porphyromonas gingivalis* Involves Histone Modification[1]

Kenichi Imai,* Kuniyasu Ochiai,† and Takashi Okamoto[2]*

Latently infected cells harbor the HIV-1 proviral DNA genome primarily integrated into heterochromatin, allowing the persistence of transcriptionally silent proviruses. Hypoacetylation of histone proteins by histone deacetylases (HDAC) is involved in the maintenance of HIV-1 latency by repressing viral transcription. In addition, periodontal diseases, caused by polymicrobial subgingival bacteria including *Porphyromonas gingivalis*, are among the most prevalent infections of mankind. Here we demonstrate the effects of *P. gingivalis* on HIV-1 replication. This activity could be ascribable to the bacterial culture supernatant but not to other bacterial components such as fimbriae or LPS. We found that this HIV-1-inducing activity was recovered in the lower molecular mass (<3 kDa) fraction of the culture supernatant. We also demonstrated that *P. gingivalis* produces high concentrations of butyric acid, acting as a potent inhibitor of HDACs and causing histone acetylation. Chromatin immunoprecipitation assays revealed that the corepressor complex containing HDAC1 and AP-4 was dissociated from the HIV-1 long terminal repeat promoter upon stimulation with bacterial culture supernatant concomitantly with the association of acetylated histone and RNA polymerase II. We thus found that *P. gingivalis* could induce HIV-1 reactivation via chromatin modification and that butyric acid, one of the bacterial metabolites, is responsible for this effect. These results suggest that periodontal diseases could act as a risk factor for HIV-1 reactivation in infected individuals and might contribute to the systemic dissemination of the virus. *The Journal of Immunology,* 2009, 182: 3688–3695.

Expression of the HIV-1 gene is the major determinant of the viral replication rate leading to disease progression of AIDS. After HIV-1 infection, integrated HIV-1 proviral DNA is incorporated into nucleosomes, and the transcriptional activity of the provirus from its long terminal repeat (LTR)[3] is under the control of the regional nucleosomal structure (1–4). Induction of HIV-1 gene expression is triggered by signal transduction from various extracellular factors such as cytokines (1–4). Similarly, it was demonstrated that histone deacetylase (HDAC) inhibitors such as trichostatin A and sodium butyrate could induce viral gene expression (5–8). In contrast to productively infected cells, latently infected cells harboring HIV-1 genomes integrated into heterochromatin structures that allow persistence of transcriptionally silent proviruses (9, 10). Hyperacetylation of core histone proteins adjacent to the HIV-1 provirus was correlated with transcriptional activation from HIV-1 LTR (5, 6, 11), whereas hypoacetylation mediated by HDAC was correlated with its repression (12–16). For example, we reported that AP-4 acts as a transcriptional repressor by recruiting HDAC molecules and is involved in the maintenance of viral latency (14). However, at present, no clinical observation has been reported suggesting the involvement of HDAC activity in the maintenance of HIV latency and the causal link between HDAC inhibition and the breakdown of viral latency.

A causal link between infection by various microbes in HIV-infected individuals and disease progression of AIDS has been often documented in the context of the ability of coinfecting microbes and their products to augment HIV replication. For example, coinfection with other pathogens, such as infection with mycobacteria and herpesviruses, is associated with increased viral replication (17, 18). Inflammation is also known to stimulate HIV-1 gene expression and replication, given that infection of these pathogens usually involves production of proinflammatory cytokines that are associated with NF-κB activation (17, 18).

Periodontal diseases are initiated by bacteria, including *Porphyromonas gingivalis*, and are associated with chronic inflammation (19–21). They are highly prevalent, affecting 50–90% of the worldwide population (19). Mounting evidence has accumulated supporting a role of *P. gingivalis* infection as a risk factor for several systemic diseases, including preterm birth, heart disease, diabetes, and atherosclerosis (19, 22–27). These associations indicate a possible role for *P. gingivalis* infection in the etiology of various systemic diseases.

Oral manifestation is one of the early signs of AIDS or HIV infection (28, 29). However, it has not been established whether or not periodontal diseases are implicated in the etiology of HIV infection. It is known that expression of the HIV-1 receptors/coreceptors was increased by chronic periodontitis (30). Moreover, it was recently demonstrated that *P. gingivalis* could up-regulate

*Department of Molecular and Cellular Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; and †Department of Microbiology, Nihon University School of Dentistry, Tokyo, Japan

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[2] Address correspondence and reprint requests to Dr. Takashi Okamoto, Department of Molecular and Cellular Biology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, Aichi 467-8601, Japan. E-mail address: tokamoto@med.nagoya-cu.ac.jp

[3] Abbreviations used in this paper: LTR, long terminal repeat; Ac, acetylated; BHI, brain-heart infusion; ChIP, chromatin immunoprecipitation; csp, culture supernatant of *Porphyromonas gingivalis* FDC381; HAT, histone acetyltransferase; HDAC, histone deacetylase; SCFA, short-chain fatty acid; YY, Yin Yang.

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CCR5 in oral keratinocytes (31) and thus facilitate the subsequent infection of HIV into permissive cells such as macrophages in a CCR5-dependent manner (32). In addition, there was a significant correlation between the clinical stage of periodontitis and the HIV-1 proviral DNA load in gingival crevicular fluid as well as the HIV-1 RNA viral load in plasma (33, 34) and saliva (34). More *P. gingivalis* organisms were found in the HIV-1-positive individuals than in the otherwise healthy controls (35, 36).

In this study, we examined the effects of *P. gingivalis* on HIV-1 replication. Our results suggest that *P. gingivalis* infection can strongly induce the reactivation of latent HIV-1 proviruses via chromatin modification by producing butyric acid. This is the first demonstration of a molecular link between a bacterial metabolite and AIDS progression.

Materials and Methods

Cell culture

ACH-2 and U1 cells were obtained from the AIDS Research and Reference Reagent Program (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). These cells were maintained at 37°C in RPMI 1640 (Sigma-Aldrich) with 10% FBS (Sigma-Aldrich). To maintain the latency of HIV-1 in ACH-2 and U1, 20 μM azidothymidine (Sigma-Aldrich) was added in the culture medium and excluded before the experiments. For stimulation experiments, cells (0.5×10^6 cells/ml or 1.0×10^6 cells/2.0-ml well) were treated with bacteria, culture supernatant of *P. gingivalis* (csp) or TNF-α (Roche). Human embryonic kidney 293T cells were purchased from the American Type Culture Collection and were grown at 37°C in DMEM (Sigma-Aldrich) with 10% heat-inactivated FBS.

Reagents

The *P. gingivalis* components fimbriae and LPS were obtained as described (37, 38). Arginine-specific (KYT1) or lysine-specific (KYT36) gingipain inhibitors were obtained from the Peptide Institute, and short-chain fatty acids (SCFA) were purchased from Sigma-Aldrich. Neutralizing Abs against human TNF-α and human IL-1β were obtained from R&D Systems.

Bacterial strains and culture conditions

Periodontopathic bacteria including *P. gingivalis* FDC381, W83, and ATCC 33277 as well as *Prevotella nigrescens* (ATCC 33653) were grown in brain-heart infusion (BHI) broth (Difco Laboratories) supplemented with 5% FBS, 5 μg/ml hemin, and 0.4 μg/ml menadione in an anaerobic system (5% CO_2, 10% H_2, and 85% N_2 at 37°C using a model 1024 anaerobic chamber; Forma Scientific) for 48 h. The supernatant was collected by centrifugation at 10,000 × *g* for 20 min at 4°C to remove the bacteria and filter sterilized through a 0.22-μm pore size membrane filter. The supernatant pH values ranged from 6.8–7.0 for *P. gingivalis* and 5.2 for *Prevotella nigrescens*. The resulting supernatants were used at a 1/2.5–1/10 dilution (25–100 μl/ml of cell culture medium). Bacteria were washed three times with PBS (pH 7.2) and standardized to 2×10^6 CFU/ml with serum-free RPMI 1640. Concentrated bacterial suspensions (0.2×10^6 CFU suspended in 100 μl of RPMI 1640) were used as *P. gingivalis* bacterial cells which were added to each well (0.5×10^6 cells/ml) as indicated.

Quantitation of SCFAs

SCFA determinations in culture supernatants of various bacteria including *P. gingivalis* were performed as follows: 1 ml of culture filtrate was acidified by adding 2.0 μl of phosphoric acid followed by addition of 2.0 ml of acetonitrile. After centrifugation to precipitate peptides, the supernatant was injected into a gas chromotograph column (Shimadzu GC-2010). Next, the SCFA were separated by a free fatty acid phase capillary column (15 m long, 0.32 mm inner diameter, and 0.25 μm film thickness). Column oven temperatures were programmed from 70°C–200°C at a rate of 10°C/min, and a flame ionization detector was used to measure SCFA concentrations.

Immunoblot assay

The experimental procedures for immunoprecipitation and immunoblotting were performed as previously described (14, 39). Briefly, cells were harvested with lysis buffer (25 mM HEPES-NaOH (pH 7.9), 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.3% Nonidet P-40, 1 mM DTT, 0.5 mM PMSF), the proteins separated by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond C; Amersham). For analysis of histone 4, the cell lysate were prepared by acid extraction as described previously (40). To detect HIV-1 proteins, the cell lysates were subjected to immunoblotting using sera from AIDS patients. To evaluate the levels of histones and their acetylated (Ac) forms, cells were treated with the lysis buffer and the cell lysates were then analyzed by immunoblotting with anti-Ac-histones 3 and 4 (Upstate Biotechnology) or anti-histone 4 (Cell Signaling Technology) Abs.

Luciferase assay

The luciferase expression plasmid under the transcriptional control of HIV-1 LTR, CD12-luc (containing the HIV-1 LTR U3 and R), was previously described (14). The 293 cells (2×10^5 cells/1 μl well) cultured in 12-well plates were transfected using Fugene-6 transfection reagent (Roche). The transfected cells were subjected to luciferase assays with the Luciferase Assay System (Promega). All of the experiments were conducted in triplicate, and the data were presented as the fold increase in luciferase activities (mean ± SD) relative to the control of three independent transfections.

Preparation of mRNA and RT-PCR

Total cellular RNA was prepared from each cell clone using RNeasy (Qiagen), and RT-PCR was performed as described (39). For cDNA synthesis, 2 μg of total RNA were reverse transcribed using random primers and Superscript Reverse Transcriptase III (Invitrogen). The cDNA was then amplified from each RNA sample with *Taq* Mastermix (Qiagen). The primer sequences for each amplified gene were as follows: *gag*, forward (5′-TTGCCAAAG AGTGACCTGAGGGAA-3′) and reverse (5′-GGGGGGACATCAAGC AGCCATGC-3′); *env*, forward (5′-CTTGCTCTCCACCTTCTTCTTC-3′) and reverse (5′-CCAATTCCCATACATTATTGTG-3′); *β-actin*, forward(5′-CAGCAAGCAGGAGTATGACGA-3′)andreverse(5′-GTGGAC TTGGGAGAGGACTGG-3′). PCR products were separated on 1.5% agarose gels and visualized by ethidium bromide staining.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed according to the provider's protocol (Upstate Biotechnology) with some modifications as previously described (14, 41). In brief, chromatin from cross-linked cells was sheared by sonication 13 times for 10 s at one-third of the maximum power of a Microson XL sonicator (Wakenyaku) with 20 s of cooling on ice between each pulse and incubated with specific Ab followed by incubation with protein A-agarose beads saturated with salmon sperm DNA. The precipitated DNA was analyzed by PCR (31–33 cycles) with *Taq* Mastermix and primers for HIV-1 LTR (−176 to +61; forward, 5′-CGAGACCTGCATCCGGAGTA-3′; reverse, 5′-AGTTTTATTGAGGCTTAAGC-3′). For each reaction, 10% of the recovered DNA was used as an input control.

Antiviral assay and measurement of viral p24 Ag

The p24 Ag level in the cell culture supernatant was measured by a p24 Ag capture ELISA assay using a commercial kit (RETRO-TEK HIV-1 p24 Ag ELISA kit; Zepto Metrix; Refs. 14 and 41).

Results

Activation of HIV-1 by the periodontopathic bacterium P. gingivalis

We examined the effects of *P. gingivalis* on the level of viral replication from cells latently infected with HIV-1. We used human cell lines known to harbor latent HIV-1 proviruses, ACH-2 and U1 cells, derived from human CD4 T and macrophage cell lines, respectively, which were the best studied models of postintegration latency (42–45). As demonstrated in Fig. 1, upon treating these cell lines with csp, the levels of viral production in the supernatant or in the cytoplasm were substantially increased. Similar effects were observed using another cell line, OM10.1 (46), a monocyte cell line latently infected with HIV-1 (data not shown). Induction of viral production from these latently infected cells was observed in a dose-dependent manner relative to csp concentration (Fig. 1, *A* and *B*). The extents of stimulation were comparable with that elicited by TNF-α as a positive control. No such effects were observed when cells were treated solely with bacterial medium or *P. gingivalis* bacterial cells cultured in a nonoptimal growth medium

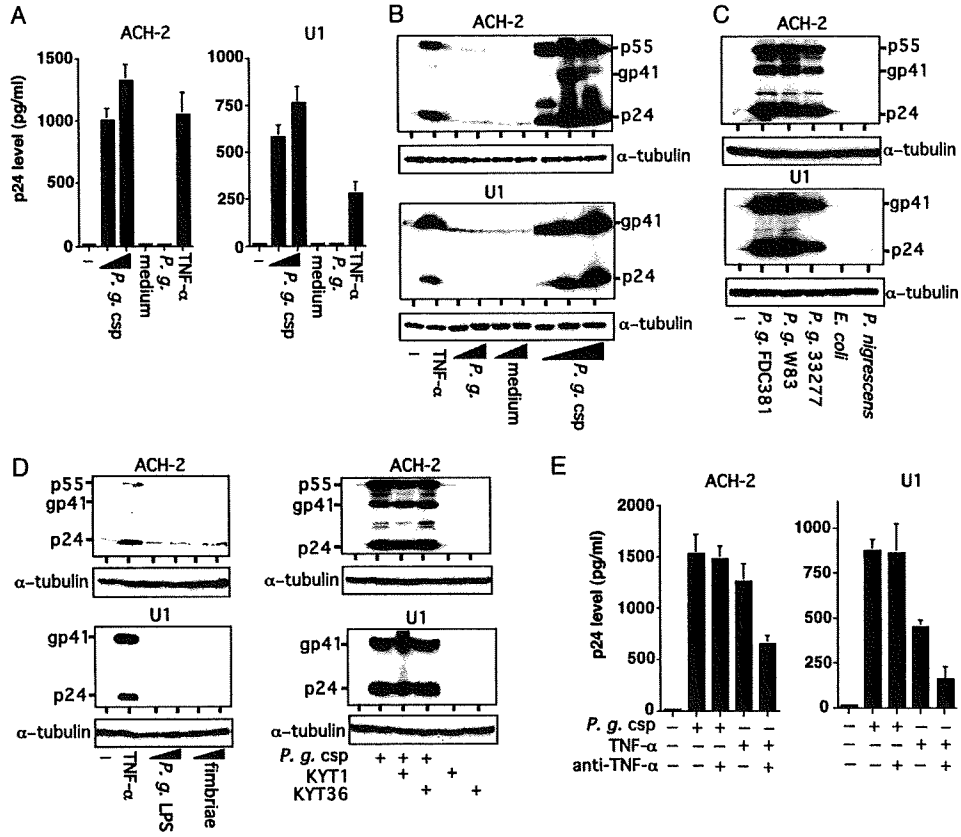

FIGURE 1. Activation of HIV-1 by the periodontopathic bacterium *P. gingivalis* (P. g.). *A–C*, csp activates the latently HIV-1-infected cells. Latent HIV-1-infected ACH-2 and U1 cells were incubated either with or without csp (25, 50, or 100 μl/ml), *P. gingivalis* FDC381 bacilli, bacterial growth medium (BHI broth) alone (medium), or TNF-α (1.0 ng/ml) for 24 h. The culture supernatant and the cell lysate were analyzed for p24 Ag levels by ELISA (*A*) and detection of virus proteins by immunoblotting with AIDS patient sera (*B*). Positions of HIV-1 proteins are indicated on the right. The α-tubulin was used as an internal control. *C*, Stimulation of latent HIV-1 replication by culture supernatants of various bacterial strains. The experiments were similarly performed as in *B*. DH5α, *Prevotella nigrescens*. *D*, Effects of various bacterial virulence factors on the HIV-1 activation. *Left*, ACH-2 and U1 cells were incubated with LPS (0, 0.5 or 5 μg/ml) or fimbriae (1 or 5 μg/ml) for 24 h. The cell lysates were collected and subjected to detection of viral proteins by immunoblots with collected sera from AIDS patients. Similarly, the effects of specific protease inhibitors were examined (*right*). The cells were treated with final concentrations of 5.0 μM arginine-specific (KYT1) or lysine-specific (KYT36) protease inhibitors. After 1 h, cells were treated with csp (100 μl/ml, 10% v/v) and incubated for an additional 24 h. The cells were then harvested, and detection of virus proteins was performed by immunoblot as in *B*. *E*, Effects of Abs to TNF-α. ACH-2 and U1 cells were pretreated with csp (100 μl/ml), and anti-TNF-α Ab (10 μg/ml) was added after 30 min. The cells were then harvested after 24 h and tested for the HIV-1 p24 Ag levels in the culture supernatant. Experiments were repeated at least three times, and representative data are shown. For the data in *A* and *E*, each data point represents the mean ± SD of three independent experiments.

wherein bacteria are in a nonproliferating state. Fig. 1*C* shows that the culture supernatant of all three representative *Porphyromonas* strains, including FDC381, W83, and 33277, could exert such effects. We also examined the effects of several *P. gingivalis* virulence factors, including LPS, fimbriae, and proteases, on the replication of HIV-1. No such activity was found with either the fimbriae or the LPS components of *P. gingivalis* (Fig. 1*D*, *left*). Because *P. gingivalis* is known to produce additional virulence factors such as Arg-specific and Lys-specific gingipains, we treated csp with the corresponding inhibitors, namely, KYT1 and KYT36 (47), respectively. However, the HIV-1-inducing activity was not affected by these gingipain inhibitors (Fig. 1*D*, *right*). Furthermore, because infection of *P. gingivalis* is associated with induction of proinflammatory cytokines such as TNF-α and IL-1β (21), we examined whether Ab against TNF-α or IL-1β could block the effect of csp. As demonstrated in Fig. 1*E*, anti-TNF-α Ab could not eliminate this effect. Similar observations were also obtained with anti-IL-1β Ab (data not shown). These findings indicate that the HIV-1-inducing activity was not ascribable to these bacterial virulence factors of high molecular mass (LPS, fimbriae, proteases, etc.) or cellular proinflammatory cytokines induced by *P. gingivalis* infection.

Involvement of butyric acid in the reactivation of latent HIV-1

We further investigated the nature of the HIV-1-inducing factor contained in csp. When csp was fractionated by filtration through YM3 filters (Millipore), the HIV-1-inducing activity was recovered in the pass-through lower-molecular-mass (<3000 Da) fraction (Fig. 2*A*). Moreover, when csp was extensively heated at 80°C for 96 h with helium gas, such activity was completely lost (Fig. 2*B*). These findings suggested that the active component contained in csp could be a heat sensitive, volatile, low-molecular-mass (<3000 Da) component. In addition, it was previously reported that the extracellularly secreted SCFA species of *P. gingivalis*, most likely butyric acid, could be involved in periodontal diseases (48, 49). Moreover, the presence of high concentrations of butyric acid in periodontal pockets was demonstrated by others (50, 51).

To examine the effects of SCFA on HIV replication from the latently infected cells, we measured the concentrations of each

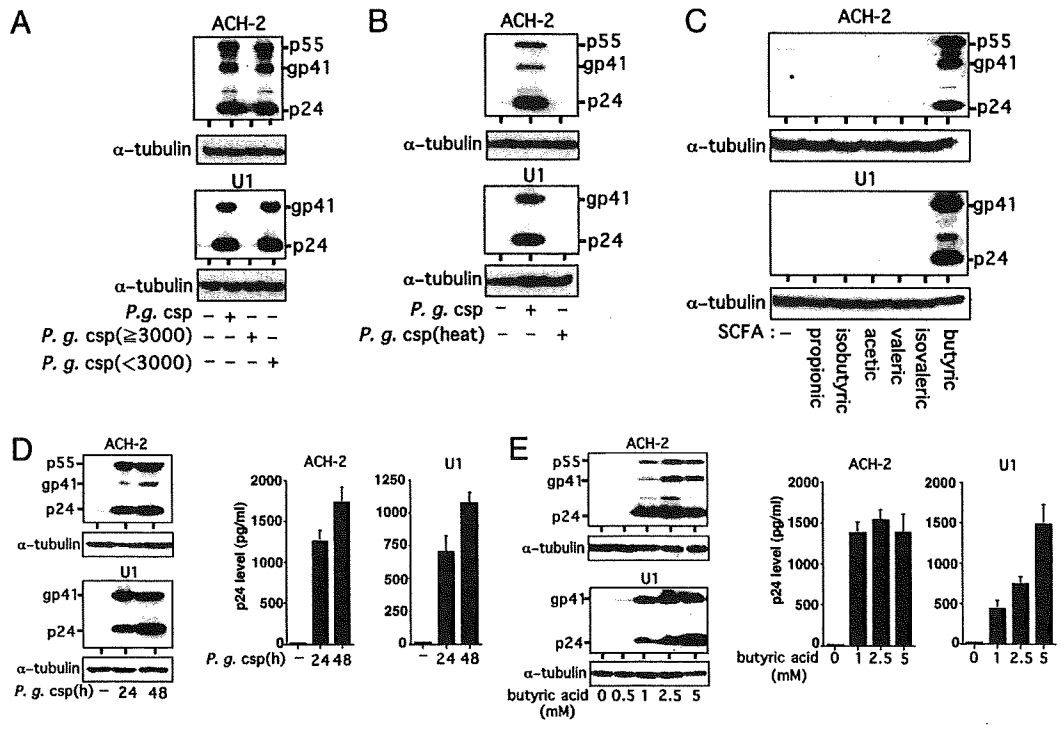

FIGURE 2. Butyric acid is the active component of the *P. gingivalis* culture supernatant (csp). *A*, Fractionation of csp via molecular sieve. The culture supernatant of *P. gingivalis* (P. g.) FDC381 (csp) was fractionated by filtration using centrifugal filters (YM3 with an exclusion molecular mass of 3000 Da; Millipore). The HIV-1-inducing activity was examined with the filtered (<3000 Da) or nonfiltered fractions (≧3000 Da) of csp. ACH-2 and U1 cells were incubated in the presence of these samples (100 μl/ml, 10% v/v) for 24 h. The HIV-1 proteins were detected by immunoblots as in Fig. 1*B*. *B*, Heat treatment of csp. The csp was heated at 80°C for 96 h with helium gas aeration. ACH-2 and U1 cells were incubated with the heat-treated csp or its control for 24 h, and the cell lysates were analyzed for viral proteins. *C*, Effects of various SCFA on the induction of HIV-1 replication from latently infected cells. ACH-2 and U1 cells were incubated either with or without SCFA (2.0 mM) for 24 h, and viral proteins were detected as above. *D* and *E*, Dose-dependent effects of butyric acid in inducing HIV-1 reactivation from the latently infected ACH-2 and U1 cells. *D*, ACH-2 and U1 cells were incubated with csp (obtained from the 24- or 48-h bacterial cultures) for 24 h. *E*, ACH-2 and U1 cells were incubated with butyric acid for 24 h. The cell culture supernatants and cell lysates were collected and subjected to the determination of p24 Ag levels by ELISA and detection of virus proteins by immunoblots with sera from AIDS patients.

SCFA in the bacterial culture supernatants by gas-liquid chromatography. Table I shows production of different SCFA species from various *Porphyromonas* bacteria as metabolites. Butyric acid was the major SCFA species produced by pathogenic *Porphyromonas* strains. However, the weakly pathogenic *Prevotella nigrescens* or the control bacterium *Escherichia coli* did not produce much butyric acid. *P. gingivalis* culture supernatant contained acetic, isovaleric, and butyric acids in high concentrations, from 6.7 to 27 mM. Propionic or isobutyric acids were produced in moderate concentrations, from 2.2 to 7.5 mM. No detectable concentration (<0.4 mM) of valeric acid was detected.

These findings prompted us to examine the effects of various SCFA species on the induction of HIV-1 protein synthesis in latently infected cells. Fig. 2*C* shows that the HIV-1-inducing activity was solely attributable to butyric acid among various SCFAs secreted by *P. gingivalis*. Therefore, the absence of HIV-1-inducing activity in culture supernatants of *Prevotella nigrescens* or *E. coli* (Fig. 1*C*) is likely due to their inability to produce butyric acid (Table I). In fact, the levels of butyric acid in the pass-through fraction (Fig. 2*A*) or in the heat-treated csp (Fig. 2*B*) correlated well with their HIV-inducing activities (Table II). Similarly, csp prepared from more mature cultures exhibited higher activity in

Table I. *Concentration of SCFA in culture supernatant of each bacterial strain*

Culture Supernatants	SCFA Levels (mM)[a]					
	Propionic	Isobutyric	Acetic	Valeric	Isovaleric	Butyric
BHI[b]	n.d.	n.d.	4.9 ± 0.2	n.d.	n.d.	0.5 ± 0.1
P. gingivalis 381 (24 h)[c]	2.2	4.7 ± 0.3	12.6 ± 0.4	n.d.	9.8 ± 0.5	18.1 ± 0.6
P. gingivalis 381 (48 h)	5.9 ± 0.2	7.5 ± 0.3	12.7 ± 0.3	n.d.	14.7 ± 0.3	27.1 ± 0.9
P. gingivalis W83	5.8 ± 0.1	6.5 ± 0.1	10.2 ± 0.1	n.d.	12.6 ± 0.2	25.4 ± 0.5
P. gingivalis 33277	2.5	2.8	8.5 ± 0.2	n.d.	6.7 ± 0.1	14.9 ± 0.5
E. coli DH5α	0.4	n.d.	6.1 ± 0.2	n.d.	n.d.	0.4
Prevotella nigrescens	n.d.	1.5	15.4 ± 0.7	n.d.	4.5 ± 0.1	n.d.

[a] Each measurement was based on the results of three independent experiments; results are mean ± SD; n.d., not detectable (<0.4 mM).
[b] Control.
[c] *P. gingivalis* FDC381 cultures grown for either 24 or 48 h.

Table II. *Concentration of butyric acid*

Treatments	Butyric Acid (mM)[a]
csp (untreated)	31.0 ± 0.3
csp ≧3000 Da[b]	2.1 ± 0.1
csp <3000 Da[c]	31.0 ± 0.3
csp heat treated[d]	2.5 ± 0.1

[a] Each measurement was based on the results of three independent experiments; results are the mean ± SD.
[b] csp was fractionated by filtration with YM3 (with exclusion molecular mass of 3000 Da) yielding the pass through (<3000) and the retained fractions.
[c] Fractions (<3000) were separated.
[d] csp was heated at 80°C for 96 h.

inducing latent HIV-1 (Fig. 2*D*), which could be explained by the elevated levels of butyric acid secreted during growth (Table I). It was also demonstrated that these effects could be attributable solely to butyric acid alone when added exogenously (Fig. 2*E*). That butyric acid is the major component of csp in inducing the reactivation of latent HIV-1 is consistent with the findings demonstrated in Fig. 1.

Hyperacetylation of histones by butyric acid produced from P. gingivalis

Butyric acid inhibits the enzymatic activity of HDAC by competing with the HDAC substrate at the enzyme active site pocket containing its catalytic center (52, 53), thus stimulating transcription of various genes including HIV-1 (7, 54–57). On the other hand, HIV-1 viral latency is maintained by transcriptional factors such as Yin Yang (YY)-1, p50 homodimer, C-promoter binding factor-1, c-Myc, and AP-4 which in turn recruit HDAC proteins into the vicinity of HIV-1 proviral DNA (12–16). Thus, it is possible that the effect of csp could be through the inhibition of HDAC. We then examined the effect of csp on the viral mRNA levels in the cells latently infected with HIV-1. In these cell lines, csp was shown to induce expression of viral genes (Fig. 3*A*, *left*). Similar results were observed with butyric acid (Fig. 3*A*, *right*). In the latter experiments, equivalent amounts of butyric acid contained in csp were compared.

In Fig. 3*B*, we examined the effects of csp and butyric acid on gene expression from the HIV-1 LTR. As demonstrated here, transient luciferase expression under the control of HIV-1 LTR clearly showed that csp or similar concentrations of butyrate exerted similar effects and that no other SCFA or quiescent *P. gingivalis* bacilli could exert such effects. Given that the effect of butyrate on HDAC does not require de novo protein synthesis (57, 58), this activity is considered to be a direct action of butyrate and not by inducing intermediate genes such as proinflammatory cytokines.

In Fig. 3, *C* and *D*, we examined the effects of csp and butyric acid on histone acetylation. To test the effect of csp on histone acetylation, cell extracts containing histone proteins prepared from

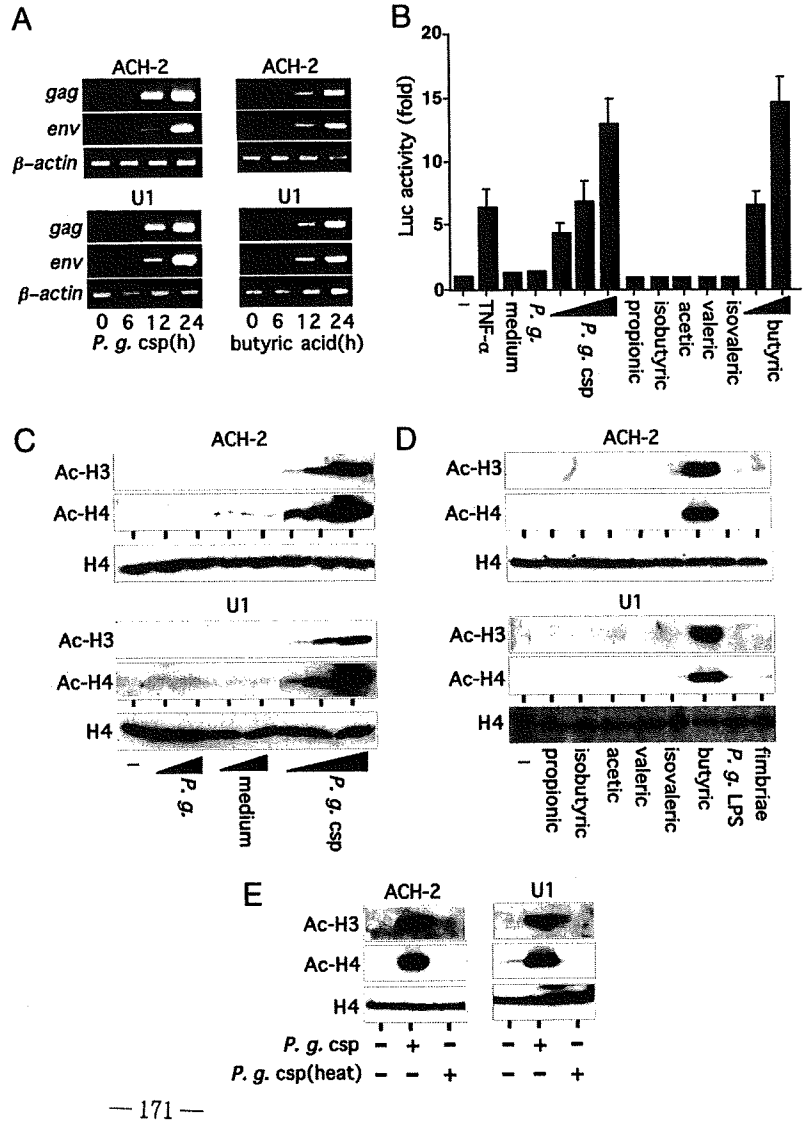

FIGURE 3. Hyperacetylation of histones by csp. *A*, Effects on the viral mRNA levels in latently infected cells. ACH-2 and U1 cells were treated with csp (100 μl/ml, 10% v/v; *left*) or butyric acid (2.0 mM; *right*) for the indicated times. RT-PCR was performed to detect viral mRNA expression with specific primers for the *gag* and *rev* genes. *B*, Activation of HIV-1 transcription by csp. The CD12-luc reporter construct, expressing the *luciferase* gene under the control of HIV-1 LTR (14), was transfected into 293 cells, incubated for 24 h, and stimulated with csp (100 μl/ml), quiescent *P. gingivalis* (P. g.) bacilli, TNF-α (1.0 ng/ml), or SCFA (1.0–2.5 mM) for another 24 h. The luciferase activity of each cell lysate was then measured. *C* and *D*, Induction of histone acetylation by csp or butyric acid. ACH-2 and U1 cells were incubated with the indicated samples for 24 h and analyzed for the Ac histone proteins by immunoblots using Ab specific for Ac-H3 or Ac-H4. The unmodified H4 protein was used as control. *E*, Heat treatment of csp. The csp was heated at 80°C for 96 h with helium gas aeration. ACH-2 and U1 cells were incubated with the heat-treated *P. gingivalis* culture supernatant or its control for 24 h.

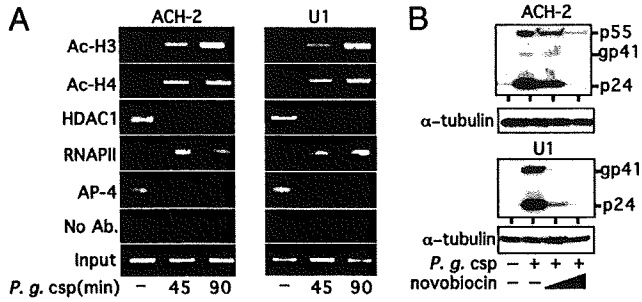

FIGURE 4. *P. gingivalis* (P. g.) facilitates HIV-1 reactivation via chromatin remodeling. *A*, ChIP assays detecting acetylated histone proteins on HIV-1 LTR DNA in ACH-2 and U1 cells. Cells were either untreated or treated with csp (100 μl/ml; i.e., 10% v/v) for the time indicated (in min) and subjected to ChIP assays as described in *Materials and Methods*. The Abs used in the ChIP assays were as indicated on the left. Input DNA (Input) represents 10% of total input chromatin DNA while immunoprecipitation without Ab (No Ab) serves as a negative control. *B*, Effects of novobiocin. ACH-2 and U1 cells were pretreated with the topoisomerase II inhibitor, novobiocin (100 and 200 μg/ml). After 1 h of pretreatment, cells were stimulated with csp (100 μl/ml) and incubated for an additional 24 h; the viral proteins were detected as in Fig. 1*B*.

both ACH-2 and U1 cells were examined by Western blotting with Abs specific for acetylated histones 3 and 4. Although there was no effect with *P. gingivalis* or control culture medium, both csp and butyric acid among various SCFAs could induce histone acetylation. In contrast, no such effect was observed with the LPS or fimbriae components of *P. gingivalis*. Consistent with these findings, when csp was subjected to extensive heat treatment, this activity was totally abolished (Fig. 3*E*; see also Table II). These results clearly show that the HIV-1-inducing activity of csp is exerted by histone acetylation, and this activity could be ascribed to butyric acid.

P. gingivalis *facilitates HIV-1 reactivation via chromatin remodeling*

As demonstrated above, we found that csp could induce histone acetylation and activate latent HIV-1 at the transcriptional level. In Fig. 4*A*, we performed ChIP assays to further examine these effects. We observed that the amounts of Ac histone proteins, Ac-H3 and Ac-H4, bound to the core promoter region (from −176 to +61) within HIV-1 LTR were increased by the treatment of cells with csp. Furthermore, acetylation of these histone proteins occurred concurrently with the recruitment of RNA polymerase II, whereas cellular factors that are negatively involved in HIV-1 gene expression such as HDAC1 and AP-4 repressor (14) were dissociated from the HIV-1 LTR. Essentially the same results were observed with butyric acid at similar concentrations (data not shown).

To determine whether the effects of csp depend on chromatin remodeling, cells were pretreated with novobiocin, a topoisomerase II inhibitor (59). Topoisomerase II is a nuclear matrix-associated enzyme responsible for the cleavage and religation of dsDNA, a prerequisite for the structural reorganization of nuclear chromatin, and thus plays an essential role in the structural remodeling of nucleosomal chromatin (60, 61). We therefore examined whether the activity of csp might involve chromatin remodeling and, if so, could be affected by novobiocin. As demonstrated in Fig. 4*B*, we found that the activity of csp in inducing HIV-1 viral gene expression from ACH-2 or U1 cells was totally abolished after treatment with novobiocin. In contrast, there was no effect on the transcriptional levels of constitutively expressed genes such as α-tubulin.

Discussion

In this study, we explored the biological actions of the culture supernatant of the Gram-negative anaerobic bacterium *P. gingivalis* implicated in periodontal diseases. We found that its culture supernatant, abbreviated as csp, could efficiently induce HIV-1 reactivation, indicating a possible pathophysiological link between periodontal diseases and clinical progression of AIDS in HIV-1-infected individuals. Moreover, our findings have highlighted the role of biochemical modification of nucleosomal histone proteins, acetylation or deacetylation, in the transcriptional activity of the integrated HIV-1 proviruses in that histone acetylation is induced by the metabolite produced from *P. gingivalis* and thus actively involved in the breakdown of viral latency. In fact, butyric acid is regarded as one of the most potent inhibitors of histone deacetylases (52, 53) and is known to modulate expression of a large number of viral and cellular genes (7, 54–57). Here we provide evidence that csp containing butyric acid can inhibit HDACs thus increasing the level of histone 3 and 4 acetylation. This is the first demonstration that butyric acid, contained in the *P. gingivalis* culture supernatant, acts as a strong inducer of transcription from the latent HIV-1 provirus. This was confirmed by ChIP assays and Western blotting with specific Abs against acetylated histones.

The cell lines used in this study contain one (ACH-2) or two (U1) integrated HIV-1 full-length genomes (42–45) and express little viral mRNA, presumably due to a block at the step of transcriptional initiation (62). Further analyses revealed that HIV-1 transcription in these cells could be induced by treatment with HDAC inhibitors such as trichostatin A and sodium butyrate, cytokines or phorbol ester (5–8, 42–45).

Regarding the latency status of HIV infection, other studies (3–6) reported the presence of specific nucleosome configurations of the proviral DNA in these cell lines, which were considered responsible for the maintenance of latency. Two nucleosomes (called Nuc-0 and Nuc-1) were considered present at the viral 5′-LTR (3–6). In particular, the Nuc-1 (+1 to +155 relative to the start site of HIV-1 gene expression) corresponds to a critical promoter region which is involved in transcriptional repression and induction because of the presence of binding sites for a number of host transcriptional regulators such as late SV40 binding factor (LPS-binding protein-1 or upstream binding factor-1), C-terminal fragment/NF1, T cell-specific factor-1, and AP-1 (63, 64). For example, LSF binds YY1 which recruits transcriptional corepressor HDAC1, thus maintaining the transcriptional latency (12). However, other transcription factors binding to the adjacent regions of Nuc-1, such as AP-4 (14), p50 homodimers (13), C-promoter binding factor-1 (15), and Sp1-cMyc complex (16), are known to recruit HDAC proteins leading to transcriptional silencing. Upon stimulation of cells such as by TNF-α, transcriptional activators including NF-κB are recruited together with coactivators exhibiting histone acetyltransferase (HAT) activity. Local histones are then acetylated, the above mentioned negative regulators are disengaged from the promoter region together with HDAC proteins, and thus transcription is initiated. In addition to such directed transcriptional activation involving localized nucleosomal rearrangement near the target sequence for positive transcription factors, a number of reports (5, 6, 8, 11) demonstrated the involvement of the HDAC-HAT equilibrium in regulating the global transcriptional activity and reactivation of latent genes including HIV-1 proviruses. This was demonstrated by using HAT or HDAC inhibitors such as trichostatin A, trapoxin, and sodium butyrate.

Our findings presented here demonstrate that butyric acid produced from *P. gingivalis* could promote gene expression of the latent HIV-1, thus suggesting that infection with *P. gingivalis*

could be one of the risk factors for promoting AIDS progression. More than adequate concentrations of butyric acid for gene activation were detected in the range of 4.7–13.8 mM in the affected dental plaques (65–67) and a mean of 2.6 ± 0.4 mM in periodontal pockets of patients with periodontal disease (50). This suggests that butyric acid may play a role in the initiation of HIV-1 reactivation in individuals latently infected with HIV-1 and could contribute to the clinical progression of AIDS. In agreement, a report by Kashanchi et al. (68) demonstrated the augmentation of HIV-1 replication by butyric acid in latently infected primary mononuclear cells from HIV-1-infected individuals as well as ACH-2 and U1 cells. Our present findings further suggest that other bacteria producing butyric acid, such as *Clostridium*, *Fusobacterium*, and *Eubacterium* in the intestine (69), might also be involved in the accelerated replication of HIV-1. In addition, some bacteria resident in vaginal mucous membranes such as *Peptostreptococcus vaginalis, Peptostreptococcus asaccharolyticus*, and *Peptostreptococcus tetradius* produce butyric acid as well (70–72). This suggests the possibility that the presence of these butyrate-producing bacterial strains may have relevance in the preferred HIV transmission through sexual contact.

In conclusion, because *P. gingivalis* infection is endemic throughout the world, our findings suggest the potential relevance of epidemiological studies to examine the causal link among *P. gingivalis* infection, periodontal diseases, and AIDS.

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Disclosures

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Inhibition of Human Immunodeficiency Virus Type 1 Replication by Blocking IκB Kinase with Noraristeromycin

Kaori Asamitsu[1], Tsuyoshi Yamaguchi[2], Kenji Nakata[1,3], Yurina Hibi[1], Ann-Florence B. Victoriano[1], Kenichi Imai[1], Kikuo Onozaki[3], Yukio Kitade[2] and Takashi Okamoto[1,*]

[1]Department of Molecular and Cellular Biology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, Aichi 467-8601; [2]Department of Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193; and [3]Department of Molecular Health Sciences, Nagoya City University Graduate School of Pharmaceutical Sciences, 3-1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467-8603, Japan

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Nuclear factor κB (NF-κB) is one of the critical transcription factors in inflammatory responses and replication of viruses such as human immunodeficiency virus (HIV). In fact, it has been demonstrated that various NF-κB inhibitors could block HIV replication. To explore more potent NF-κB inhibitors, we focused on carbocyclic adenine nucleosides that had been reported to have anti-inflammatory effects. We synthesized 15 carbocyclic adenine nucleoside compounds and examined their effects on the NF-κB-dependent gene expression using HEK293 cell line. Among these compounds, noraristeromycin (NAM) exhibited the most potent inhibitory effect on the NF-κB activity under the non-cytotoxic concentrations. NAM-inhibited IκBα phosphorylation and degradation upon stimulation of cells with tumour necrosis factor-α (TNF-α). In addition, NAM prevented p65 phoshorylation. These findings suggested that both IκB kinase-α (IKK-α) and -β were targeted by NAM. Interestingly, *in vitro* kinase assay revealed that NAM inhibited the kinase activity of IKK-α more potently than that of IKK-β. When we treated the cell lines, OM10.1 and Molt4/IIIB, in which HIV-1 is latently and chronically infected, we found a strong suppressive effect of NAM on HIV-1 viral replication upon stimulation with TNF-α.

Key words: NF-κB, IKK, noraristreromycin, phosphorylation, transcription.

Abbreviations: ACHP, 2-amino-3-cyano-4-alkyl-6-(2-hydroxyphenyl)pyridine; AZT, 3′-azido-3′-deoxythymidine; cPA, 9-[(1*S*,3*R*)-*cis*-cyclopentan-3-ol]adenine; CREB, cAMP-responsive element binding protein; HIV, human immunodeficiency virus; IKK, IκB kinase; NAM, noraristeromycin; NF-κB, nuclear factor κB; TNF-α, tumour necrosis factor α.

Nuclear factor κB (NF-κB) is an inducible cellular transcription factor that regulates a wide variety of cellular genes involved in the control of the inflammatory and immune response, cellular proliferation, apoptosis and cell-cycle progression (*1–3*). In addition to these kinds of genes, NF-κB is also a potent cellular activator of human immunodeficiency virus type 1 (HIV-1) gene expression (*4*, *5*) and thus positively controls viral replication in the infected cells. In cells chronically infected with HIV-1, activation of NF-κB together with constitutive active Sp1 could trigger the transcription of viral genes including the transactivator Tat that mediates transcriptional elongation of viral expression, culminating in the explosive replication of HIV-1. Thus, down-regulation of NF-κB activity is expected to have beneficial effects on the clinical development of AIDS in HIV infected individuals (*6*).

NF-κB is a hetero- or homo-dimer consisting of Rel family proteins, p65 (RelA), RelB, c-Rel, p50/p105 and p52/p100, and normally present in the cytoplasm in association with its inhibitor protein IκB (*1–3*). Stimulation by the inflammatory cytokines such as TNF-α and IL-1β activates IκB kinase (IKK) complex by upstream kinases such as mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/3 (MEKK1/3) and NF-κB-inducing kinase (NIK) (*7*). IKK is a large molecular weight complex consisting of at least two catalytic subunits, IKK-α, IKK-β and the associated regulatory subunit IKK-γ/NEMO. Activated IKK complex phosphorylates IκB on two specific serine residues (Ser32/36 in IκBα, and this phosphorylation leads to polyubiquitination of IκB, its subsequent degradation by 26S proteasome and thus nuclear translocation of NF-κB (*7*). In addition, we and others have demonstrated that IKK-α and IKK-β could also phosphorylate NF-κB p65 subunit at Ser536, which is crucial for the NF-κB-mediated transactivation of target genes (*8*, *9*).

A number of low-molecular weight compounds have been reported to block NF-κB activity through inhibiting various steps of NF-κB signalling. For example, dehydroxymethylepoxyquinomycin and magnolol inhibit nuclear translocation of NF-κB (*10*, *11*). A serine/threonine kinase inhibitor fasudil hydrochloride blocks HIV-1 replication from latently infected cells through its

*To whom correspondence should be addressed. Tel: +81-52-853-8205, Fax: +81-52-859-1235,
E-mail: tokamoto@med.nagoya-cu.ac.jp

inhibitory effect on the signal transduction of NF-κB by presumably inhibiting IKK (*12*). Moreover, monovalent gold salt compounds, such as aurothioglucose and aurothiomalate, inhibit the DNA binding of NF-κB dimer through its redox mechanism and are thus considered to exhibit clinical benefit in the treatment of rheumatoid arthritis (*13*, *14*). In addition, inhibitors of 26S proteasome such as bortezomib were shown to inhibit IκB degradation and NF-κB activation (*3*, *15*). However, clinical applicability of these compounds is limited due to their relatively poor specificity and/or low bioavailability.

IKK is thought to be a focal target of drug development and several IKK inhibitors have been reported (*16–19*). Carbocyclic adenine nucleosides have been reported to show anti-inflammatory effects by inhibiting TNF-α production from macrophage (*20–22*). One of such compounds, 9-[(1*S*,3*R*)-*cis*-cyclopentan-3-ol]adenine (cPA) was previously reported to inhibit LPS-induced NF-κB activation (*23*) although its effect on IKK has not been explored. In this study, the effects of newly synthesized 15 carbocyclic adenine nucleosides including cPA have been examined on NF-κB-dependent gene expression and IKK. We demonstrate that noraristeromycin (NAM), among these compounds, exhibited the most potent inhibitory effect on the NF-κB activity by blocking the IKK activity at non-cytotoxic concentrations. Furthermore, we also demonstrate a strong suppressive effect of NAM on HIV-1 viral replication from the latently infected OM10.1 and the chronically infected Molt4/IIIB cells.

MATERIALS AND METHODS

Synthetic Carbocyclic Adenine Nucleosides (Fig. 1)—cPA (compound **1**) (*22*), NAM (compound **2**) (*24*) and carbocyclic adenine nucleosides such as compounds **3** (*25*), **4–7** (*24*), **8** (*22*), **9** (*26*) and **15** (*27*) were synthesized as previously described. Compound **10** was easily prepared by osmium-oxidation of 9-[(1′*R*, 4′*R*)-4′-hydroxy-2′-cyclopenten-1′-yl]-9-*H*-2-fluoroadenine (*25*), which was obtained by the Mitunobu reaction of 2-fluoroadenine with ((1*S*, 4*R*)-*cis*-4-acetoxy-2-cyclopenten-1-ol. On the other hand, compound **11** was obtained by osmium oxidation of 9-[(1′*R*, 4′*R*)-4′-hydroxy-2′-cyclopenten-1′-yl]-9-*H*-2,6-diaminopurine, which was prepared by the palladium-coupling reaction of 2-amino-6-chloropurine with (1*S*, 4*R*)-*cis*-4-acetoxy-2-cyclopenten-1-ol and subsequent ammonolysis. Analogous palladium-coupling reaction of 8-azaadenine with (1*S*, 4*R*)-*cis*-4-acetoxy-2-cyclopenten-1-ol and subsequent osmium oxidation gave compound **12**. Ammonolysis of 9-[(1′*R*, 4′*S*)-4′-hydroxy-2′-cyclopenten-1′-yl]-9-*H*-N^6-benzoyladenine (*24*, *27*) and 9-[(1′*R*, 4′*R*)-4′-acetoxy-2′-cyclopenten-1′-yl]-9-*H*-adenine (*27*) afforded the corresponding compounds **13** and **14**, respectively.

Other Reagents and Plasmids—Recombinant human TNF-α, forskolin and 3′-azidodeoxythimidine were purchased from Roche (Penzberg, Germany), Wako (Tokyo, Japan) and Sigma (St.Louis, MO, USA), respectively. Antibodies against p65 (C20), IκBα (C21), IKK-α (H744) and α-tubulin (H300) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against the phosphorylated form of p65 at Ser536 and the phosphorylated form of IκBα at Ser32 and Ser36 were obtained from cell signalling. Horseradish peroxidase-conjugated secondary antibodies against mouse IgG and rabbit IgG were purchased from Amersham Biosciences (Buckinhamshire, UK). Anti-Flag M2 affinity gel was purchased from Sigma.

The reporter plasmid expressing firefly luciferase under the control of NF-κB (pGL3-κB luc) was described previously (*8*). pCRE-luc, expressing firefly luciferase under the control of cAMP-responsive element binding protein (CREB), and pRL-TK were purchased from Stratagene (La Jolla, CA, USA) and Promega (Madison, WI, USA), respectively. Flag-IKK-α and Flag-IKK-β expression vectors (*9*) were kindly provided from Dr H. Nakano. pGST-IκBα(*1-54*) (*9*) was a gift from Dr H. Sakurai.

Cell Culture and Transfection—HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 humidified air. Cells were transfected using Fugene-6 transfection reagent (Roche) according to the manufacture's instructions as described previously (*8*). OM10.1, latently infected macrophage/monocyto cell line with HIV-1, and Molt4/IIIB cells, chronically infected with HIV-1, were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 humidified air. To maintain the latency of the HIV-1 in OM10.1, 20μM 3′-azido-3′-deoxythymidine (AZT) was added in the culture medium and was excluded prior to experiments (*28*).

Luciferase Assay—Transient luciferase assays were performed as described previously (*8*). Briefly, HEK293 cells were transfected with a total of 0.5 μg of plasmid DNA using Fugene-6 transfection reagent (Roche). Blank control plasmid pUC19 was used to equalize the amount of DNA for each transfection. After 24 h of transfection, cells were pre-treated with compounds of indicated concentrations for 30 min, and stimulated with or without TNF-α for additional 24 h. To stimulate protein kinase A and eventually activate the transcriptional activity of CREB, pCRE-luc plasmid was transfected and treated with forskolin for 24 h. The cells were then harvested with 500 μl of lysis buffer (Promega) and the same amount of lysates (~3 μg) was prepared for the luciferase assay. Luciferase activity was measured by the Luciferase Assay System (Promega) as previously described (*8*). Transfection efficiency was monitored by *Renilla* luciferase activity with pRL-TK plasmid (Promega) containing TK promoter as an internal control. The data are presented as the fold increase in luciferase activities (means ± SD) relative to control of three independent transfections.

Measurement of Cell Viability—In order to assess the cytotoxicity of NAM and its derivatives, WST-1 assay (Roche) was performed as described previously (*28*). HEK293 cells were seeded at a density of 1×10^5 cells/ml in a 96-well plate (in 100 μl/well) and were cultured for 24 h at 37°C. The cytotoxicities of NAM to OM10.1 and Molt4/IIIB cells were similarly evaluated. NAM was added with indicated concentrations and cells were treated for additional 24 h. After 48 h incubation, 10 μl of WST-1 reagent were added, the cells were further incubated for 4 h and absorbance at 450 nm was

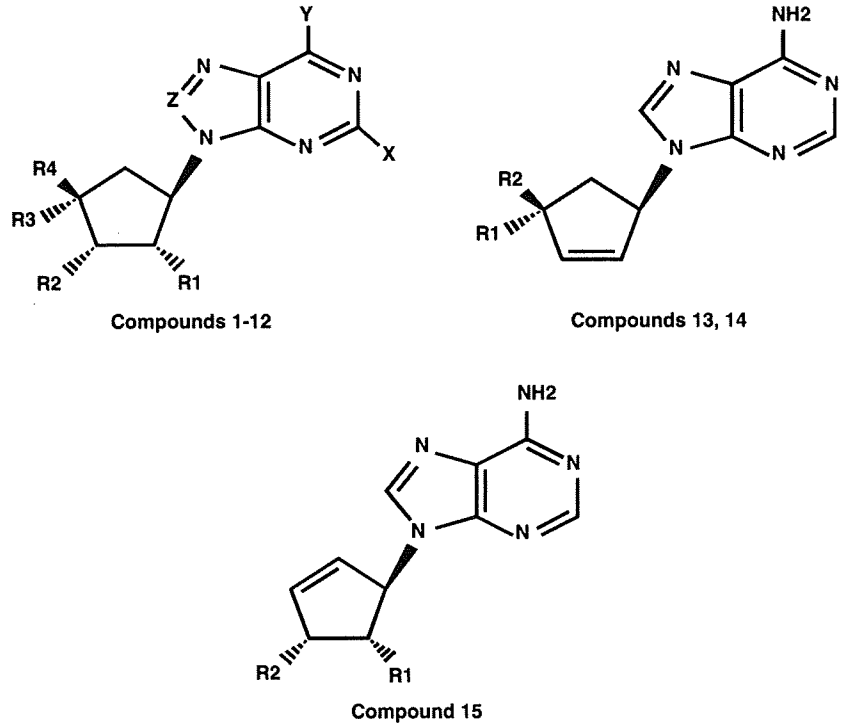

Fig. 1. **Structural formulae of synthetic carbocyclic adenine nucleosides.**

measured with the aid of multi-plate reader using plain medium as blank.

Immunoblot Analysis—Immunoblot analysis was performed as described previously (*8*, *18*). Briefly, HEK293 cells were pre-treated with indicated concentrations of NAM for 24 h, and then stimulated with 1 ng/ml TNF-α for indicated time. After washing with ice cold PBS, cells were harvested with ice cold lysis buffer (25 mM HEPES–KOH, pH 7.5, 200 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% Nonidet P-40, 1 μM dithiothreitol, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4 and 20 mM NaF) supplemented with protease inhibitor cocktail (Roche). Equal amounts of samples (30 μg) were resolved by 10% SDS–PAGE and transferred on PVDF membrane (Immobilon-P; Millipore, Bedford, MA, USA). The membranes were probed with the indicated primary antibodies, and horseradish peroxidase-conjugated secondary antibodies. Immunoreactive proteins were visualized by enhanced chemiluminescence (SuperSignal; Pierce, Rockford, IL, USA).

In Vitro *Kinase Assay*—The enzymatic activity of the endogenous IKK complex was assessed as following ('endogenous' assay): approximately 6×10^5 HEK293 cells were either stimulated or unstimulated with 20 ng/ml TNF-α, washed with PBS after 15 min, cells were harvested for preparation of cell lysate and the IKK complex was immunoprecipitated with anti-IKK-α antibody. The cell lysates from both TNF-α-treated or non-treated control were prepared and the IKK activity was measured with one-tenth of either cell lysate *in vitro* with the IKK substrate GST-IκBα(1-54) (*9*, *29*). In 'exogenous' assay, the enzymatic activities of IKK-α and IKK-β were measured according to Fiorentino *et al.* (*29*). Briefly, HEK293 cells (6×10^5) were transfected with 2 μg of Flag-IKK-α or Flag-IKK-β expression vectors by using Fugene-6 transfection reagent (Roche) according to the manufacture's instructions. At 24 h after transfection, cells were left untreated or treated with 20 ng/ml TNF-α for 15 min to stimulate the IKK activity. The transfected cells were harvested and resuspended in kinase lysis buffer [50 mM Tris–KOH (pH 7.5), 200 mM NaCl, 2 mM EDTA, 1% Brij97, 10% glycerol, 0.5% TritonX-100] supplemented with a protease inhibitor cocktail (Roche), 2 mM phenylmethanesulphonyl fluoride-α-toluenesulphonyl fluoride, 50 μM dithiothreitol and 1 mM Na_3VO_4. These cell lysates were immunoprecipitated either with anti-Flag or anti-IKK-α antibody for 3 h at 4°C. The immunoprecipitates were washed three times with kinase lysis buffer and then washed once with kinase reaction buffer [20 mM HEPES–KOH (pH 7.5), 5 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol, 0.1 mM Na_3VO_4, 20 mM NaF and 10 mM β-glycerophosphate). The IKK substrate GST-IκBα(*1-54*) protein was purified from *Escherichia coli* expressing this protein according to the method previously described (*9*). Kinase assays were performed in 30 μl of kinase reaction buffer containing 5 μM ATP, 5 μCi of [γ-^{32}P]ATP and 1.0 μg of GST-IκBα(*1-54*) in the presence or absence of various concentrations of NAM. After incubation at 30°C for 20 min, reactions were terminated by adding 6 μl of the SDS sample buffer and boiling for 5 min. Equal amounts of samples were resolved by 10% SDS–PAGE and gels

Table 1. **Effects of carbocyclic nucleoside compounds on NF-κB dependent transcription.**

Compound No.	X	Y	Z	R_1	R_2	R_3	R_4	(%) inhibition	
								Control	TNF-α
1 (cPA)	H	NH_2	C	H	H	H	OH	−7±12	30±6
2 (NAM)	H	NH_2	C	OH	OH	H	OH	−5±5	56±4
3	F	NH_2	C	OH	OH	H	OH	19±8	56±3
4	Br	NH_2	C	OH	OH	H	OH	31±2	60±0
5	NH_2	NH_2	C	OH	OH	H	OH	−1±6	39±3
6	CH_3	NH_2	C	OH	OH	H	OH	17±7	37±5
7	NH_2	Cl	C	OH	OH	H	OH	−8±6	10±5
8	H	NH_2	C	H	H	OH	H	14±11	23±17
9	H	NH_2	C	OH	OH	OH	H	13±11	47±4
10	F	NH_2	C	OH	OH	OH	H	−15±10	−11±3
11	NH_2	NH_2	C	OH	OH	OH	H	2±9	15±4
12	H	NH_2	N	OH	OH	OH	H	17±5	20±7
13	–	–	–	H	OH	–	–	−6±18	7±4
14	–	–	–	OH	H	–	–	3±8	19±2
15	–	–	–	OH	OH	–	–	14±21	55±2

HEK293 cells were transfected with 4×κB-luc reporter plasmid. Twenty-four hours after transfection, cells were pre-treated with 100 μM of each synthetic carbocyclic adenine nucleoside compound for 30 min and stimulated with 1 ng/ml of TNFα. Forty-eight hours after transfection, cells were lysed and luciferase activity was determined. The data are presented as the percentage of inhibition in luciferase activities relative to control transfection. Values are the means ± SD of three independent transfections.

were vacuum dried. Radioactive signals were detected with BAS-1800 II (Fuji film, Tokyo, Japan) and quantified with Image Gauge V 4.0 (Fuji film) software.

Measurement of Viral p24 Antigen—The viral replication levels of HIV-1 from latently infected cells were measured as previously described (*28*). Briefly, OM10.1 cells were seeded at a density of 2×10^5 cells/ml in 12-well plates and maintained at 37°C in 5% CO_2 humidified air. Thirty minutes after treatment with NAM, cells were stimulated by 0.2 ng/ml TNF-α, incubated for 24 h and culture supernatants were collected to measure the HIV-1 p24 antigen level using a commercial kit (RETRO-TEK HIV-1 p24 Antigen ELISA kit; Zepto Metrix Corp., Buffalo, NY, USA) as previously reported (*28*).

RESULTS

Effects of Carbocyclic Adenine Nucleosides on NF-κB Transactivation—Since it is known that high concentrations of cPA could inhibit NF-κB activation, we have synthesized 15 carbocyclic adenine nucleoside derivatives with modifications at the second position of adenine and the 2′, 3′ and 4′ positions of carbocyclic moiety. In order to examine the effects of these compounds on the NF-κB-mediated transcription, HEK293 cells were pre-treated with each compound at 100 μM for 30 min and stimulated with TNF-α for 24 h. The effects of these compounds were evaluated by performing transient luciferase assay with NF-κB-dependent luciferase reporter plasmid. We assessed the non-specific effects, mostly the cytotoxic effect, of each compound by measuring the effect of each compound on basal transcription without TNF-α stimulation ('Control' in Table 1). As shown in Table 1, whereas cPA at 100 μM exhibited ~30% inhibition of NF-κB-mediated transcription, greater inhibitory effects were demonstrated with eight compounds (compounds **2–6**, **8**, **9** and **15**). The dose-dependent inhibition of the basal as well as the TNF-α-stimulated and NF-κB-mediated transcription by compounds **1**, **8**, **9**, **13–15** were observed (data not shown). Among these compounds, we dropped six compounds (compounds **3**, **4**, **6**, **8**, **9** and **15**) because of their strong effects on the basal transcription level, reflecting the non-specific cytotoxicity. Two strong inhibitors compound **2** (NAM) and compound **5** (2-amino-NAM), shared structural characteristics including (*1*) OH modifications of three cyclic carbons (trihydroxylation in R_1, R_2 and R_4) and (*2*) H- or NH_2-modification of adenine nucleoside at the second position (X). However, NAM exhibited higher anti-NF-κB effect than 2-amino-NAM and similar non-specific effect (Table 1). It is also noted that halogen [F (compound **3**) or Br (compound **4**)] modification of adenine nucleoside at the second position (X) showed strong non-specific inhibitory effects as well as the inhibitory effects on the NF-κB-mediated transactivation even when cyclic carbons are equally trihydroxylated. On the other hand, methylation or amination at the second position (X) did not enhance the inhibitory effect on NF-κB-mediated transcription.

The NF-κB-specific Inhibition by NAM—Thus, our initial screening elucidated NAM as a potential anti-NF-κB inhibitor. Figure 2A shows the dose-dependent inhibitory effect of NAM on the NF-κB-mediated transcription. The 50% inhibition was obtained at the concentration of 2.7 μM (IC_{50}) with NAM; whereas, 50% cytotoxicity of NAM was 730 μM (CC_{50}) (Fig. 2C), thus therapeutic window of NAM for NF-κB inhibition was ~270-fold. The apparent lack of inhibitory effect of NAM on the basal transcription level (Fig. 2A, see the experiments with no TNF-α) suggested that the inhibitory effect of NAM is specific for NF-κB. In order to further examine the specificity of NAM, we tested whether it could inhibit gene expression by another transcription factor CREB, whose activity is controlled by protein kinase A pathway. As shown in Fig. 2B, NAM exhibited no significant effect on CREB-dependent transcription induced by forskolin. In addition, NAM

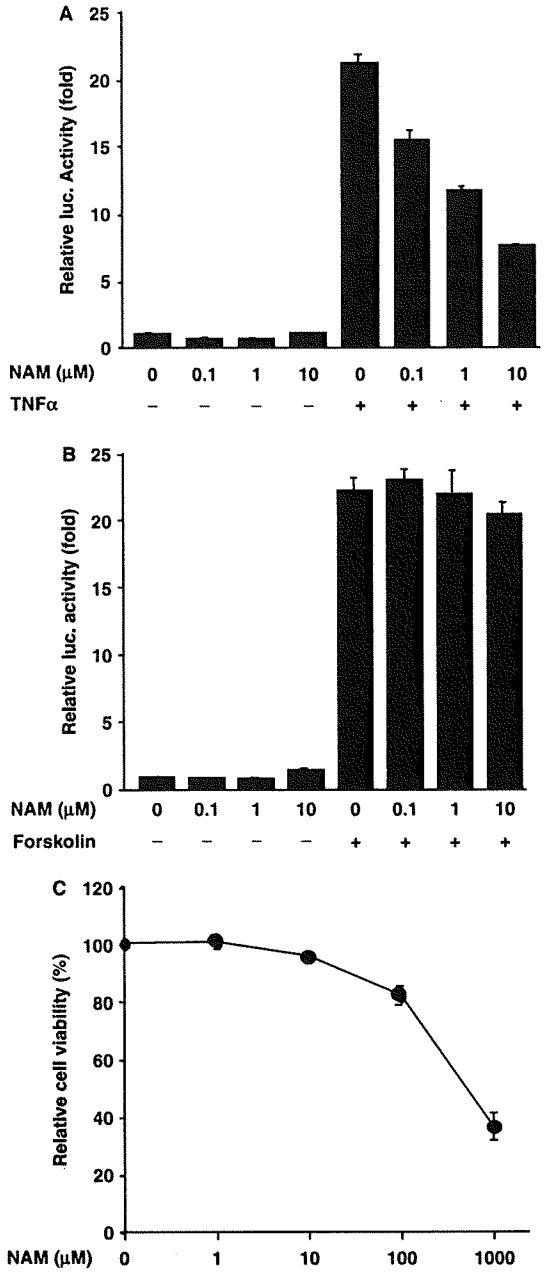

Fig. 2. **Inhibition of NF-κB-dependent transcription by NAM.** (A and B) HEK293 cells were transfected with 4 × κB-luc (A) or 4 × CRE-luc (B) reporter plasmids. Twenty-four hours after transfection, cells were pre-treated with the indicated concentrations of NAM for 30 min and stimulated with 1.0 ng/ml of TNF-α (A) or 10 μM of forskolin (B). Forty-eight hours after transfection, cells were lysed and luciferase activity was determined. The data are presented as the fold increase in luciferase activities relative to control transfection (no stimulation). Values are the means ± SD of three independent transfections. Insets show expanded ranges of NAM effects on the basal transcription from 4 × κB-luc and CRE-luc reporter plasmids. (C) The cytotoxic effects of NAM. The HEK293 cell viability was determined by WST assay. This experiment was performed in triplicates and the means ± SD are shown.

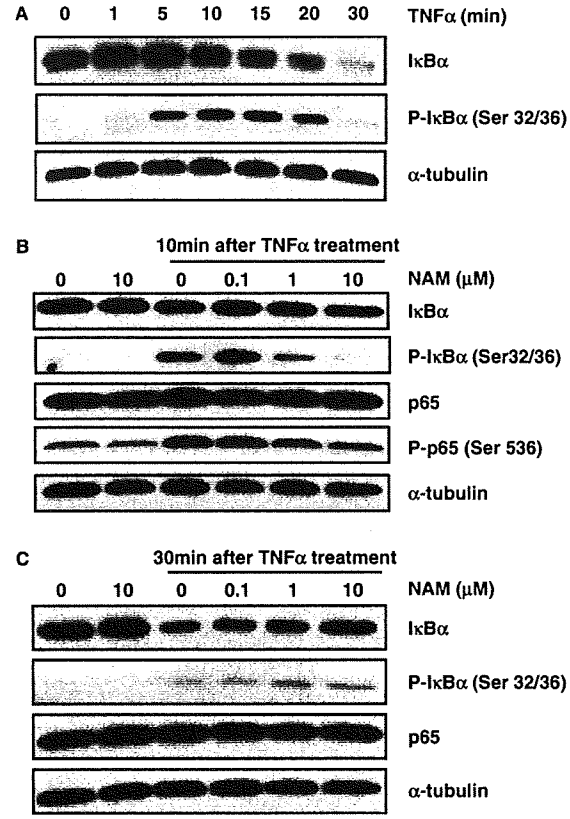

Fig. 3. **Inhibition of IκBα and p65 phosphorylation and IκBα degradation by NAM *in vivo*.** (A) Time course of IκBα phosphorylation and its degradation following TNF-αstimulation. HEK293 cells were stimulated with 1.0 ng/ml of TNF-α for indicated periods (min). It was noted that maximum IκBα phosphorylation and degradation were observed after 10 and 30 min of TNF-α stimulation, respectively. (B and C) Inhibition of IκBα phosphorylation by NAM. Cells were cultured in the presence of the indicated concentrations of NAM for 24 h, stimulated with TNF-α (1.0 ng/ml) for 10 (B) or 30 min (C), and harvested for western blot analyses. Equal amounts of lysate (30 μg) were immunoblotted with specific antibodies against IκBα, phospho-IκBα (at Ser32/36), p65 and phospho-p65 (at Ser536). Anti-α-tubulin antibody was used as an internal control.

did not inhibit TNF-α-induced AP1 transactivation (data not shown). Collectively, these results indicate that NAM specifically inhibits the signalling pathway that leads to NF-κB activation under non-cytotoxic concentrations.

Inhibition of IκBα and p65 Phosphorylation and IκBα Degradation by NAM In Vivo—In order to investigate the mechanism by which NAM inhibits NF-κB activation pathway, we first examined the effect of NAM on the level of phosphorylation of IκBα at Ser32/36 residues, known to be crucial in the NF-κB activation pathway (2). As shown in Fig. 3A, the phosphorylation of IκBα was observed as early as 1 min after TNF-α stimulation and reached a peak at 10 min of stimulation. IκBα was subsequently degraded and almost disappeared after 30 min of stimulation by TNF-α. In Fig. 3B and C, the effect of NAM on the IκBα phosphorylation was examined at 10 and

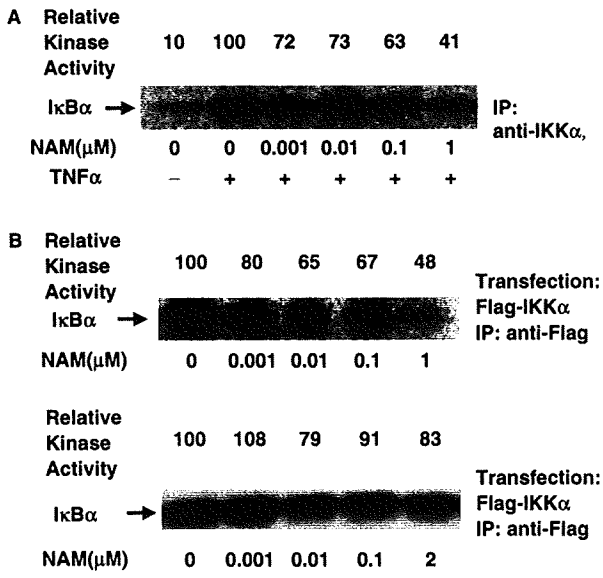

Fig. 4. **Inhibition of IKK by NAM *in vitro*.** (A) Effects of NAM on IKK complex *in vitro*. HEK293 cells were either stimulated or unstimulated with TNF-α (20 ng/ml) and the endogenous IKK complex was immunoprecipitated from the whole cell lysate by anti-IKK-α antibody. The kinase activity of the immunoprecipitated IKK complex was evaluated *in vitro* with purified recombinant GST-IκBα(1-54) protein as a substrate in the presence of various concentrations of NAM. (B) Effects of NAM on IKK-α and IKK-β *in vitro*. HEK 293 cells were transfected with expression vectors for Flag-IKK-α (upper panel) or IKK-β (lower panel). Twenty-four hours after transfection, cells were stimulated with 20 ng/ml of TNF-α for 15 min. Whole cell lysates were immunoprecipitated with anti-Flag antibody, and kinase activities of IKK-α or IKK-β were determined *in vitro* similarly as in A. *In vitro* kinase assays were performed in 30 μl of kinase reaction buffer containing 5 μM ATP, 5 μCi of [γ-^{32}P]ATP and 1.0 μg of GST-IκBα(1-54) for 20 min at 30°C. The relative kinase activities were quantified by densitometric measurement and are indicated below each gel band. These experiments were performed more than three times and the representative results are shown.

30 min after TNF-α stimulation. A dose-dependent inhibition of the IκBα phosphorylation by NAM is shown in Fig. 3B. In addition, Fig. 3C shows a dose-dependent suppression of IκBα degradation by NAM (at 30 min after TNF-α stimulation). We also found the inhibitory effect of NAM on the phosphorylation of NF-κB p65 subunit at Ser536 residue that is known to stimulate the transcriptional competence of NF-κB via 'non-canonical pathway' (*8*, *9*, *30*) (Fig. 3B). These results suggest that NAM suppresses the NF-κB-mediated transcription by inhibiting the phosphorylation of IκBα and p65.

Inhibitory Effects of NAM on IKK Activity In Vitro—Since the kinase molecules responsible for the phosphorylation of IκBα and p65 are IKK subunits, especially IKK-α and IKK-β, we performed *in vitro* kinase assay and asked which IKK subunit is responsible for the inhibitory effect of NAM. In Fig. 4A, the endogenous IKK complex was immunoprecipitated from HEK293 cells by incubation with anti-IKK-α antibody and the kinase activity was determined with GST-IκBα(*1-54*) as a substrate and the effects of NAM was examined. NAM inhibited the endogenous IKK activity following the TNF-α stimulation in a dose-dependent manner. The 50% inhibition thus obtained *in vitro* was ~0.64 μM. We then examined the effect of NAM on the kinase activities of IKK-α and IKK-β. In Fig. 4B, HEK293 cells were transfected with the expression vectors for Flag-IKK-α or Flag-IKK-β and stimulated with TNF-α to stimulate the upstream kinase cascade. The cell lysates were immunoprecipitated with anti-Flag antibody. Each one-tenth of the immunoprecipetate, containing the TNF-α-activated IKK, was dissolved in the kinase buffer, various amounts of NAM were added together with the same amount of GST-IκBα substrate. The effects of NAM on the kinase activities of immunoprecipitated IKK-α and IKK-β were then determined. As shown in Fig. 4B, NAM strongly inhibited the kinase activity of IKK-α but not IKK-β. The IC_{50} value of NAM for IKK-α was 0.37 μM and was similar to that with the endogenous IKK complex (Fig. 4A). On the other hand, the effect of NAM on the IKK-β activity was very weak and NAM inhibited not >70% even at 100 μM.

Suppression of HIV-1 Replication from the Latently Infected Cells by NAM—Since the HIV-1 replication from the latently infected cells highly depends on NF-κB activity (*5*), we examined the effect of NAM on the NF-κB-dependent HIV-1 replication from one such cell line, OM10.1 (*28*). In Fig. 5A, OM10.1 cells were preincubated with various concentrations of NAM for 30 min, HIV-1 replication was induced by TNF-α, and the amounts of viral production were determined. The inhibitory effect of NAM was thus evaluated by measuring the viral p24 antigen levels in the cell culture supernatants of OM10.1 cells. As shown in Fig. 5A, NAM strongly suppressed the expression level of viral p24 protein induced by TNF-α in a dose dependent manner; whereas, no significant effect was observed on the basal level of HIV-1 production (without TNF-α stimulation). Similarly, the effect of NAM on chronic HIV production was examined in Fig. 5B. When Molt4/IIIB cells, chronically infected with HIV-1 and continuously producing the virions, were stimulated with TNF-α, the level of viral production was significantly augmented as previously reported (*28*). When Molt4/IIIB cells were pretreated with NAM at various concentrations, a significant reduction of HIV production was observed. Under these concentrations of NAM, no significant cytotoxicity was observed (Fig. 5A and B, right panels). Thus, NAM was effective in repressing the HIV-1 viral replication in chronically infected cells.

DISCUSSION

In this study, we demonstrated that NAM showed the most potent inhibitory effect on the NF-κB-mediated transactivation under non-cytotoxic concentrations among newly synthesized 15 carbocyclic adenine nucleosides. Moreover, NAM strongly suppressed the HIV-1 replication in latently infected cells, implicating its efficacy during the maintenance therapy of HIV-1 infection (*4*, *5*). It is likely that this anti-HIV activity of NAM is mediated by inhibiting the NF-κB activation cascade. We found that NAM inhibited the TNF-α-induced phosphorylation and

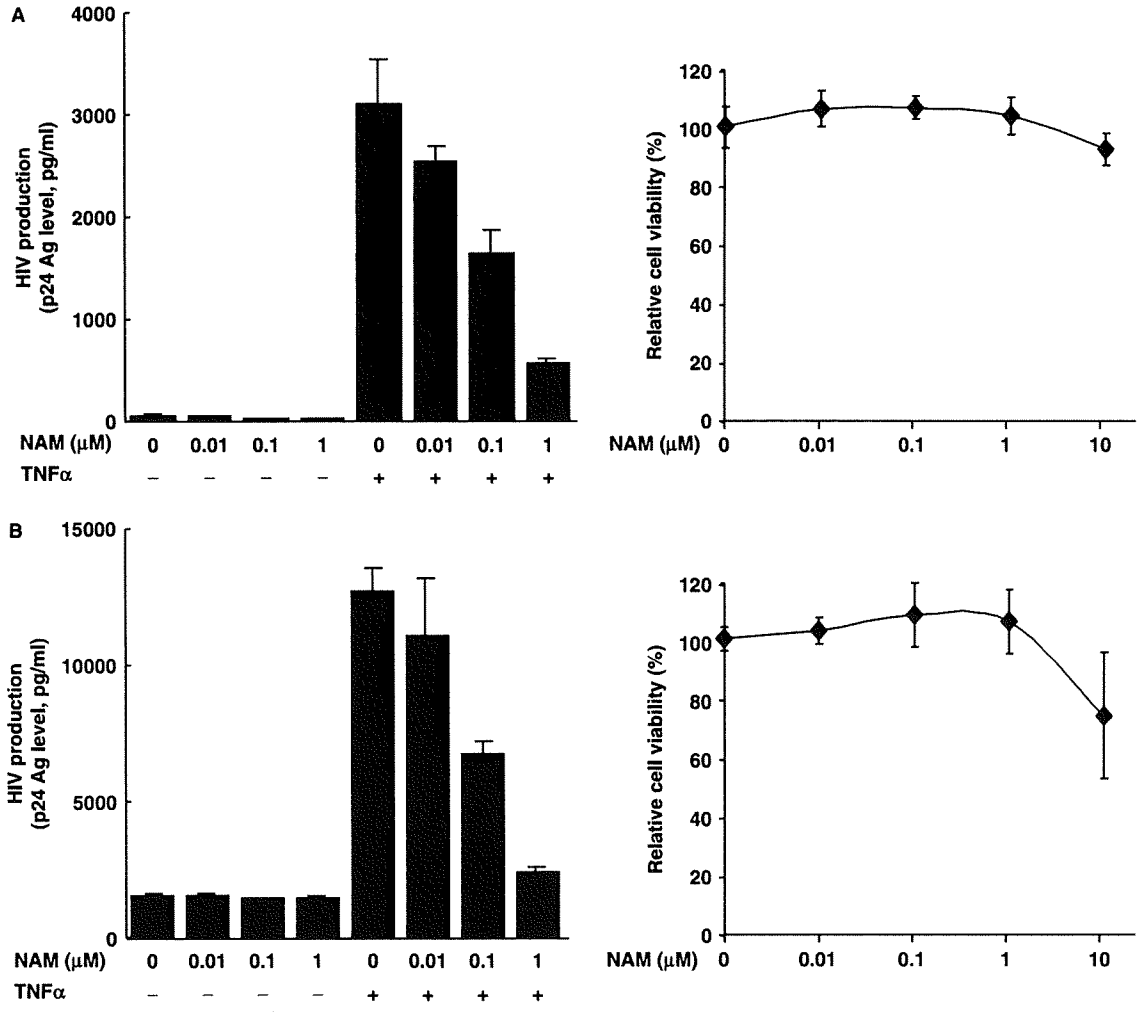

Fig. 5. **The effect of NAM on HIV-1 replication from chronically infected cells.** (A) Effects of NAM on the viral production from OM10.1, latently infected with HIV-1. (B) Effects of NAM on the viral production of Molt4/IIIB cells, chronically infected with HIV-1. Cells were seeded at a density of 5×10^5 cells/ml in 12-well plates, pre-treated with the indicated concentrations of NAM for 1 h, and were then stimulated with or without TNF-α (0.2 ng/ml). After further 24 h incubation, culture supernatants were collected and HIV-1 p24 antigen levels were measured using a commercial ELISA kit. The data are presented as the fold increase in p24 antigen levels relative to the untreated control. The cell viability in the presence of various concentrations of NAM was determined by WST-1 assay. These experiments were performed in triplicates and the means ± SD are shown.

subsequent degradation of IκBα. In addition, NAM also inhibited the phosphorylation of p65 at Ser536 that is a hallmark of IKK-α action via 'non-canonical pathway' (*8, 30*). We further explored the NAM action and found that the inhibitory effects of NAM are through inhibition of the kinase activity of IKK. The *in vitro* kinase assay revealed that whereas IKK-β was not responsive to the NAM-mediated inhibition, NAM inhibited the kinase activity of IKK-α subunit at a concentration similar to its effect for the endogenous IKK complex. These findings suggest that NAM is a novel inhibitor of IKK-α and is a potential drug for the treatment of AIDS and other diseases in which NF-κB plays a major role (*3, 31*).

IKK inhibitors previously reported are classified into two categories with their mechanisms of action; (i) ATP analogues and (ii) allosteric inhibitors. For example, 2-amino-3-cyano-4-alkyl-6-(2-hydroxyphenyl)pyridine (ACHP) and its derivatives act as ATP analogues and inhibit IKK (*17, 18*). We have previously demonstrated that ACHP shows a strong inhibitory effect on the constitutive NF-κB activation in multiple myeloma (*18*) and adult T-cell leukaemia (*19*) cells and effectively induces apoptotic cell death. Similarly, ACHP was effective in suppression of the cytokine-mediated HIV-1 replication from the latently infected cells, which is dependent on NF-κB (*28*). It was demonstrated that ACHP shows greater inhibition on IKK-β than IKK-α (*17*). However, no compound except NAM has been demonstrated to specifically inhibit IKK-α. NAM appears to inhibit IKK activity as an ATP analogue because of its structural similarity although detailed studies are needed. Other ATP analogue IKK inhibitors such as AS602868 (*32*) and