of KIR-incompatible, T-cell-depleted alloreactive donor NK cells [55]. At the present time, graft-versus-host disease is also controlled in the transplanted patient. Moreover, the remission rates were significantly high when KIR-mismatched alloreactive NK cells were used in studies on the transfer of haploidentical NK cells [56]. Higher sensitivity against KIR-incompatible NK cells is seen *in vitro* in solid tumors such as renal cell carcinoma or melanoma [57]. Thus, effective treatments can be expected by preparing sufficient amounts of highly purified allogenic NK cells.

THE POSSIBILITY OF NK CELL THERAPY AND FURTHER ISSUES

Although NK cells are recognized as cells that widely attack cells with missing-self molecules, such as tumor cells and virus-infected cells, the details of their action mechanisms remained unknown for a long time. However, the details of NK cell function have been clarified over the past few years by the increased number of molecular biological analyses of receptors and immunologic studies of innate immunity, and the significance of these cells has been re-recognized. Based on new findings regarding NK cells, the possibility of cellular immunotherapy using NK cells has become attractive. However, a sufficiently large amount of clinical data for NK cell therapy is not yet available. In addition, the quality and quantity of immune cell cultures differ according to individual institutions, and it is important to make appropriate evaluations of differences in cell quality, including their safety, after adjustment for each institution [58].

To date, treatments for cancers or viral infections have focused on how to remove the tumor cells or virus from the body using inhibitory drugs. However, *in vivo* immunity should be used to the maximum extent in the future. For this purpose, the possibility of cellular immunotherapy using NK cells has been further introduced through the progress in elucidating their molecular biology and involvement in innate immunity as well as the development of a culture method for NK cells.

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Original article

Statin-induced inhibition of HIV-1 release from latently infected U1 cells reveals a critical role for protein prenylation in HIV-1 replication

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Abstract

Latent infection of human immunodeficiency virus type 1 (HIV-1) represents a major hurdle in the treatment of acquired immunodeficiency syndrome (AIDS) patients. Statins were recently reported to suppress acute HIV-1 infection and reduce infectious virion production, but the precise mechanism of inhibition has remained elusive. Here we demonstrate that lypophilic statins suppress HIV-1 virion release from tumor necrosis factor alpha-stimulated latently infected U1 cells through inhibition of protein geranylgeranylation, but not by cholesterol depletion. Indeed, this suppression was reversed by the addition of geranylgeranylpyrophosphate, and a geranylgeranyltransferase-1 inhibitor reduced HIV-1 production. Notably, silencing of the endogenous Rab11a GTPase expression in U1 cells by RNA interference destabilized Gag and reduced virion production both in vitro and in NOD/SCID/γc^{null} mice. Our findings thus suggest that small GTPase proteins play an important role in HIV-1 replication, and therefore could be attractive molecular targets for anti-HIV-1 therapy.

Keywords: Statins; Prenylation; HIV-1; Rab11a; Small GTPases

1. Introduction

Infection with human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), is characterized clinically by a long asymptomatic period of latency preceding the development of AIDS. Even during this period of latency, the virus is continuously replicating and causing *de novo* infection. Recent studies using combination anti-retroviral therapy have revealed a population of latently infected cells that are refractory to antiviral therapy, which is believed to be a leading cause of the persistence of infection [1]. Although patients treated successfully with the highly active anti-retroviral therapy (HAART) achieved undetectable levels of virus load, viremia recurred in almost every patient when the drug therapy was stopped, because latent virus in reservoir cells is not susceptible to this anti-retroviral therapy or host immune responses [2,3]. Thus, HIV-1 infection remains incurable and new therapeutic approaches need to be developed.

Recent studies have suggested that lypophilic statins have direct anti-HIV effects. del Real et al. showed that lovastatin reduced acute infection by HIV-1 NL4-3.Luc.R.E. pseudotyped with HIV-R5 or X4 envelopes, but not that by the virus pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) envelope. Lovastatin treatment of HEK 293T producer cells also reduced HIV-1-X4-enveloped infectious virus production, but not that of VSV-G-pseudotyped virus. The

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proposed mechanism was that statins targeted Rho GTPases and affected the actin cytoskeleton re-arrangement necessary for virus entry or budding [4,5]. It was also reported that statins suppressed virion-associated intercellular cell adhesion molecule 1–leukocyte function antigen 1 interactions that are required for viral entry [6]. Audoly et al., using inhibitory toxins, proposed that small GTP-binding proteins are involved in the assembly of HIV-1 Gag in their acute infection model [7]. Quite recently, Nabatov et al. reported that statins disrupt CCR5 and RANTES expression levels in CD4+ T lymphocytes in vitro and preferentially decrease infection of R5 versus X4 HIV-1 [8]. However, the effect of statins in chronically HIV-1-infected cells and its precise mechanism remain to be uncovered.

Statins, which are used to treat hypercholesterolemia, inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis in the liver catalyzing the conversion of HMG-CoA to mevalonic acid [9,10]. In addition to inhibiting cholesterol synthesis, statins also block the synthesis of isoprenoid intermediates such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGpp). Both FPP and GGpp serve as important lipid attachments for the post-translational modification of variety of proteins, including heterotrimeric G proteins and small GTP-binding proteins such as the Ras, Rho, Rap, and Rab GTPase family proteins [11,12]. This modification, called protein prenylation, is a common mechanism for membrane association of approximately 0.5% of all intracellular proteins. Prenylation consists of the covalent attachment, via thioether linkage, of a C15 (farnesyl) or C20 (geranylgeranyl) isoprenoid group to a C-terminal cysteine residue in the context of a 'prenylation motif'. Farnesyl and geranylgeranyl moieties can bind covalently to several low molecular weight GTPase proteins, and this binding is catalyzed by three prenyltransferases: farnesyltransferase (FTase), geranylgeranyltransferase-1 (GGTase-I) or geranylgeranyltransferase-2 (GGTase-2, also called Rab GGTase). Thus, inhibition of the mevalonate pathway or geranylgeranyltransferases leads to impairment of protein prenylation.

Protein prenylation is critical for intracellular localization and function of small GTPase proteins. In general, modification with FPP is necessary for proper localization of Ras family proteins, whereas GGpp is required for Rho, Rab, and Rap family proteins. Among them, Rab GTPase proteins form the largest family within the Ras-like GTPase superfamily [13,14]. More than 50 Rab proteins have been identified in mammalian cells. Each Rab is believed to be localized to a specific subcellular compartment, reflecting the complexity and variety of trafficking events found in mammalian cells. Rab proteins, unlike other small GTPases, exhibit a variety of prenylation motifs at their C-termini, containing either one or more frequently, two cysteine residues, both of which are modified by geranylgeranyl groups [15]. It was recently reported that siRNA-mediated silencing of Rab9 expression in JC53 HeLa-derived indicator cells inhibited HIV replication, as did silencing expression of other genes that facilitate the late-endosome-to-*trans*-Golgi vesicular transport [16]. Interestingly, acute HIV-1 replication in JC53 cells was also affected, although less profoundly, by silencing expression of Rab11a. It has been well documented that Rab11a is mainly located on pericentriolar recycling endosomes and plays a key role in regulating vesicle trafficking through recycling endosomes to the plasma membrane as well as in exocytosis [17,18].

Here we investigated the effect of statins on virus production in chronically HIV-1-infected promonocytic U1 cells, and showed a critical role for protein prenylation in the late phase of HIV-1 replication.

2. Materials and methods

2.1. Reagents and cells

Simvastatin and lovastatin were purchased from LKT Laboratories, Inc. (MN, USA), and activated by dissolving in ethanol and treatment with 0.1 M NaOH. The pH was then adjusted to 7.0 with HCl. GGTI-298 and FTI-277 were purchased from Calbiochem (Darmstadt, Germany). Anti-Rab11a monoclonal antibody was purchased from BD transduction laboratories (Japan). The serum derived from an HIV-1-infected patient was described previously [19]. Anti-mouse IgG (H&L), anti-human rabbit HRP-linked antibody was obtained from American Qualex manufactures (CA, USA). DMRIE-C reagent for transfection was purchased from Invitrogen (CA, USA). All other reagents including anti-tubulin (T-9026) monoclonal antibody, squalene, GGpp, cycloheximide, TNF-α and phorbol-12-myristate-13 acetate (PMA) were purchased from Sigma (MO, USA). U1 and HEK 293T cells were grown in RPMI 1640 and DMEM, respectively, supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and streptomycin at 37 °C.

2.2. Treatment and stimulation of cells

Cells were treated with or without simvastatin or lovastatin for 2 days and equivalent numbers of viable cells were stimulated with TNF-α or PMA for additional 2 days in the presence or absence of statins, and then intracellular and extracellular Gag (p24 and p55) antigen was quantified. More than 80% of cells were found viable after treatment with 1 μM of simvastatin, and we normalized the levels of Gag protein (p24 and p55) based on the number of viable cells in each sample. The amount of Gag per viable cell was calculated by dividing the Gag value with the number of viable cells. In some of the experiments, GGpp (1 μM), squalene (50 μg/ml) or GGTI-298 (1 μM) was added during the entire course of the experiment.

2.3. HIV-1 Gag quantification

Culture supernatant was collected after centrifugation and subjected to quantification of the HIV-1 Gag (p55 and p24) antigen by automated enzyme-linked immunosorbent assay (ELISA) (Fuji Rebio Inc., Tokyo, Japan). Cell pellets were

washed three times with PBS, re-suspended with the p24 lysis buffer (0.5% Triton X-100 in PBS), put on ice for 30 min, and then the Gag antigen was quantified by using auto-ELISA system. The amount of Gag was normalized by dividing the Gag value with the number of viable cells. The relative amounts of Gag were expressed as percentages of that for cells simply stimulated with TNF-α or PMA (arbitrarily set at 100%). The ratio of Gag amount in culture supernatant to that in cells was calculated by dividing the normalized Gag amount in supernatant with that in cell lysate.

2.4. Western blotting

Cells were lysed in a lysis buffer (20 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulphonylfluoride (PMSF), 0.1% aprotinin, and 0.1% leupeptin) for preparation of whole-cell extracts. Thirty micrograms aliquots of protein, determined by the Bradford assay, were resolved by SDS–PAGE and detected by standard immunoblotting procedures using the specific primary antibodies.

2.5. Transmission electron microscopy

Cells were fixed with 2.5% glutaraldehyde in PBS for 2 h, washed and fixed overnight at 4 °C in the same buffer and post-fixed with 1% OsO_4 buffered with PBS for 2 h. The cells were then dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin (90 nm) sections were cut on an ultracut S microtome (Reichert, Vienna, Austria), double-stained with uranyl acetate and lead citrate, and then examined by transmission electron microscopy (H-7100, Hitachi, Hitachinaka, Japan).

2.6. Lentivirus vectors

Annealed oligonucleotides containing the targeting *rab11a* (5′-GAGCGATATCGAGCTATAA-3′) or *Renilla luciferase* (5′-GTAGCGCGGTGTATTATAC-3′) sequence were first inserted immediately downstream of the H1 promoter of the pSuperRetro vector (Oligoengine), generating pSR-Rab11a-i and pSR-Ctrl-i, respectively. The shRNA expression cassettes were then transferred to a newly constructed lentivirus vector, pCS-puro-PRE, carrying a puromycin resistance gene expressed under the control of the phosphoglycerate kinase (PGK) promoter. Construction details for pCS-puro-PRE will be described elsewhere (Saitoh et al., unpublished). EcoRI–XhoI fragments containing the H1 promoter and targeting sequence from pSR-Rab11a-i or pSR-Ctrl-i were inserted between the EcoRI and XhoI sites of pCS-puro-PRE, generating pCS-puro-Rab11a-i and pCS-puro-Ctrl-i, respectively.

2.7. Transfection and infection

The VSV-G-pseudotyped lentivirus was produced by co-transfection of HEK 293T cells with pCS-puro-Rab11a-i or pCS-puro-Ctrl-i, pHCMV-VSV-G encoding the vesicular stomatitis virus glycoprotein (VSV-G) and a packaging construct pCMVΔR8.2 (a kind gift from ISY Chen, USA), using FuGENE 6 (Roche Diagnostics, IN, USA) according to the manufacturer's instructions. Culture supernatants of 293T cells were collected 48 h post-transfection, filtered through 0.20-μm pore-size filters, supplemented with polybrene (10 μg/ml), and used immediately for infection of U1 cells. Infected cells were selected in the presence of 3 μg/ml of puromycin.

2.8. Animal experiments

NOD/SCID/γc^{null} (NOG) mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). All mice were maintained under specific pathogen-free conditions in the animal center of Tokyo Medical and Dental University (Tokyo, Japan). The Ethical Review Committee of the Institute approved the experimental protocol. NOG mice were inoculated intraperitoneally with approximately 2.5×10^6 Rab11a-depleted or control U1 cells per mouse as described previously [20]. Blood and ascites were examined for HIV-1 p24 amount 2 weeks after cell inoculation.

3. Results

3.1. Statins suppressed HIV-1 release from U1 cells

We used U1 cells that do not constitutively produce or release HIV-1 virions to the culture supernatant. U1 cells are derived from U937 promonocytic cells that survived the cytopathic effect associated with the acute infection by HIV-1 LAI/IIIB. U1 cells contain two integrated copies of proviral HIV-1 DNA and are characterized by low constitutive levels of virus expression that can be up-regulated by several cytokines and phorbol esters. Upon stimulation of U1 cells with PMA or with cytokines such as TNF-α, a dramatic increase in HIV-1 gene expression and robust virion release can be induced. Virions were shown to be released from U1 cells in a manner similar to that for cells of monocytic lineage [21,22]. U1 cells were treated with or without simvastatin for 48 h and then stimulated with TNF-α. We found that treatment of U1 cells with 1 μM of simvastatin, which is within the clinically relevant range, suppressed TNF-α-induced release of p24 to culture supernatant (Fig. 1A). Conversely, intracellular Gag protein (including both p55 and its processed form p24) was increased after the treatment with simvastatin and TNF-α. As a result, the ratio of Gag in culture supernatant to Gag in cell lysate was reduced (Fig. 1B). The release of p24 from cells treated with simvastatin and PMA (Fig. 1A,B) was not profoundly suppressed, but this treatment increased intracellular level of Gag. Essentially similar results were obtained with another lipophilic statin lovastatin (Fig. 1C,D), but not with hydrophilic paravastatin (data not shown), suggesting that lypophilic statins, such as simvastatin and lovastatin, successfully entered cultured cells and worked as HMG-CoA reductase inhibitors.

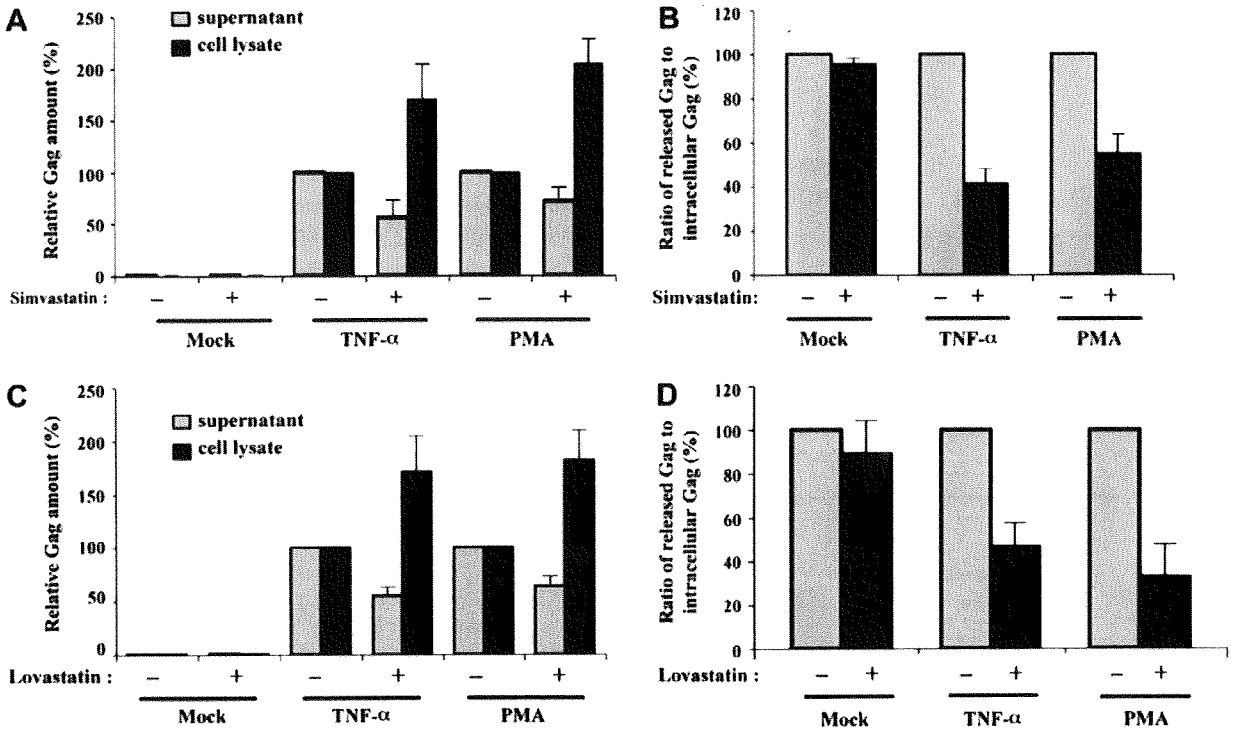

Fig. 1. Statins reduced virus release and increased intracellular Gag in U1 cells. U1 cells were treated or not with simvastatin (A) or lovastatin (C) for 2 days, followed by stimulation with TNF-α (1 ng/ml) or PMA (1 ng/ml) for additional 2 days in the continued presence or absence of simvastatin or lovastatin. Gag (p24 and p55) in culture supernatants (gray bars) and in cell lysates (filled bars) were then quantified. (A) and (C) The relative amounts of Gag in statin-treated (+) cultures are shown in percentage of that of cells (−) stimulated with TNF-α or PMA alone (arbitrarily set at 100%). (B) and (D) Ratios of released Gag to intracellular Gag for statin-treated cells are shown in percentage of the ratio obtained for cells simply stimulated with TNF-α or PMA (arbitrarily set at 100%). Results shown are mean ± SD values of three independent experiments.

3.2. Geranylgeranylation is required for HIV-1 replication in U1 cells

A previous report showed that treatment of 293T cells with lovastatin reduced production of wild type, but not VSV-G-pseudotyped HIV-1, and that this inhibition was reversed by the addition of GGpp [4]. To examine if the reduced p24 release from U1 cells treated with simvastatin resulted from impaired production of geranylgeranyl, we treated U1 cells with simvastatin and TNF-α in the presence of 1 μM GGpp (Fig. 2A). No cytotoxicity was observed after the treatment with GGpp. Addition of GGpp restored the p24 release to the level for control cells stimulated with TNF-α. In contrast, the amount of Gag in cells treated with simvastatin and TNF-α in the presence of GGpp remained higher than that in cells treated with simvastatin and TNF-α. Squalene, one of the metabolites in the cholesterol biosynthesis from FPP, did not interfere with simvastatin-induced inhibition of virion release or intracellular Gag protein accumulation (Fig. 2B). To further investigate the importance of protein prenylation in HIV-1 replication in U1 cells, we tested if geranylgeranyltransferase-1 inhibitor (GGTI) could inhibit virus replication. GGTI was not toxic to U1 cells at 1 μM, whereas farnesyltransferase inhibitor (FTI) was too toxic to be tested in U1 cells (data not shown). GGTI reduced both p24 release and intracellular Gag in TNF-α-stimulated U1 cells (Fig. 2C), suggesting that geranylgeranylation of small GTPase proteins plays a critical role in HIV-1 production.

3.3. Simvastatin enhances intracellular Gag accumulation in U1 cells

We next examined how simvastatin modifies expression of intracellular HIV-1 Gag-related proteins, p55 and its processed form p24. Immunoblotting with anti-HIV-1 Gag antiserum that detected both p55 and p24 revealed that p24 was increased in the presence of simvastatin, while the amount of p55 remained almost unchanged (Fig. 3A). Since the results shown in Fig. 2A suggested the importance of geranylgeranylation, we examined the prenylation status of Rab11a, one of the Rab family small GTPases known to be involved in trafficking of recycling endosomes and exocytosis. As shown in Fig. 3A, treatment with 1 μM simvastatin resulted in almost complete upward shifting of the Rab11a band, indicating accumulation of the non-prenylated form of Rab11a. Besides, GGpp counteracted the simvastatin effect on the prenylation of Rab11a (Fig. 3B). This suggests that simvastatin inhibited the biosynthesis of geranylgeranyl, leading to impaired prenylation of small GTPases involved in intracellular vesicle trafficking. To further gain insight into the effects of simvastatin on HIV-1 replication, we performed transmission electron microscopic (TEM) analysis. Many virus particles were found to be

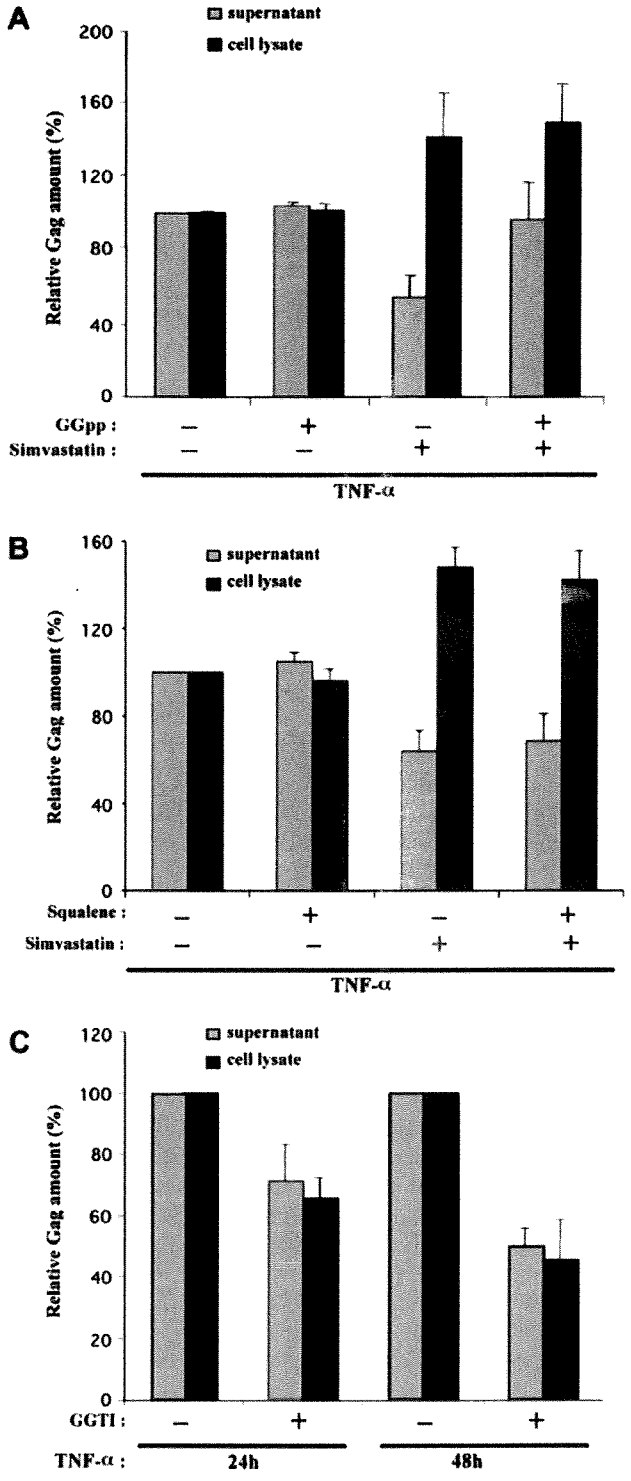

Fig. 2. GGpp restored simvastatin-inhibited virion release, and GGTI suppressed HIV-1 Gag production in U1 cells. U1 cells were cultured for 2 days in the presence (+) or absence (−) of simvastatin, GGpp (A), squalene (B), and GGTI (C). Cells were then stimulated with TNF-α (1 ng/ml) for additional 1 or 2 days, and Gag in supernatants (hatched bars) and cell lysates (filled bars) was quantified. The relative amounts of Gag are shown in percentage of that of cells simply stimulated with TNF-α (arbitrarily set at 100%). Data shown are mean ± SD values of three independent experiments.

released from TNF-α-stimulated U1 cells in the mature form (Fig. 4A,B), but only a few from simvastatin- and TNF-α-treated U1 cells. In contrast, many mature virus particles could be seen in intracellular vesicles of U1 cells treated with simvastatin and TNF-α, whereas it was difficult to find mature virions in vesicles of U1 cells treated with TNF-α alone (Fig. 4C,D). These results suggested impaired release or intracellular trafficking of virions.

3.4. Rab11a mediates HIV-1 replication in U1 cells

In order to further investigate the role of Rab11a in HIV-1 replication in U1 cells, we suppressed the expression of endogenous Rab11a by RNA interference. Immunoblotting analyses (Fig. 5A) demonstrated that the level of Rab11a expression was reduced by ~80–90% in cells expressing Rab11a-specific shRNA (Rab11a-i) compared to cells expressing control shRNA (Ctrl-i). While Rab11a depletion did not affect the growth of U1 cells (data not shown), it reduced the release of p24 as well as intracellular Gag expression induced by TNF-α (Fig. 5B). Immunoblotting analyses revealed that both p24 Gag and p55 Gag are decreased in Rab11a-depleted cells compared to control cells (Fig. 5C). These findings indicate that HIV-1 requires Rab11a for its efficient replication in U1 cells. We next examined if Rab11a depletion affects the stability of Gag, using a protein synthesis inhibitor cycloheximide. As shown in Fig. 5C, the levels of p55 Gag and p24 Gag in Rab11a-depleted cells were generally lower than those in control cells. Importantly, while the expression of p55 Gag remained almost unchanged up to 12 h after CHX treatment in control cells, p55 Gag in Rab11a-depleted cells rapidly decreased with a half-life of ~6 h (Fig. 5D,E). The expression of p24 Gag in Rab11a-depleted cells was lost even more rapidly following cycloheximide treatment, while p24 Gag was only marginally reduced in control cells. These results indicate that Rab11a depletion reduced the stability of Gag, which led to inefficient viral replication in U1 cells.

3.5. Rab11a depletion affects HIV-1 replication in NOG mice

The significant suppression by Rab11a depletion of TNF-α-induced HIV-1 replication in cultured U1 cells prompted us to examine whether depletion of Rab11a in U1 cells can also suppress virus replication in NOG mice. We inoculated Rab11a-depleted or control U1 cells in the peritoneal cavity of immune-deficient NOG mice. Blood and ascites were recovered 2 weeks after inoculation, and then the Gag amounts were determined. Knockdown of Rab11a expression in U1 cells did not apparently influence the growth of cells in mice, but efficiently suppressed HIV-1 replication *in vivo* as revealed by Gag amounts in both serum and ascites (Fig. 6A,B).

4. Discussion

The inhibition of HIV-1 replication by statins was previously reported in acute HIV-1 infection models, and three

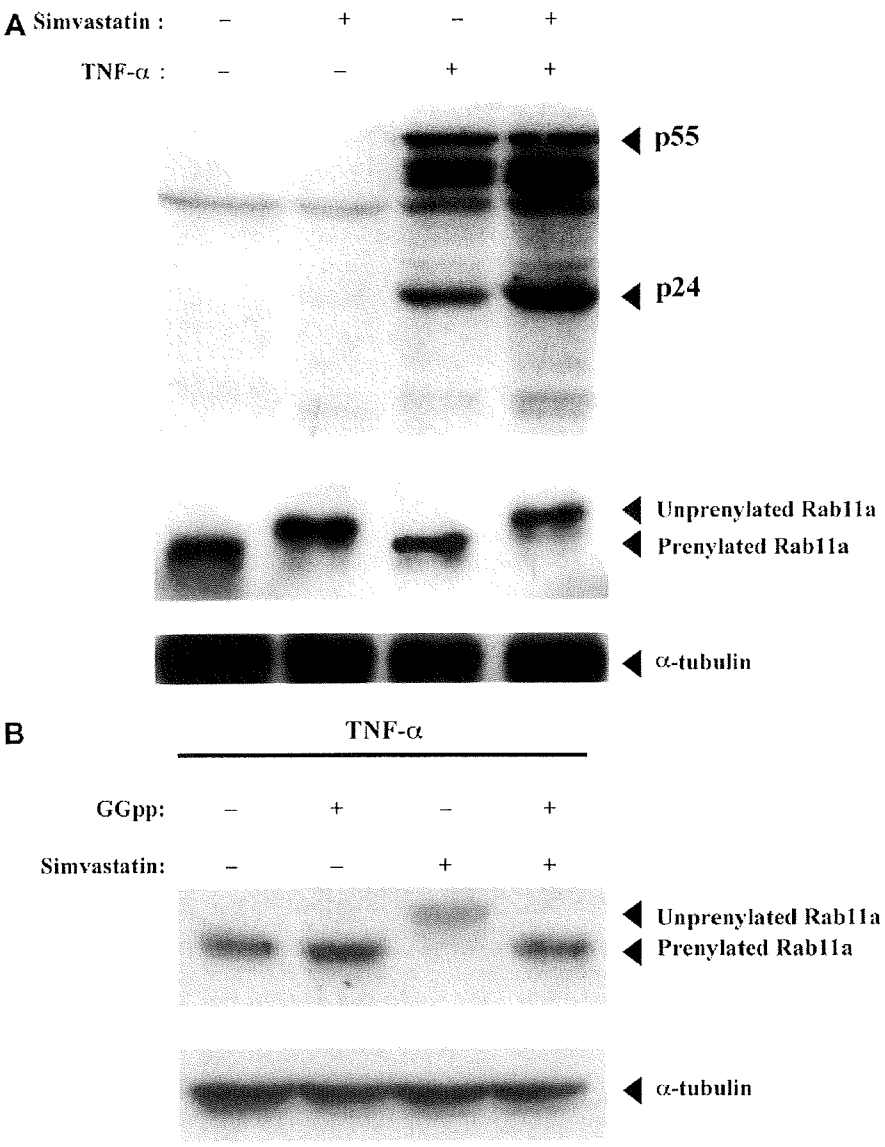

Fig. 3. Simvastatin increased p24 and ablated prenylation of Rab11a in U1 cells. U1 cells were incubated for 2 days with or without simvastatin (A) or GGpp (B), and then stimulated with TNF-α in the continued presence or absence of simvastatin or GGpp for additional 2 days. Whole-cell lysates were prepared and subjected to immunoblotting with serum derived from an HIV-1-infected patient or antibodies to Rab11a or α-tubulin.

different mechanisms were proposed. First, inhibition of HMG-CoA reductase activity resulted in impaired synthesis of GGpp required for prenylation of a small GTPase protein Rho [4]. Second, direct binding of statins to lymphocyte-function-associated antigen 1 (LFA-1) diminished HIV-1 attachment to target cells by preventing the interaction between virion-associated host intercellular adhesion molecule 1 and its natural cell surface ligand LFA-1 [6]. Third, statins disrupted CCR5 and RANTES expression [8]. In this report, we showed statin-induced increase in intracellular Gag and decrease in virus release from chronically HIV-1 infected cells, and defined diminished geranylgeranylation as a principal mechanism of statin-induced inhibition of virus release. The inhibition was associated with nearly a complete loss of prenylation of a small GTPase protein Rab11a, which facilitates vesicle trafficking to the plasma membrane from both the *trans*-Golgi network and recycling endosomes. Indeed, RNA interference-mediated silencing of Rab11a expression also led to a marked reduction in both intracellular and secreted Gag protein. These observations are not limited to TNF-α-induced HIV-1 production in vitro, because the silencing of Rab11a expression also reduced p24 release from U1 cells inoculated in immune-deficient mice.

The effects of simvastatin on HIV-1 replication in U1 cells, increase in intracellular Gag and decrease in virus release, cannot solely be explained by the loss of functional small GTPases involved in vesicle trafficking, because supplementing GGpp in the presence of simvastatin, indeed, restored virus release, but did not normalize the level of intracellular Gag. The increase in intracellular Gag by simvastatin treatment

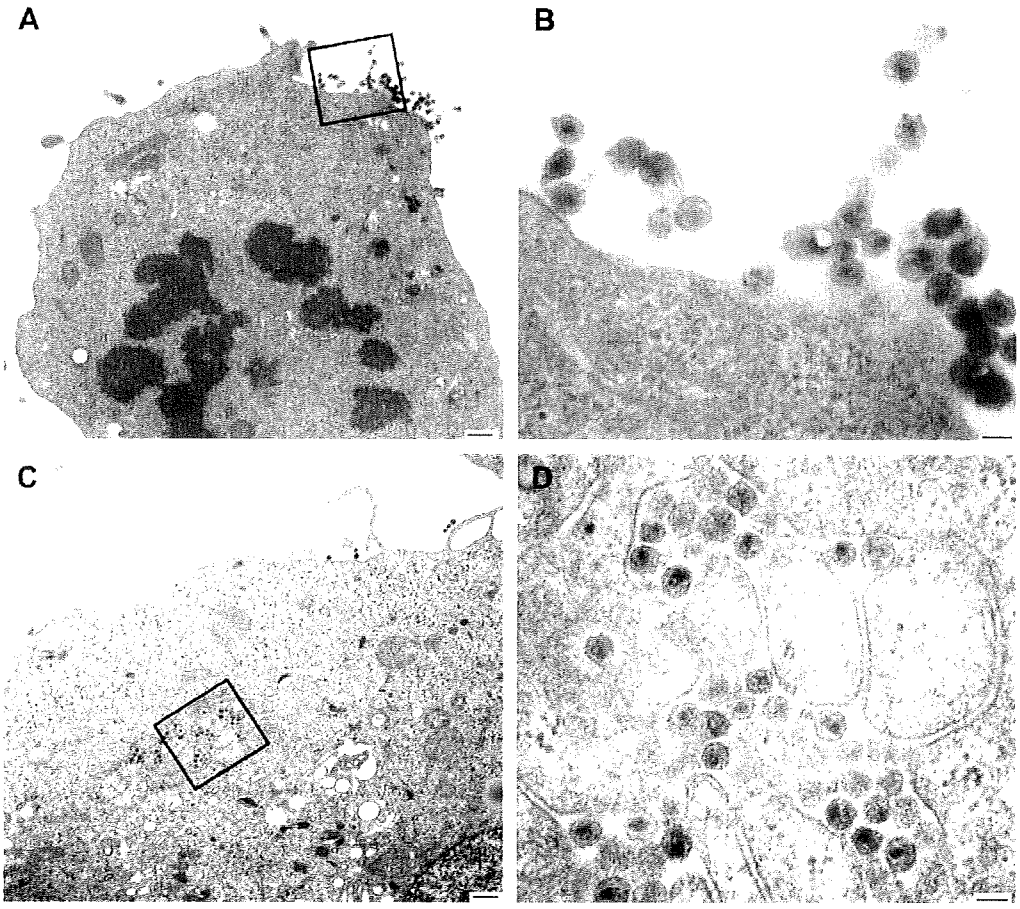

Fig. 4. Transmission electron microscopic (TEM) images of U1 cells. (A) TNF-α-stimulated U1 cell. Many HIV-1 particles are visible at the cell surface. Bar indicates 500 nm (10,000×). (B) Higher magnification of the area indicated by the square in (A). The cone-shaped core structure is evident. Bar indicates 100 nm (60,000×). (C) U1 cell treated with simvastatin and TNF-α. Large vesicles near the Golgi zone contain maturated HIV-1 particles. Bar indicates 500 nm (10,000×). (D) Higher magnification of the area indicated by the square in (C). Bar indicates 100 nm (60,000×).

might simply be a result of accumulation of virions due to impaired virion release, but the results of more specified inhibition by GGTI or gene silencing indicate that loss of prenylation or depletion of Rab11a GTPase reduces both intracellular and extracellular Gag. Thus, simvastatin appears to have yet unknown actions to increase intracellular p24 in U1 cells. In this regard, simvastatin may potentially enhance production of Gag as lovastatin was previously reported to augment HIV-1 LTR-directed transcription in Jurkat cells [4]. The inhibition of virus release by simvastatin would, therefore, be due to loss of prenylation of a yet unidentified protein.

Recent reports support a model of intracellular Gag trafficking common to a variety of cell types in which Gag localizes initially to perinuclear clusters, and then to late endosomes and MVBs and/or MVB-like compartments [23,24]. Both in macrophages and dendritic cells, HIV-1 Gag can be detected in CD63-positive late endosomes and viral exit proceeds through TSG101-dependent budding into the lumen of late endosomes to form multivesicular bodies, followed by the export of viral particles as exosomes [25–28]. However, the transport mechanism of endosomal compartments or MVBs to the cell surface during the course of viral maturation and budding remains to be fully elucidated. Small GTPase proteins have been reported to be involved in vesicle trafficking and actin polymerization. It should be noted that Rab11a is mainly located on pericentriolar recycling endosomes and regulates vesicle trafficking through recycling endosomes to the plasma membrane as well as release of exosomes [29]. In the present report, depletion of Rab11a resulted in an obvious destabilization of Gag p55 and p24, suggesting that Gag failed to traffic through the endosomal compartments or MVBs and could be directed to lysosomal degradation.

Since lipophylic statins cannot be used for HIV-1 infected patients due to its pharmacokinetic interaction with protease inhibitors, inhibiting prenylation of small GTPases involved in Gag trafficking by GGTIs could represent an alternative strategy for effective anti-HIV-1 therapy. GGTI used in this study was previously reported to arrest human tumor cells in G0/G1 and induces $p21^{WAF1/CIP1/SDI1}$ expression in a p53-independent manner and was considered potentially useful in cancer therapy [30]. Perhaps, specific inhibition of individual GTPases involved in HIV-1 replication such as Rab11a would

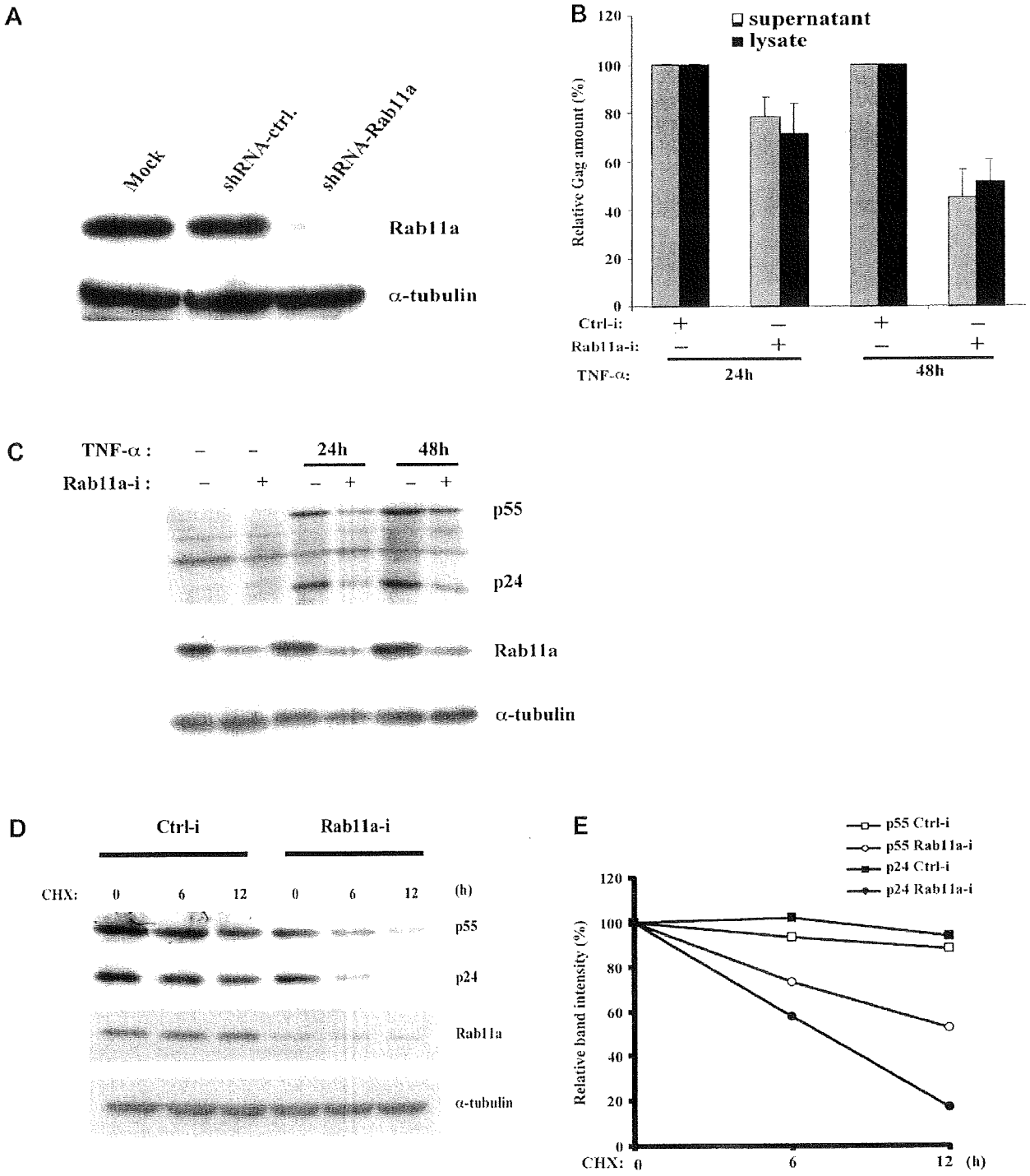

Fig. 5. Depletion of Rab11a destabilized HIV-1 Gag in U1 cells. (A) Whole-cell lysates (30 μg) prepared from U1 cells expressing control (Ctrl-i) or *rab11a*-specific (Rab11a-i) shRNA were subjected to 12% SDS–PAGE and immunoblotting with anti-Rab11a and anti-α-tubulin antibodies. (B) U1 cells expressing Ctrl-i or Rab11a-i were stimulated with TNF-α (1 ng/ml) for 24 or 48 h. Gag in supernatants and cell lysates was quantified, and the relative amounts of Gag are shown in percentage of that for cells expressing Ctrl-i (arbitrarily set at 100%). (C) Whole-cell lysates (30 μg) were prepared 24 or 48 h after TNF-α stimulation, and subjected to immunoblotting with the HIV-1-infected patient's serum, anti-Rab11a or anti-α-tubulin antibodies. (D) U1 cells expressing Ctrl-i or Rab11a-i were stimulated with TNF-α (1 ng/ml) for 24 h. Cells were then treated with cycloheximide (50 μM) for the indicated periods of time and whole-cell lysates (30 μg) were subjected to immunoblotting with the HIV-1-infected patient's serum, anti-Rab11a or anti-α-tubulin antibodies. (E) The relative band intensities of Gag (p55 and p24) normalized by that of α-tubulin are shown in percentage of that of cells before the addition of cycloheximide (arbitrarily set at 100%).

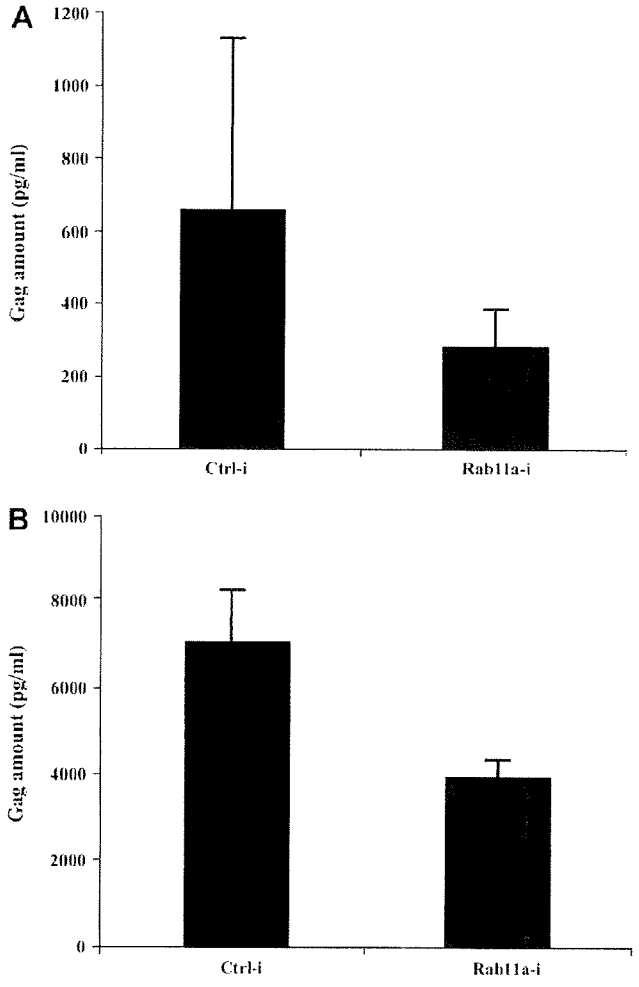

Fig. 6. Depletion of Rab11a reduced HIV-1 production in NOG mice. NOG mice (four mice in each group) were injected intraperitoneally with approximately 2.5×10^6 U1 cells expressing either Ctrl-i or Rab11a-i. The amounts of Gag in serum (A) and ascites (B) were quantified 2 weeks after cell inoculation.

be therapeutically more beneficial, because GGTIs do not work specifically on particular GTPases.

In summary, the present study revealed a critical role for protein geranylgeranylation in HIV-1 virion release from chronically HIV-1-infected promonocytic cells, and suggest that geranylgeranyltransferase-1 or its substrates, small GTPases involved in Gag trafficking, could be attractive molecular targets for controlling HIV-1 replication.

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Overexpressed NF-κB–inducing kinase contributes to the tumorigenesis of adult T-cell leukemia and Hodgkin Reed-Sternberg cells

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The nuclear factor-κB (NF-κB) transcription factors play important roles in cancer development by preventing apoptosis and facilitating the tumor cell growth. However, the precise mechanisms by which NF-κB is constitutively activated in specific cancer cells remain largely unknown. In our current study, we now report that NF-κB–inducing kinase (NIK) is overexpressed at the pretranslational level in adult T-cell leukemia (ATL) and Hodgkin Reed-Sternberg cells (H-RS) that do not express viral regulatory proteins. The overexpression of NIK causes cell transformation in rat fibroblasts, which is abolished by a super-repressor form of IκBα. Notably, depletion of NIK in ATL cells by RNA interference reduces the DNA-binding activity of NF-κB and NF-κB–dependent transcriptional activity, and efficiently suppresses tumor growth in NOD/SCID/γc^{null} mice. These results indicate that the deregulated expression of NIK plays a critical role in constitutive NF-κB activation in ATL and H-RS cells, and suggest also that NIK is an attractive molecular target for cancer therapy. (Blood. 2008;111:5118-5129)

Introduction

The nuclear factor-κB (NF-κB) transcription factors are known to regulate the expression of a wide range of genes involved in development, immune responses, apoptosis, and carcinogenesis as dimers of the REL family members, RelA, RelB, c-Rel, p50, and p52.[1] The p50 and p52 proteins are generated by proteasome-mediated processing of their precursors, p105 and p100, respectively. In resting cells, Rel proteins are sequestered in the cytoplasm through their interactions with the ankyrin repeats of the inhibitory proteins IκBα, -β, and -ϵ, as well as the precursor proteins p105 and p100. On stimulation, signals converge at the multiprotein IκB kinase (IKK) complex, which is composed of 2 catalytic subunits, IKK1/α and IKK2/β, and the scaffolding proteins, NF-κB essential modulator (NEMO, also known as IKKγ) and ELKS.[2] Phosphorylation by the IKK complex of specific serine residues on the IκB or precursor proteins results in their poly-ubiquitination and proteasome-dependent degradation or processing.[2] Released NF-κB then translocates to the nucleus and regulates expression of target genes.

NF-κB signaling pathways are largely classified as either canonical or noncanonical based on the stimuli and targets of the IKK complex.[2] Canonical activation is induced by stimuli, such as tumor necrosis factor-α (TNFα) and interleukin-1β, and involves NEMO- and IKK2/β-dependent phosphorylation and the subsequent degradation of IκB proteins. Noncanonical NF-κB pathways are activated after the stimulation of a range of TNF receptor family members, such as B-cell activating factor belonging to the TNF family (BAFF) receptor, lymphotoxin-β receptor, Fn14 and CD40, and direct NF-κB–inducing kinase (NIK)- and IKK1/α-dependent phosphorylation and subsequent processing of p100, leading to activation of NF-κB complexes containing RelB.[2,3] Of note in this context, the noncanonical pathways operate in a delayed fashion and are sensitive to protein synthesis inhibition.[4,5]

Compared with the mechanisms underlying the transduction of ligand-induced signaling to NF-κB activation, much less is known about how NF-κB is constitutively activated in a variety of cancer cells.[6] Constitutively high NF-κB activity has typically been demonstrated in human hematopoietic cancer cells, including adult T-cell leukemia (ATL), Hodgkin lymphoma, and multiple myeloma cells.[7,8] We have previously reported the aberrant expression of p52 in ATL and Hodgkin Reed-Sternberg (H-RS) cells that do not express viral regulatory proteins, such as Tax of the human T-cell leukemia virus or latent membrane protein 1 of the Epstein-Barr virus.[9,10] In addition, IKK activation in ATL and H-RS cells was found to be sensitive to protein synthesis inhibition.[10,11] These results indicate that the noncanonical pathways of NF-κB activation operate in these cancer cells. Aberrant p52 expression has also been reported in other types of cancer cells, including breast,[12] prostate,[13] pancreas,[14] and colon.[15] However, the actual triggers of noncanonical NF-κB activation in these cancer cells remain largely unknown except for certain multiple myeloma cells that have mutations in the *NIK*, *TRAF3*, and related genes.[16,17]

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NIK is a serine-threonine kinase that is an essential participant in the induction of the IKK1-dependent processing of p100 as well as IκB degradation in response to stimuli, such as CD70, CD40 ligand, and BAFF.[18] It has also been reported previously that the IKK complex is recruited to CD27 in a manner dependent on NIK function. However, the mechanism by which NIK activity is regulated thereafter was unknown until it was recently demonstrated that these stimuli protect basally translated endogenous NIK protein from proteasome-mediated degradation.[19,20] Liao et al reported that the interaction of NIK with TNF receptor–associated factor 3 (TRAF3) is responsible for the rapid degradation of NIK and that noncanonical NF-κB stimuli induce the degradation of TRAF3 and the elevation of NIK expression.[19] In a separate study, Qing et al have demonstrated that noncanonical NF-κB stimuli stabilize the NIK protein but do not modify its RNA expression or protein translation.[20] The findings of these studies explain the delay in triggering the noncanonical pathway and its high sensitivity to protein synthesis inhibition.

Because NIK is a central regulator of the noncanonical pathway of NF-κB activation, we have investigated in our current study how this kinase is regulated in hematopoietic cancer cells, in which IKK is constitutively activated in the absence of viral regulators.

Methods

Cell culture

ED40515(−),[21] ATL-43Tb(−),[22] and TL-Om1[23] are human T-cell leukemia virus type-I (HTLV-I)–infected T-cell lines established from the leukemic cells of ATL patients. The H-RS cell lines, HDLM-2, L428, and L540, were purchased from the German Collection of Micro-organisms and Cell Cultures (Braunschweig, Germany). CEM[24] and Jurkat[25] are HTLV-I–free human T-lymphoblastic leukemia cell lines. A human B-cell line, Romas RG69,[20] was a kind gift from Dr Gutian Xiao (State University of New Jersey, Piscataway, NJ). Primary leukemia cells derived from ATL patients were obtained under informed consent at Imamura Bun-in Hospital and supplied through the Joint Study on Predisposing Factors of ATL Development. The patients were diagnosed with ATL on the basis of clinical and hematologic features and the presence of antibodies to ATL-associated antigens in serum and of the HTLV-I proviral genome in the leukemia cells. Use of peripheral blood lymphocytes from ATL patients for research purposes was approved by the institutional review board of each institute. Peripheral blood mononuclear cells (PBMCs) derived from healthy donors were also obtained under informed consent. PBMCs were isolated from both ATL patients and healthy donors by density gradient separation with Ficoll-Plaque PLUS (Amersham Biosciences, Uppsala, Sweden). Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate; 5R is a NEMO-deficient subline of the Rat-1 cell line and has been described previously.[26] B5 and h12 are sublines of Rat-1 and 5R, respectively, express the blasticidin deaminase gene under the control of an NF-κB–dependent promoter, and have also been described previously.[26,27] Plat-E packaging cells were described previously.[28] B5, h12, Plat-E, 293T cells, and mouse embryonic fibroblasts were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate. Anchorage-independent cell growth was examined essentially as described previously.[29] Images were captured using an inverted microscope (IX70, Olympus, Tokyo, Japan) and processed with Openlab 3.0.2 software (Improvision, Coventry, United Kingdom). Cells used in this study were all maintained at 37°C in air containing 5% CO_2.

Virus infection and transfection

Plat-E cells were transfected with pMRX-HA-NIK-ires-puro, pMRX-HA-kd-NIK-ires-puro, or pMRX-HA-ires-puro (EV1) (Document S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article) using the calcium phosphate precipitation method. Culture supernatants were collected 48 hours after transfection and filtered. B5 and h12 cells were infected for 2 hours in the presence of 10 μg/mL polybrene. Infected cells were then cultured in medium containing 2 μg/mL puromycin, and cell clones were isolated. Rat fibroblasts expressing SR-IκBα or its empty control vector (EV2) were established essentially as described previously.[10] For production of lentiviruses, 293T cells were cotransfected with pCS-puro-Ctli, pCS-puro-NIKi-1, or pCS-puro-NIKi-2 (Document S1) together with the pCMVΔR8.2 packaging construct and pHCMV-VSV-G (kind gifts from Dr I.S.Y. Chen) using FuGENE 6 (Roche Applied Science, Indianapolis, IN). Culture supernatants were collected 48 hours after transfection and filtered. ED40515(−) and ATL-43Tb(−) cells were infected once or twice with 24 hours interval with these lentiviruses for 6 hours in the presence of 10 μg/mL polybrene. At 48 hours after the infection, cells were cultured in medium containing 2 μg/mL puromycin for an additional 48 hours. These infectants were subjected to immunoblotting, electrophoretic mobility shift assay (EMSA), and transient transfection with 2 μg of IgκCona-luc[30] and pEF1-LacZ[26] using DMRIE-C (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Assays for luciferase and β-galactosidase were performed 48 hours after transfection by standard methods. Luciferase activity was normalized on the basis of β-galactosidase activity. The growth of lentivirus-infected cells was determined by the trypan blue staining method.

Immunoprecipitation

For the immunoprecipitation of endogenous NIK, approximately 2×10^7 cells were lysed in buffer A (20 mM Tris-HCl, pH7.5, 0.5% Nonidet P-40, 150 mM NaCl supplemented with 1 μg/mL aprotinin, 1 μg/mL leupeptin, 0.57 mM phenylmethanesulphonylfluoride, 10 μM MG132, 10 μM MG115) followed by preclearing with purified rabbit IgG (Cedarlane Laboratories, Hornby, ON) and protein G-Sepharose beads (Pierce Biotechnology, Rockford, IL). After centrifugation at 14000 rpm for 3 minutes, supernatants were subjected to immunoprecipitation with purified nonimmune rabbit IgG or anti-NIK antibody (#4994) (Cell Signaling Technology, Danvers, MA). Immunoprecipitates were washed 3 times with TNT buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 1% Triton X-100). Endogenous NIK proteins were detected by immunoblotting with anti-NIK antibody (#4994). For the immunoprecipitation of HA-tagged NIK, 750 μg cell lysates prepared with buffer A was subjected to immunoprecipitation with anti-HA antibody (12CA5, a kind gift from Dr A. Israël, Institut Pasteur Paris, Paris, France). Immunoprecipitates were washed 3 times with TNT buffer. HA-tagged NIK proteins were detected by immunoblotting with anti-NIK antibody. For immunoprecipitation of endogenous IKK1/2, 1500 μg cell lysates prepared with buffer A were subjected to immunoprecipitation with anti-IKK1 monoclonal antibody (B78-1; BD PharMingen, San Diego, CA) or purified mouse IgG2b (MI10-104; Bethyl Laboratories, Montgomery, TX). Immunoprecipitates were washed 3 times with TNT buffer. Expression of endogenous proteins was detected by immunoblotting with antiphospho-IKK1/IKK2 (Ser180/Ser181) (#2681; Cell Signaling Technology), anti-IKK1 (H-744), or anti-IKK2 (H-470; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies.

Quantitative RT-PCR

Total RNA was extracted using Isogen reagents (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Quantitative RT-PCR amplifications were performed with 100 ng total RNA, 0.3 μM of each primer, and 0.25 μM TaqMan probe using an ABI-7700 Sequence Detector (Applied Biosystems, Foster City, CA); reverse transcription was performed at 48°C for 30 minutes, Taq DNA polymerase was activated at 95°C for 10 minutes, followed by 45 amplification cycles of 95°C for 15 seconds, and annealing and extension at 60°C for 1 minute. The *NIK*, *VEGF*, *ICAM-1*, and *MMP-9* mRNA levels were normalized based on the amount of *18S* ribosomal RNA determined simultaneously by the real-time RT-PCR.

Mice and inoculation of cells

NOD/SCID/γc^{null} (NOG)[31] mice were purchased from the Central Institute for Experimental Animals (Kawasaki, Japan). All mice were maintained under specific pathogen-free conditions in the Animal Center of Tokyo Medical and Dental University (Tokyo, Japan). The Ethical Review Committee of the institute approved the experimental protocol. ED40515(−) cells expressing Ctli or NIKi-1 and -2 were washed twice with serum-free RPMI 1640 and resuspended in the same medium. Mice were anesthetized with ether and inoculated subcutaneously in the postauricular region with 5×10^6 cells per mouse, as described previously.[31] We measured tumor size and weight 2 weeks after cell inoculation.

Statistics

Statistical significance was evaluated using a 2-tailed, unpaired Student's *t* test. *P* values less than .05 were considered to be significant.

Results

NIK is aberrantly expressed in both adult T-cell leukemia and Hodgkin Reed-Sternberg cells

The constitutive processing of p100 to p52 in ATL and H-RS cells[9,10] prompted us to examine whether NIK is aberrantly expressed in both established and primary ATL cells. Immunoblotting of whole-cell lysates prepared from ATL or H-RS cells did not show any detectable NIK signal (data not shown); however, when endogenous NIK was immunoprecipitated from approximately 20 million of these cells and subjected to immunoblotting, NIK was specifically detectable in anti-NIK immunoprecipitates from ATL and H-RS cells, but not from control cells, such as CEM and Jurkat (Figure 1A). Previous studies revealed that inhibition of the proteasome function allowed for detection of endogenous NIK in simple whole-cell lysates of B-cell lines.[19,20] Treatment of ED-40515(−) cells with the MG132 proteasome inhibitor for 3 hours before harvesting enabled us to observe robust endogenous NIK expression at the expected position (Figure 1B). Lysates of 293T cells with or without exogenous NIK expression were used as the positive and negative controls, respectively. We next examined the NIK expression levels as well as those of p100 phosphorylated at serine residues 866 and 870 in a panel of ATL, H-RS, and control cells (Figure 1C). No appreciable NIK expression could be observed in control CEM and Jurkat T-cell lines treated with MG132, in which NF-κB is not constitutively activated. Proteasome inhibition induced strong NIK expression in other Tax-negative ATL-derived cell lines, ATL-43Tb(−) and TL-Om1. Proteasome inhibition also strongly augmented NIK expression in H-RS cells, but only weakly so in the control B-cell lines, RG69. These results indicate that the steady-state levels of NIK of the authentic size are elevated in ATL and H-RS cells, and suggest that NIK may be abundantly produced in ATL and H-RS cells, but is rapidly degraded by the proteasome. The levels of NIK expression correlated well with those of phosphorylated p100 (Figure 1C). Moreover, p52 and the phoshorylated form of IκBα were also abundant in ATL and H-RS cell lines, but not in the control T-cell lines (Figure 1C). These results indicate that the overexpression of NIK is closely linked to the downstream events leading to constitutive activation of the canonical and noncanonical NF-κB pathways in ATL and H-RS cells. A previous study suggested that L428 cells express a C-terminally truncated form of IκBα and that the phosphorylated form of this protein was accumulated after treatment of the cells with proteasome inhibitor or dexamethasone.[32,33] In agreement with this, we did not detect IκBα expression with the antibody used in this study, which recognizes the C-terminus of the protein, but detected the phoshorylated form of this IκBα only after treatment with MG132 (data not shown).

We next investigated *NIK* expression at the mRNA level by quantitative PCR (Figure 1D) and found that that *NIK* transcripts were at between 20- and 100-fold higher levels in ATL and H-RS cells, compared with CEM cells. Next, actinomycin D was used to block new mRNA synthesis, so that decay of existing transcripts could be detected. Quantitative PCR analyses revealed that the half-life of *NIK* mRNA was approximately 3 hours both in the ATL and control T cells (Figure 1E). Essentially similar results were obtained with the other cell lines shown in Figure 1D, including H-RS cell lines (data not shown). A previous report has demonstrated that NF-κB is constitutively activated in primary ATL cells in the peripheral blood.[34] We therefore quantified the *NIK* mRNA levels in PBMCs from both healthy donors and ATL patients (Figure 2A), and found that *NIK* mRNA is overexpressed in PBMCs of 15 of 21 ATL patients. Actinomycin D treatment of PBMCs further revealed that *NIK* mRNA was not apparently stabilized in primary ATL cells (Figure 2B). Moreover, fluorescence in situ hybridization studies on primary ATL cells failed to detect amplification or translocation of the *NIK* gene (Figure S1; Table S2). Finally, when PBMCs were cultured for 3 hours in the presence of MG132, NIK protein was detectable in cells from an ATL patient showing abundant *NIK* mRNA expression, but not in those from a healthy donor (Figure 2C).

NIK transforms rat fibroblasts in an NF-κB–dependent manner

To further explore the roles for NIK during cell transformation, we infected the 3T3-like rat fibroblast cell line Rat-1 with a retroviral vector expressing human NIK and examined its oncogenic activity. As expected, cells transduced with this NIK vector exhibited strong NF-κB DNA binding activity within 36 hours (data not shown). Rat-1 cells transduced with a control retrovirus became resistant to the selection marker puromycin approximately 24 hours after infection and continued to proliferate rapidly. In contrast, Rat-1 cells transduced with the NIK expression vector expressed a readily detectable level of NIK, had a transformed morphology, but ceased proliferating and died within 3 to 4 days after becoming resistant to puromycin. Cells that survived 2 weeks of puromycin selection after NIK transduction eventually appeared indistinguishable from those transduced with the control vector and showed no detectable NIK expression or NF-κB DNA binding activity (data not shown).

Based on these observations, we speculate that the retroviral overexpression of NIK is toxic to the cells so that only cells that had lost its expression could emerge from the puromycin-resistant pools. To address this problem, we used B5 and h12 cells carrying an integrated Igκ2bsrH plasmid that confers resistance to the antibiotic blasticidin S when cells are constitutively expressing active NF-κB.[26] B5 cells are derived from Rat-1 cells, and h12 cells are from 5R cells that lack NEMO expression. When the B5 and h12 cells were transduced with the wild-type NIK retroviral expression vector and subjected to selection with both puromycin and blasticidin S, the majority of the resultant cell clones maintained detectable NIK expression (Figure 3A), elevated catalytic activity of IKK (Figure 4), and the initial transformed morphology (Figure 5B). On the other hand, when B5 and h12 cells were transduced with a retrovirus vector expressing a catalytically inactive mutant form of NIK and selected with puromycin alone, the cells successfully

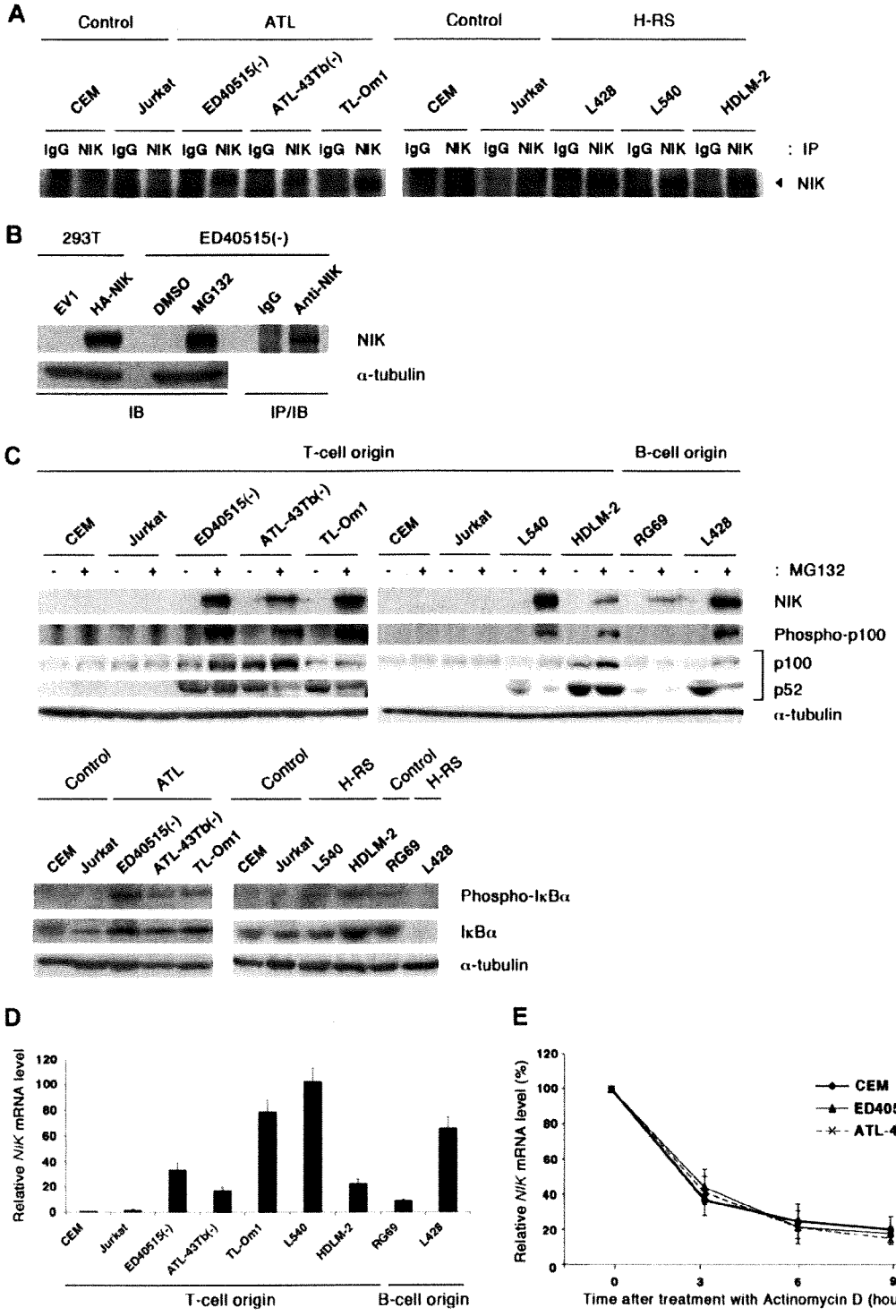

Figure 1. NIK protein is overexpressed in established ATL and Hodgkin Reed-Sternberg cells. (A) Steady-state levels of NIK expression in the ATL and H-RS cell lines were revealed by immunoprecipitation-coupled immunoblotting. Approximately 2 10^7 cells were lysed with buffer A. After preclearing, immunoprecipitation was performed at 4°C, using anti-NIK antibody (NIK) or its isotype IgG (IgG). After 3 washes with TNT buffer, immune complexes were analyzed by immunoblotting with anti-NIK antibody. (B) 293T cells were transfected with pMRX-HA-iresPuro or pMRX-HA-NIKiresPuro for 24 hours. Whole-cell lysates were used as negative and positive controls. ED40515(−) cells were pretreated with (+) or without (−) MG132 (20 μM) for 3 hours, lysed with RIPA buffer, and subjected to immunoblotting with anti-NIK or anti-α-tubulin antibodies. Immunoprecipitation-coupled immunoblotting was performed as in panel A. (C) Top panels: control T-cell lines (CEM and Jurkat), leukemic cell lines derived from ATL patients that do not express Tax (ED40515(−), ATL43-Tb(−), and TL-Om1, a control B-cell line (RG69), and H-RS cell lines (HDLM-2 and L540) were pretreated with (+) or without (−) MG132 (20 μM) for 3 hours, and 30 μg of the whole-cell extracts were subjected to Western blot analysis with the antibodies to the indicated proteins. Bottom panels: Whole-cell extracts from the indicated cell lines were analyzed by Western blotting with the antibodies to the indicated proteins. (D) Total RNA was extracted from the indicated cell lines and subjected to real-time RT-PCR to quantify the *NIK* mRNA levels. The *NIK* mRNA levels were normalized to *18S* RNA. The relative *NIK* mRNA levels shown represent the fold increases in mRNA abundance, relative to that of the CEM cells (arbitrarily set at 1). (E) Cells were cultured in the presence of actinomycin D (5 μg/mL) for the times indicated, and then total RNA was isolated and subjected to quantitative RT-PCR as in panel D. Data are expressed as mean plus or minus SD of 3 independent experiments. The relative amounts of *NIK* mRNA shown represent the percentages in mRNA abundance, relative to that of each cell line before the addition of actinomycin D (arbitrarily set at 100%). IB indicates immunoblotting; IP, immunoprecipitation.