a family with partial androgen insensitivity syndrome can reduce the efficiency of protein translation (Choong *et al.*, 1996). Finally, it has been reported recently that a Graves'-disease–associated SNP that substitutes T for C at position −1 upstream from the open frame ATG codon of the CD40 gene enhances translation and could predispose to disease (Jacobson *et al.*, 2005). However, all these SNPs are located near the authentic translation initiation ATG codon of the proteins and directly affect the translation efficiency from the open frame ATG codon. In the case of the IL-7 gene, however, the mutation is located at −29 to −27 upstream from the open frame ATG codon, rather than near the authentic translation initiation codon. Nevertheless, it could up-regulate the IL-7 gene expression probably by changing the translation efficiency from the upstream, out-of-frame ATG codon. Our data showed that the consensus sequence for translation initiation is important, not only for the open-frame initiation ATG codon, but also for the upstream, out-of-frame ATG that is thought to reduce translation efficiency from the downstream initiation ATG. Similar to mutations within the consensus sequence of the open-frame initiation ATG codon, nucleotide substitution within the consensus sequence of the upstream, out-of-frame ATG can also modulate translation efficiency.

Our data also showed that the 1470-bp upstream non-coding region of the IL-7 gene exhibited extremely low levels of diversity in both Japanese and Thai populations. Also, no non-synonymous polymorphism has yet been identified in the IL-7 coding region. The reason for the low levels of diversity of the IL-7 gene is not clear at present, but is probably the result of its importance for the survival, development and proliferation of B and T cells. Experiments with IL-7 deficient mice proved that IL-7 is a non-redundant cytokine (von Freeden-Jeffry *et al.*, 1995). It is therefore reasonable to assume that low levels of diversity of the upstream non-coding region of the IL-7 gene that regulates the transcription of this gene are needed to provide a stable condition for IL-7 production.

In conclusion, we have identified a polymorphism in the upstream non-coding region of the IL-7 gene that could up-regulate gene expression. Although the frequency of this allele is very low in Japan and Thailand, it would be interesting to analyse this polymorphism in HIV-1-infected individuals with different rates of immune reconstitution after treatment with a highly active antiretroviral therapy. It would be important to analyse this polymorphism in other ethnic groups. On the other hand, an elevation of plasma IL-7 has also been reported in lymphopenia cases, including patients undergoing bone marrow transplantation or chemotherapy for cancer, or patients with idiopathic $CD4^+$ lymphopenia (Fry & Mackall, 2005; Bolotin *et al.*, 1999). It would also be of interest to analyse this polymorphism in those patients.

Acknowledgements

We thank J Sakuragi and S Sakuragi for their helpful discussions, S Bandou for technical assistance and N Teramoto for help. We also thank the Japanese and Thais who kindly donated blood for this study.

References

Beq, S., Rannou, M.T., Fontanet, A., Delfraissy, J.F., Theze, J. & Colle, J.H. (2004) HIV infection: pre-highly active antiretroviral therapy IL-7 plasma levels correlate with long-term CD4 cell count increase after treatment. *AIDS*, **18**, 563.

Bolotin, E., Annett, G., Parkman, R. & Weinberg, K. (1999) Serum levels of IL-7 in bone marrow transplant recipients: relationship to clinical characteristics and lymphocyte count. *Bone Marrow Transplantation*, **23**, 783.

Choong, C.S., Quigley, C.A., French, F.S. & Wilson, E.M. (1996) A novel missense mutation in the amino-terminal domain of the human androgen receptor gene in a family with partial androgen insensitivity syndrome causes reduced efficiency of protein translation. *Journal of Clinical Investigation*, **98**, 1423.

Dean, M., Carrington, M., Winkler, C., Huttley, G.A., Smith, M.W., Allikmets, R. *et al.* (1996) Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. *Science*, **273**, 1856.

von Freeden-Jeffry, U., Vieira, P., Lucian, L.A., McNeil, T., Burdach, S.E. & Murray, R. (1995) Lymphopenia in interleukin (IL) -7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. *Journal of Experimental Medicine*, **181**, 1519.

Fry, T.J. & Mackall, C.L. (2002) Interleukin-7: from bench to clinic. *Blood*, **99**, 3892.

Fry, T.J. & Mackall, C.L. (2005) The many faces of IL-7: from lymphopoiesis to peripheral T cell maintenance. *Journal of Immunology*, **174**, 6571.

Fry, T.J., Connick, E., Falloon, J., Lederman, M.M., Liewehr, D.J., Spritzler, J. *et al.* (2001) A potential role for interleukin-7 in T-cell homeostasis. *Blood*, **97**, 2983.

Gonzalez-Conejero, R., Corral, J., Roldan, V., Martinez, C., Marin, F., Rivera J. *et al.* (2002) A common polymorphism in the annexin V Kozak sequence (−1C>T) increases translation efficiency and plasma levels of annexin V, and decreases the risk of myocardial infarction in young patients. *Blood*, **100**, 2081.

Grabstein, K.H., Namen, A.E., Shanebeck, K., Voice, R.F., Reed, S.G. & Widmer, M.B. (1990) Regulation of T cell proliferation by IL-7. *Journal of Immunology*, **144**, 3015.

Heufler, C., Topar, G., Grasseger, A., Stanzl, U., Koch, F., Romani, N. *et al.* (1993) Interleukin 7 is produced by murine and human keratinocytes. *Journal of Experimental Medicine*, **178**, 1109.

Jacobson, E.M., Concepcion, E., Oashi, T. & Tomer, Y. (2005) A Graves' disease-associated Kozak sequence single-nucleotide polymorphism enhances the efficiency of CD40 gene translation: a case for translational pathophysiology. *Endocrinology*, **146**, 2684.

Kopka, J., Mecikovsky, D., Aulicino, P.C., Mangano, A.M., Rocco, C.A. & Bologna, R. (2005) High IL-7 plasma levels may induce and predict the emergence of HIV-1 virulent strains in pediatric infection. *Journal of Clincal Virology*, **33**, 237.

Kostrikis, L.G., Huang, Y., Moore, J.P., Wolinsky, S.M., Zhang, L., Guo, Y. *et al.* (1998) A chemokine receptor CCR2 allele delays HIV-1 disease progression and is associated with a CCR5 promoter mutation. *Nature Medicine*, **4**, 350.

Kozak, M. (1986) Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell*, **44**, 283.

Liu, H., Chao, D., Nakayama, E.E., Taguchi, H., Goto, M., Xin, X. *et al.* (1999) Polymorphism in RANTES chemokine promoter affects HIV-1 disease progression. *Proceedings of the National Academy of Sciences USA*, **96**, 4581.

Llano, A., Barretina, J., Gutierrez, A., Blanco, J., Cabrera, C. & Clotet, B. (2001) Interleukin-7 in plasma correlates with CD4 T-cell depletion and may be associated with emergence of syncytium-inducing variants in human immunodeficiency virus type 1-positive individuals. *Journal of Virology*, 75, 10319.

Lupton, S.D., Gimpel, S., Jerzy, R., Brunton, L.L., Hjerrild, K.A., Cosman, D. *et al.* (1990) Characterization of the human and murine IL-7 genes. *Journal of Immunology*, 144, 3592.

Michael, N.L., Louie, L.G., Rohrbaugh, A.L., Schultz, K.A., Dayhoff, D.E., Wang, C.E. *et al.* (1997) The role of CCR5 and CCR2 polymorphisms in HIV-1 transmission and disease progression. *Nature Medicine*, 3, 1160.

Nakayama, E.E., Hoshino, Y., Xin, X., Liu, H., Goto, M., Watanabe, N. *et al.* (2000) Polymorphism in the interleukin-4 promoter affects acquisition of human immunodeficiency virus type 1 syncytium-inducing phenotype. *Journal of Virology*, 2000 (74), 5452.

Namen, A.E., Lupton, S., Hjerrild, K., Wignall, J., Mochizuki, D.Y., Schmierer, A. *et al.* (1988) Stimulation of B-cell progenitors by cloned murine interleukin-7. *Nature*, 333, 571.

Oshima, S., Nakamura, T., Namiki, S., Okada, E., Tsuchiya, K., Okamoto, R. *et al.* (2004) Interferon regulatory factor 1 (IRF-1) and IRF-2 distinctively up-regulate gene expression and production of interleukin-7 in human intestinal epithelial cells. *Molecular and Cellular Biology*, 24, 6298.

Plum, J., De Smedt, M., Leclercq, G., Verhasselt, B. & Vandekerckhove, B. (1996) Interleukin-7 is a critical growth factor in early human T-cell development. *Blood*, 88, 4239.

Schluns, K.S., Kieper, W.C., Jameson, S.C. & Lefrancois, L. (2000) Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nature Immunology*, 1, 426.

Signori, E., Bagni, C., Papa, S., Primerano, B., Rinaldi, M., Amaldi, F. *et al.* (2001) A somatic mutation in the 5'UTR of BRCA1 gene in sporadic breast cancer causes down-modulation of translation efficiency. *Oncogene*, 20, 4596.

Sudo, T., Nishikawa, S., Ohno, N., Akiyama, N., Tamakoshi, M. & Yoshida, H. (1993) Expression and function of the interleukin 7 receptor in murine lymphocytes. *Proceedings of the National Academy of Sciences, USA*, 90, 9125.

Wolf, S.S. & Cohen, A. (1992) Expression of cytokines and their receptors by human thymocytes and thymic stromal cells. *Immunology*, 77, 362.

Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmacology 565 (2007) 212–219
ejp
www.elsevier.com/locate/ejphar

Magnolia ovovata extract and its active component magnolol prevent skin photoaging via inhibition of nuclear factor κB

Kiyotaka Tanaka [a,b], Junichi Hasegawa [b], Kaori Asamitsu [a], Takashi Okamoto [a,*]

[a] *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*
[b] *Department of Research and Development, Ichimaru Pharcos Co., Ltd., Motosu, Japan*

Received 5 October 2006; received in revised form 27 December 2006; accepted 30 January 2007
Available online 17 February 2007

Abstract

Transcriptional activity of nuclear factor κB (NF-κB) is induced by environmental signals including inflammation, UV irradiation and oxidative stress. It was shown that the NF-κB activity greatly contributes to the skin photoaging process. Thus, it is plausible that NF-κB inhibitors could directly prevent skin photoaging. In this study, we found that *Magnolia ovovata* extract inhibited NF-κB-mediated gene expression and demonstrated that external swabbing with Magnolia extract preventing skin photoaging processes through keratinocyte hyperproliferation and degradation of collagen fibers in mice skin. We have identified magnolol as the solely responsible active compound in Magnolia extract. Magnolol effectively inhibited the NF-κB-dependent transcription, but no effect was observed with other inducible transcription factors such as activator protein-1 (AP-1) and cyclic-AMP responsive element-binding protein (CREB). In addition, magnolol was effective in inhibiting the production of basic fibroblast growth factor (bFGF) and matrix metalloprotease-1 (MMP-1) from the cells overexpressing p65, a major subunit of NF-κB. Although magnolol did not affect the phosphorylation and degradation of IκBα, it inhibited the nuclear translocation of the activated NF-κB. These findings suggest that Magnolia extract and its active component magnolol can be used to prevent the skin photoaging via inhibiting NF-κB by external topical application.

Keywords: NF-κB; Photoaging; Ultraviolet; *Magnolia ovovata*; Magnolol; Skin

1. Introduction

Ultraviolet (UV) irradiation is one of the harmful environmental factors that cause skin photoaging (Matsumura and Ananthaswamy, 2004). Chronically irradiated skins by such harmful factors are associated with abnormal cutaneous reactions such as epidermal hyperplasia, accelerated breakdown of collagen, and inflammatory responses. Our previous report (Tanaka et al., 2005) demonstrated that the UVB-induced skin photoaging processes are ascribable to the activation of a transcription factor, nuclear factor κB (NF-κB). Characteristic skin changes of the UV-irradiated skin include fine and coarse wrinkling, roughness, dryness and laxity (Chung, 2003). Among these changes, skin wrinkling, roughness, and laxity can be ascribed to keratinocyte hyperproliferation and collagen fiber degradation (Brenneisen et al., 2002). UV irradiation induces skin inflammation through production of proinflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα), that subsequently activate NF-κB and stimulate production of matrix metalloprotease-1 (MMP-1) (Chung, 2003; Sun et al., 2002; Bond et al., 1999), eventually leading to degradation of collagen fibers (Corsini et al., 1997; Yarosh et al., 2000; Barchowsky et al., 2000). It is also reported that NF-κB stimulates basic fibroblast growth factor (bFGF) production (Sabourin et al., 1993; Wakisaka et al., 2002) in dermis upon UV irradiation and is responsible for the hyperproliferation of keratinocytes.

We previously found that NF-κB is responsible for skin photoaging and its inhibitor parthenolide could prevent such processes by blocking the UVB-induced production of bFGF and MMP-1 and prevented the UVB-induced proliferation of keratinocytes and melanocytes (Tanaka et al., 2005). Similar

* Corresponding author. Tel.: +81 52 853 8205; fax: +81 52 859 1235.
E-mail address: tokamoto@med.nagoya-cu.ac.jp (T. Okamoto).

0014-2999/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2007.01.095

reports were reported from others (Sabourin et al., 1993; Wakisaka et al., 2002 and references therein). However, all these studies have been carried out with internal application of NF-κB inhibitors either intraperitoneal or *per os* applications. As such internal applications were not suitable for prevention of skin photoaging, in this study we have screened various plant extracts by topical swabbing to find such an agent.

NF-κB is normally sequestrated in the cytoplasm as an inactive complex with inhibitory molecule inhibitor κB (IκB). The signal-induced degradation of IκB liberates NF-κB into the nucleus where it stimulates expression of the target genes (Okamoto et al., 1997; Umezawa and Chaicharoenpong, 2002; Victoriano et al., 2006). In this study, we attempted to identify an active agent, contained in a plant extract, which can be applied externally.

Magnolia obovata Thunb is known as pharmaceutical plant that has anti-inflammatory activity (Chen et al., 2002; Lee et al., 2005). Magnolol is a phenolic compound and is the well-described pharmaceutical component of *Magnolia obovata* Thunb. Magnolol is known to inhibit smooth muscle contraction (Ko et al., 2003), tumour metastasis (Ikeda et al., 2003) and inflammatory responses, and exhibit anti-microbial effects (Park et al., 2004). Although it was recently shown that magnolol could inhibit the NF-κB activation pathway in the downstream of mitogen-activated protein kinase kinase 1 (MEKK-1) (Lee et al., 2005), the mechanism of action is yet to be clarified. After extensive screening trials of plant extracts with our UVB-skin photoaging system using an animal model (Tanaka et al., 2005), we have identified *Magnolia obovata* Thunb extract (Magnolia extract) being able to block the UVB-mediated skin changes via external swabbing. We clarified that this effect of Magnolia extract was through inhibition of NF-κB activation.

In this report, we demonstrate that Magnolia extract exhibits preventive effects against the UV-induced skin photoaging by external swabbing. We also found that the magnolol is the active component contained in the Magnolia extract and is responsible for the anti-NF-κB effects.

2. Materials and methods

2.1. Reagents and plasmids

Magnolia extract was made by bark of *Magnolia obovata* Thunb. The bark was soaked in 50% ethanol of 10 times volumes for 1 week, followed by filtration through a cellulose membrane of 0.45 μm (pore size). Purified magnolol was purchased from Matsuura Yakugyo (Nagoya, Japan). Recombinant human TNFα was purchased from Wako (Osaka, Japan). The reporter plasmid expressing firefly luciferase under the control of NF-κB (pGL3–4κBwt-Luc) was constructed by inserting four tandem copies of the κB sequence (GGGACTTTCC) from HIV-1 enhancer into pGL3-promoter vector (Promega, Madison, WI) as reported previously (Tetsuka et al., 2000; Tanaka et al., 2005). Construction of the mutant NF-κB reporter plasmid, pGL3–4κBm-luc, containing mutated NF-κB binding sites, was described previously (Tanaka et al., 2005; Tetsuka et al., 2000). Control luciferase reporter plasmids under controls of CRB, pCRE-luc, and AP1, pAP-1-luc, were purchased from Stratagene (Jolla, CA). The p65 expressing plasmid, pCMV-p65, was described previously (Tetsuka et al., 2000).

2.2. Cell culture

The HaCaT human keratinocyte cell line (Boukamp et al., 1988) was generously provided by N. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany). HaCaT cells were grown at 37 °C in RPMI 1640 medium supplemented with 1% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Human normal fibroblasts (KURABO, Osaka, Japan) were grown at 37 °C in DMEM supplemented with 5% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Human embryonic kidney 293 cells (Riken, Tsukuba, Japan) were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM glutamate, 100 units/ml penicillin and 100 μg/ml streptomycin. The cytotoxicity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sanda et al., 2005).

2.3. Transfection and luciferase assay

Cells were transfected with various plasmids using Fugene-6 transfection reagent (Roche, Basal, Switzerland). Briefly, cells were cultured in 12-well plates, and transfections were performed with 1.5 μl Fugene-6 transfection reagent per ml culture medium and a total of 0.5 μg of plasmid DNA as previously described (Tetsuka et al., 2000; Uranishi et al., 2001). Control plasmid pUC19 was used to equalize the amount of DNA for each transfection. Fugene-6-DNA complexes were allowed to form for 15 min at room temperature in serum-free medium before being added to the cells. After 24 h of transfection, cells were incubated for additional 24 h and then harvested. The luciferase activity was measured by the luciferase assay system (Promega, Madison, WI). The relative light units were determined with a TD-20/20 Luminometer (Promega). Transfection efficiency was monitored by *Renilla* luciferase activity with pRL-TK plasmid containing TK promoter as an internal control. All luciferase activities shown in transient transfection assays were corrected by the internal control activity of *Renilla* luciferase by pRL-TK. The assays were performed in triplicates. The results were presented as the fold increases in luciferase activities (means±S.D.) relative to the control in three independent transfections.

2.4. Quantitative determination of bFGF and MMP-1

The commercial EIA kits were used to determine the concentrations of bFGF (Cytimmune, College Park, MD) and MMP-1 (Amersham Biosciences, Uppsala, Sweden) according to the suppliers' protocol. All the measurements were performed in triplicates.

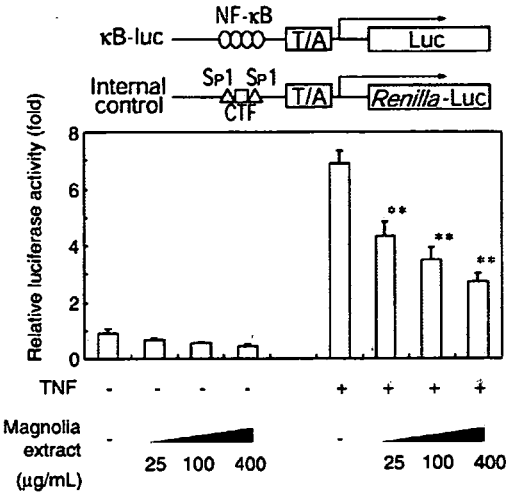

Fig. 1. Inhibition of NF-κB-dependent gene expression by Magnolia extract. Positions of cis-regulatory elements, binding sites for NF-κB, Sp1 and CTF, for gene expression of luciferase gene reporter plasmids, κB-luc and its internal control, are indicated in the top. 293 cells were transfected with κB-luc reporter plasmid, incubated for 22 h, pretreated with Magnolia extract at 25, 100 or 400 μg/ml of final concentration for 2 h, stimulated by TNFα (1.0 ng/ml), and further incubated for 24 h before the cell harvest. Relative luciferase activities were calculated based on the luciferase activities of κB-luc and those of the internal control expressing *Renilla* luciferase. These experiments were repeated for at least 6 times and constitutive results were obtained. The data represent the mean±S.D. of the results. **, $P<0.01$.

2.5. Mouse model for the UVB-irradiated skin

Fifteen male hairless mice (HR-1) of 6 weeks age were subjected to this study. All mice were randomly allocated into three groups: UV+Magnolia extract treatment, UV treatment, and control. UV treatment was performed by exposing the back of hairless mice using a FL20 S-E sunlamp with the emission maximum of 280–320 nm wavelength every other day for 10 weeks. The UVB irradiation doses were gradually increased from 36 to 216 mJ/cm^2 to prevent severe acute skin reactions and the total dose of UVB for 10 weeks was 4 J/cm^2. For the Magnolia extract treatment, 100 μl of diluted Magnolia extract for 15% with water (final concentration of magnolol was 20 μM) was treated with external swabbing with brush every other day before UVB irradiation. The same amount of 7.5% ethanol solution was treated to control groups. After 10 weeks, the skin elasticity was measured by a Cutometer SEM 474 (Nippon EUROTEC, Tokyo, Japan) in which immediate distention (Ue), final distention (Uf), and immediate retraction (Ur) were calculated from the distension kinetics. Back skin specimens were then excised for histological examination. They were paraffin-embedded for morphologic analysis of collagen fibers and the determination of skin thickness by hematoxylin and eosin staining (H&E staining). The structural integration of collagen fibers was examined by scanning electron microscopy using the t-butyl alcohol freeze-drying method (Inoue and Osatake, 1988). The thickness of epidermis was measured using a software for image analysis, Win ROOF (Mitani, Fukui, Japan). These animal experiments were performed according to the institutional regulation and were approved by the institutional review board.

2.6. Western blot analysis

293 cells were cultured at approximately 80% confluency in 100 mm dishes and added with magnolol. After 2 h, the cells were treated with TNFα and harvested in appropriate time with cold phosphate-buffered saline (PBS). The cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH7.5, 1% Nonidet P-40 [NP-40], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.5% deoxycholate and 1 mM phenylmethylsulfonyl fluoride [PMSF]). Approximately 50 μg of cell lysate was boiled at 95 °C for 5 min in the sample buffer. The samples were then separated by SDS-10% PAGE, followed by protein blotting onto a polyvinylidene fluoride (PVDF) membrane (BioRad, Hercules, CA). The protein-blotted membranes were blocked with 5% (w/v) fat-free dry milk in PBS with 0.05% Tween 20 (PBS-T) overnight at 4 °C. They were then incubated with anti-phospho IκBα antibody (Cell signaling, Beverly, MA) or anti-IκBα antibody (Santa Cruz, Delaware Avenue, CA) at 1:1000 dilution in PBS-T containing 1% bovine serum albumin overnight at 4 °C. After washing three times for 5 min with PBS-T solution, blots were further incubated for 1 h at room temperature with goat anti-rabbit IgG antibody coupled to horseradish peroxidase (Amersham Pharmacia Biotech) at 1:2000 dilution in 5% skim milk in

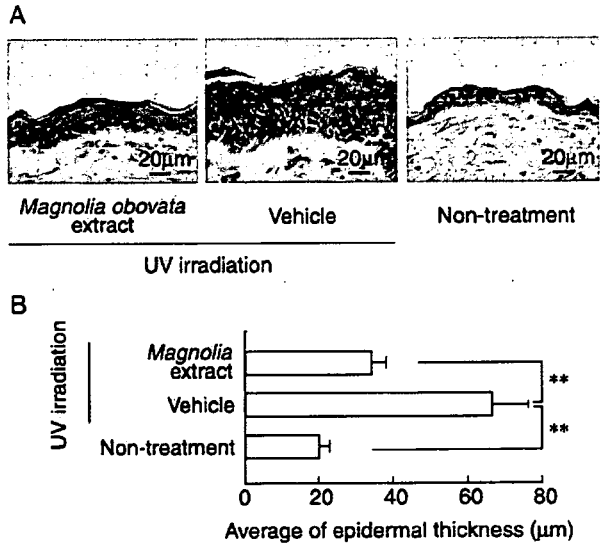

Fig. 2. Induction of epidermal hyperproliferation by UVB and inhibition by Magnolia extract. A. Epidermal hyperproliferation by UVB. UV irradiation was performed at a total dose of 4 J/cm^2. In Magnolia extract-treated group, 100 μl Magnolia extract in 7.5% ethanol was administered by external swabbing every other day before UVB irradiation. In the vehicle group, 100 μl of 7.5% ethanol was similarly administered. Tissue sections were subjected to histological examination. Scale bar=20 μm. B. Induction of epidermal hyperproliferation and the inhibitory effects of Magnolia extract. All skin tissue specimens were measured about epidermal thickness, and shown the average epidermis. This experiment was performed with at least 5 animals per treatment group and essentially the same results were obtained for each set. **, $P<0.01$.

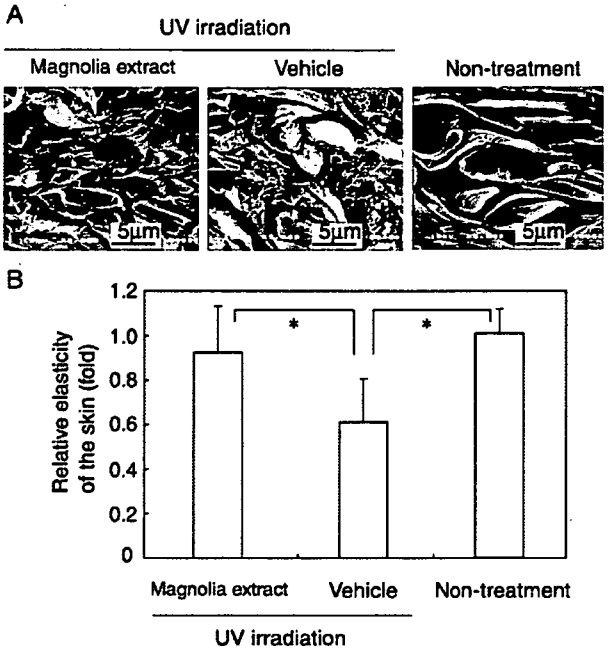

Fig. 3. Effects of Magnolia extract on the UVB-induced degradation of collagen fiber and the skin elasticity. A. Degradation of collagen fiber by UVB. The mice were treated as in Fig. 2. The structure of collagen was examined by scanning electron microscope. Scale bar=5 μm. B. The quantitative analysis of skin elasticity. The skin elasticity was measured by Cutometer (Nippon EUROTEC). This experiment was performed with at least 5 animals per treatment group and essentially the same results were obtained for each set. *, $P<0.05$.

PBS-T and washed three times in PBS-T before visualization. The expressions of the proteins were detected by ECL detection system (Amersham Pharmacia Biotech).

2.7. Localization of p65 by indirect immunofluorescence

In order to examine the cellular localization of NF-κB, 293 cells were treated with or without 10 μM magnolol, and cultured for 3 h. The cells were treated with or without 1 ng/ml of TNFα stimulation for 25 min before the harvest. Cells were immunostained with rabbit polyclonal anti-p65 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (primary antibody) and fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Cappel, Irvine, CA) (used as the secondary antibody) to demonstrate the localization of p65 as described previously (Sakurada et al., 1996).

2.8. Statistical analysis

The data were collected from at least three independent experiments. Animal experiments were performed with at least 5 animals per each treatment group. Quantitative data were expressed as the mean±S.D. Statistical significance was examined by the ANOVA and the paired Student's t test. Differences were considered statistically significant if $P<0.05$. The levels of statistical significance were indicated as the following: *, $P<0.05$; **, $P<0.01$; n.s., not significant.

3. Results

3.1. The inhibitory effects of NF-κB by Magnolia extract

In Fig. 1, the inhibitory effects of Magnolia extract on the TNFα-mediated NF-κB dependent gene expression are demonstrated in the cultured 293 cells. In this experiment, TNFα stimulated NF-κB dependent gene expression by approximately 7-folds. However, when Magnolia extract was added to the cell culture prior to the TNFα stimulation, a clear dose-dependent inhibition was observed. In addition, no significant cytotoxic effect with Magnolia extract was observed in these assays (the cell viability was 95% at 400 μg/ml).

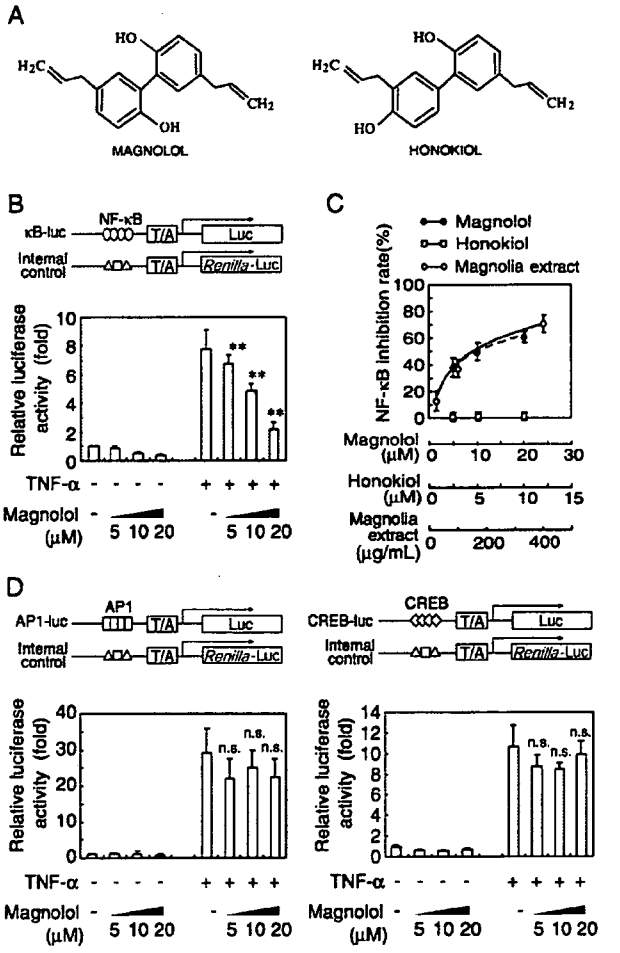

Fig. 4. Identification of magnolol as the active component in Magnolia extract responsible for NF-κB inhibition. A. Chemical formula of magnolol and honokiol. B. Effects of magnolol on NF-κB-dependent gene expression. The cells were pretreated with magnolol for 2 h prior to the TNFα (1.0 ng/ml) treatment. C. Comparison of the NF-κB inhibitory effects of Magnolia extract, magnolol and honokiol. Each data set in Figs. 1 and 4B was plotted on this figure. D. Effects of magnolol on AP-1 and CREB. Magnolol was similarly added prior to the TNFα or forskolin treatment. These data was from 5 repeated experiments and expressed as mean±S.D. **, $P<0.01$; n.s., not significant.

3.2. Effects of Magnolia extract on epidermal hyperproliferation

The results of UVB-induced epidermal hyperproliferation were examined in Fig. 2. The preventive effects of Magnolia extract on the UVB-induced skin thickening were evaluated by directly measuring the thickness of epidermis. Whereas UVB induced epidermal hyperproliferation (66 μm in average) as compared with the control untreated skin (20 μm) (thus estimated to be 3.3 fold), the treatment with Magnolia extract significantly reduced the epidermal hyperproliferation (34 μm) to 1.7 fold ($P<0.01$).

3.3. Effects of Magnolia extract on degradation of collagen fiber

Then, we examined the effects of Magnolia extract on collagen fiber degradation induced by UVB. As shown by scanning electron microscopic examination (Fig. 3A, right), normal collagen structure consists of solid bundles of collagen fibers to make smooth fascicular configuration. When the mouse skin was irradiated with UVB, total dose of 4.0 J/cm^2 for 10 weeks, the fascicular configuration of collagen fiber became loose and collagen bundles were disintegrated, suggesting the fragmentation of collagen fibers (Fig. 3A, middle). However, when the skin was topically pretreated with Magnolia extract, destruction of collagen bundles was prevented (Fig. 3A, left). In Fig. 3B, the cutaneous elasticity was quantitatively evaluated using a Cutemeter™ (Nippon EUROTEC, Tokyo, Japan) measuring skin viscoelasticity. Although the cutaneous elasticity was significantly deteriorated by UVB irradiation (the relative elasticity was reduced to 0.6 fold, $P<0.05$), pretreatment with Magnolia extract significantly prevented this effect of UVB irradiation (0.92 fold, statistically not significant as compared with the untreated control).

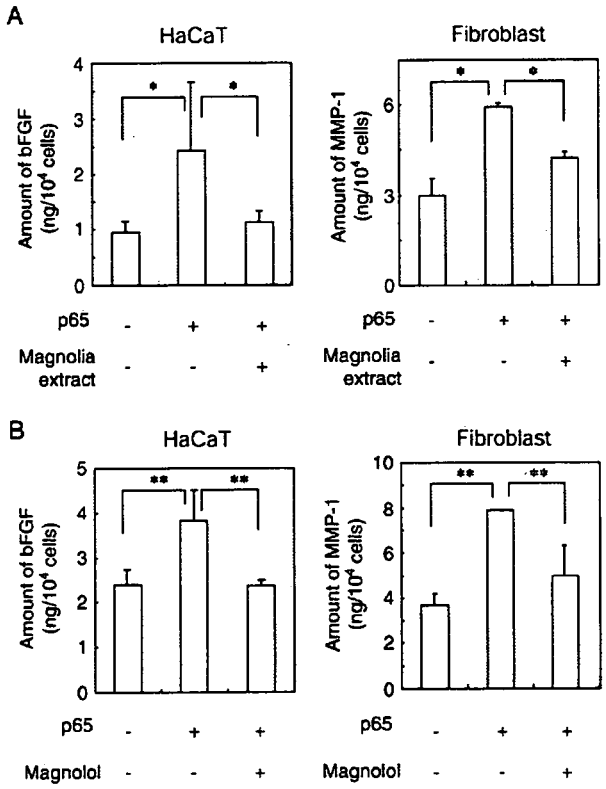

Fig. 5. Induction of bFGF and MMP-1 from cultured skin cells by p65 overexpression and inhibition by Magnolia extract or magnolol. A. Effects of Magnolia extract on bFGF production from HaCaT keratinocytes (left panel). Magnolia extract (final concentration of 150 μg/ml) was added 24 h after the transfection of pCMV-p65. Effects of Magnolia extract on MMP-1 production from human primary fibroblasts (right panel). B. Effects of magnolol on bFGF production from HaCaT keratinocytes (left panel). Magnolol (final concentration of 10 μM) was added 24 h after the transfection of pCMV-p65. Effects of magnolol on MMP-1 production from human primary fibroblasts (right panel). These data were obtained from 5 repeated experiments, and the results are expressed as mean±S.D. *, $P<0.05$; **, $P<0.01$.

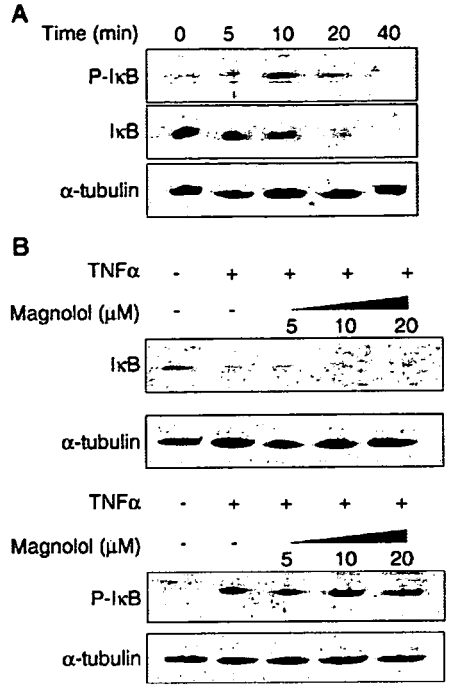

Fig. 6. Effects of magnolol on the TNFα-mediated phosphorylation and degradation of IκBα. A. Induction of phosohorylation and degradation of IκBα by TNFα. TNFα (1.0 ng/ml) were added for 0, 5, 10, 20, or 40 min and cells were harvested. B. Effects of magnolol on the phosohorylation and degradation of IκBα after treatment with TNFα for 10 min and 20 min, respectively. Magnolol was added to cell cultures 2 h prior to the addition of TNFα. IκBα (upper panel) and its phosphorylated form (lower panel) were detected by immunoblotting. These experiments were carried out for at least 3 times and constitutive results were obtained.

3.4. The inhibitory effects of NF-κB by magnolol

Since major pharmacological components with Magnolia extract were known to be magnolol and honokiol, both belonging to phenolic compounds, we then examined the effect of magnolol or honokiol on the TNFα-mediated NF-κB dependent gene expression (Fig. 4). When magnolol was added,

the NF-κB dependent gene expression was inhibited in a dose-dependent manner (Fig. 4B and C), where as no such effect was observed with honokiol (Fig. 4C). In Fig. 4B, we have described the equivalent doses of magnolol and Magnolia extract based on the HPLC quantification of magnolol content revealing that Magnolia extract solution contained 135 μM magnolol. Interestingly, the anti-NF-κB inhibitory profiles of Magnolia extract and purified magnolol in Magnolia extract were nearly identical when the equivalent doses of magnolol and Magnolia extract were plotted abscissa. This finding indicates that the major activity of Magnolia extract in inhibiting the NF-κB dependent gene expression could be ascribed to magnolol. Hence, it is suggested that magnolol is the main active ingredient of Magnolia extract in inhibiting the NF-κB activity. In contrast, no inhibitory effect was observed with honokiol. In addition, no inhibitory effect was observed on AP-1 or CREB-dependent gene expression by magnolol (Fig. 4D). Moreover, no cytotoxic effect was observed at the highest magnolol concentration (20 μM) used in these assays (the cell viability at this concentration of Magnolia extract was 97%).

3.5. Induction of bFGF and MMP-1 by NF-κB and inhibition by Magnolia extract and magnolol

It was previously shown that production of bFGF and MMP-1 is mediated by NF-κB (Bond et al., 1999; Sabourin et al., 1993; Tanaka et al., 2005). When p65 was overexpressed, mimicking actions of the activated NF-κB thus bypassing the signal transduction pathway (Tetsuka et al., 2000), production of bFGF and MMP-1 was augmented (Fig. 5A,B; second bar in each figure). However, pretreatment with Magnolia extract (Fig. 5A) or magnolol (Fig. 5B) significantly reduced the production of both bFGF and MMP-1 (third bar in each figure).

3.6. The mechanism of inhibition of NF-κB by magnolol

We then explored the mechanism by which magnolol exhibits anti-NF-κB effects. In Fig. 6, the effects of magnolol on the signal-induced IκBα phosphorylation and its degradation were examined by immunoblotting. Using the specific antibody, the phosphorylated form of IκB (P-IκB) was readily detectable after 10 min of TNFα treatment, followed by the IκBα degradation (Fig. 6A). However, the pretreatment of magnolol did not affect the phosphorylation and degradation of IκBα (Fig. 6B) after 10 min and 20 min with TNFα treatment, respectively.

In Fig. 7, we examined the effects of magnolol on the nuclear translocation of NF-κB. When the intracellular localization of NF-κB (p65) was examined by immunofluorescence, magnolol could effectively block the nuclear translocation of NF-κB (Fig. 7A). Furthermore, when p65 gene was overexpressed in 293 cells, the p65-mediated gene expression was also inhibited by magnolol in a dose-dependent manner (Fig. 7B). Similarly to the results with TNFα stimulation (Figs. 4B, 7A), magnolol could block the nuclear translocation of exogenous p65 (data not shown). These findings are consistent with the above observation that magnolol could inhibit the nuclear translocation

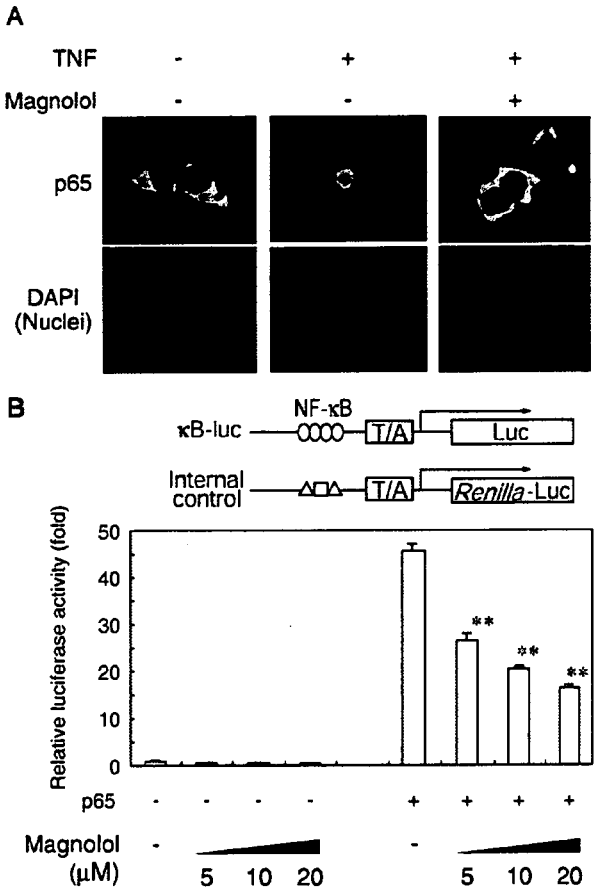

Fig. 7. Effects of magnolol on the NF-κB nuclear localization induced by TNFα. A. Nuclear localization of p65. 293 cells were treated with TNFα for 25 min in the presence or absence of 10 μM magnolol. The same cells were counter-stained by DAPI to show the cell nuclei. The results were expressed as mean±S.D. of 5 independent experiments. B. Inhibition of p65-dependent gene expression by magnolol. After 24 h of transfection with plasmids, cells were treated with magnolol, incubated for additional 24 h and the luciferase activities were measured. These experiments were repeatedly performed for 3 times and the same observations were obtained. The representative results are demonstrated. **, $P<0.01$.

of NF-κB without affecting the signal-induced phosphorylation and degradation of IκBα.

4. Discussion

NF-κB has been regarded as the major mediator involved in the UV-induced skin changes by inducing the gene expression of IL-1, TNFα, MMP-1 and bFGF (Barchowsky et al., 2000; Tanaka et al., 2005). Since IL-1 and TNFα are known to stimulate NF-κB (Okamoto et al., 1997), NF-κB is considered to play an important role in the maintenance and expansion of the skin photoaging processes although NF-κB is involved in maintaining the skin homeostasis (Pasparakis et al., 2002; Takao et al., 2003). Thus, intervention of NF-κB actions is expected to exhibit a protective effect on the skin photoaging. In fact, we previously demonstrated that a known NF-κB

inhibitor, parthenolide, could prevent the skin photoaging processes induced by UVB, suggesting a possibility that NF-κB inhibitors that could be topically administered would be more effective in preventing the skin photoaging (Tanaka et al., 2005).

In this study, we found for the first time that Magnolia extract could effectively inhibit NF-κB-mediated gene expression in cell cultures and prevent the photoaging processes such as hyperproliferation of epidermis and destruction of collagen in an experimental animal model using mice. Since the sun protecting factor (SPF) value of Magnolia extract was below the detection limit and its specific absorption wavelength was not recognizable within UVB range (data not shown), sunscreen effect of Magnolia extract is not contributable. Thus, the efficacies of Magnolia extract could be attributable to such biological effects of Magnolia extract observed in cell culture and animal studies. In vitro test, Magnolia extract was effective in blocking the induction of MMP-1 and bFGF even in cells overexpressing p65, bypassing the cellular activation cascade of NF-κB (Fig. 5A). We have subsequently identified magnolol as the major active component of Magnolia extract. Magnolol is known to be the main pharmaceutical component of *Magnolia obovata* Thunb (Chen et al., 2002; Lee et al., 2005). Although Magnolia extract also contains honokiol (Lee et al., 2005; Park et al., 2004), a chemical isomer of magnolol, honokiol did not exhibit significant effect on the NF-κB-dependent gene expression (data not shown). Magnolol is known to have a number of pharmacological effects such as anti-allergic (Ikeda et al., 2003; Wang et al., 1995), anti-inflammatory (Park et al., 2004), and anti-microbial effects (Park et al., 2004). Others suggested that magnolol might have inhibitory activity against NF-κB (Chen et al., 2002; Lee et al., 2005) although the details of its mechanism of action have not been elucidated.

We further analyzed the effects of magnolol and found that magnolol could specifically inhibit the NF-κB-dependent gene expression and did not affect the actions of other transcription factors such as AP-1 and CREB (Fig. 4). Magnolol was effective in blocking the induction of MMP-1 and bFGF even in cells overexpressing p65, bypassing the cellular activation cascade of NF-κB (Fig. 5B). Consistently, no inhibitory effect of magnolol was found on the signal-mediated IκBα phosphorylation and its degradation, a major mechanism of most NF-κB inhibitors (Dai et al., 2004; Shishodia et al., 2005; Victoriano et al., 2006). Since magnolol was shown to block the nuclear translocation of NF-κB (Fig. 7), magnolol appears to block the action of NF-κB in the downstream of signal transduction pathway thus endowing magnolol with a unique feature as an effective NF-κB inhibitor. However, the details of its molecular action are still yet to be clarified. With regard to our findings obtained with a human keratinocyte cell line, HaCaT, accumulating numbers of papers have clearly demonstrated the UV-induced NF-κB activation in this cell line (Tebbe et al., 2001; Saliou et al., 1999) although some claimed that HaCaT cells did not respond to the normal stimuli that activate NF-κB signaling (Chaturvedi et al., 2001). We have obtained HaCaT cells from the original developer (Boukamp et al., 1988), and the signal-induced NF-κB activation was reproducibly demonstrated as similarly as in other cell line 293. It is possible that this inconsistency may have arisen due to the variations or phenotypic alterations of original cell lines during cell cultures in many laboratories.

In this study we demonstrate that Magnolia extract could prevent photoaging by topical application, thus it is regarded as a feasible candidate component for anti-photoaging cosmetics. In addition, the external application could deliver a chemical(s) at high concentrations to the site of photoaging, thus a systemic adverse effect is unlikely unless it is actively absorbed in tissues. We found that Magnolia extract exhibits the selective anti-NF-κB activity and that most of its activity can be ascribed to a major component, magnolol. Interestingly, we found that magnolol could block the nuclear translocation of NF-κB whereas those of other transcription factors such as AP-1 and CREB were not affected. Thus, magnolol can be used as a selective inhibitor for NF-κB. Similar finding was reported with other compounds such as dehydroxymethylepoxyquinomicin (DHMEQ) (Umezawa and Chaicharoenpong, 2002) that was reported to block the nuclear translocation of NF-κB. Although the mechanism of magnolol and DHMEQ is not yet clarified, elucidation of the molecular mechanism should provide useful information for the treatment of some malignancies where NF-κB is known to be activated (Okamoto, 2006). Furthermore, since Magnolia extract was free from contact dermatitis or sensitizing potential (our unpublished observations), we believe it can be safely used as a component of cosmetics effective for the prevention of photoaging. It was thought that external topical application could reduce a burden of patients. Future pharmacological assessments of magnolol should clarify the efficacy of this compound as a novel NF-κB inhibitor in the treatment of cancer and leukemia (Sanda et al., 2005).

Acknowledgments

We thank Dr. N. Fusenig for generous gift of HaCaT cells and Miss. A.B. Victoriano for careful language edition of the manuscript. This work was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Japanese Human Science Foundation, and Japanese Society for the Promotion of Science.

References

Barchowsky, A., Frleta, D., Vincenti, M.P., 2000. Integration of the NF-κB and mitogen-activated protein kinase/AP-1 pathway at the collagenase-1 promotor: divergence of IL-1 and TNF-dependent signal transduction in rabbit primary synovial fibroblasts. Cytokine 12, 1469–1479.

Bond, M., Baker, A.H., Newby, A.C., 1999. Nuclear factor κB activity is essential for matrix metalloproteinase-1 and -3 upregulation in rabbit dermal fibroblasts. Biochem. Biophys. Res. Commun. 264, 561–567.

Boukamp, P., Petrussevska, R.T., Breitkreutz, D., Hornung, J., Markham, A., Fusenig, N.E., 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J. Cell Biol. 106, 761–771.

Brenneisen, P., Sies, H., Scharffetter-Kochanek, K., 2002. Ultraviolet-B irradiation and matrix methalloproteinases: from induction via signaling to initial events. Ann. N.Y. Acad. Sci. 973, 31–43.

Chaturvedi, V., Qin, J.Z., Denning, M.F., Choubey, D., Diaz, M.O., Nickoloff, B.J., 2001. Abnormal NF-kappaB signaling pathway with enhanced

susceptibility to apoptosis in immortalized keratinocytes. J. Dermatol. Sci. 26, 67–78.

Chen, Y.H., Lin, S.J., Chen, J.W., Ku, H.H., Chen, Y.L., 2002. Magnolol attenuates VCAM-1 expression in vitro in TNF-alpha-treated human aortic endothelial cells and in vivo in the aorta of cholesterol-fed rabbits. Br. J. Pharmacol. 135, 37–47.

Chung, J.H., 2003. Photoaging in Asians. Photodermatol. Photoimmunol. Photomed. 19, 109–121.

Corsini, E., Sangha, N., Feldman, S.R., 1997. Epidermal stratification reduces the effects of UVB (but not UVA) on keratinocyte cytokine production and cytoxicity. Photodermatol. Photoimmunol. Photomed. 13, 147–152.

Dai, S., Hirayama, T., Abbas, S., Abu-Amer, Y., 2004. The IkappaB kinase (IKK) inhibitor, NEMO-binding domain peptide, blocks osteoclastogenesis and bone erosion in inflammatory arthritis. J. Biol. Chem. 279, 37219–37222.

Ikeda, K., Sakai, Y., Nagase, H., 2003. Inhibitory effect of magnolol on tumour metastasis in mice. Phytother. Res. 17, 933–937.

Inoue, T., Osatake, H., 1988. A new drying method of biological specimens for scanning electron microscopy, the t-butyl alcohol freeze-drying method. Arch. Histol. Cytol. 51, 53–59.

Ko, C.H., Chen, H.H., Lin, Y.R., Chan, M.H., 2003. Inhibition of smooth muscle contraction by magnolol and honokiol in porcine trachea. Planta Med. 69, 532–536.

Lee, J., Jung, E., Park, J., Jung, K., Lee, S., Hong, S.S., Park, J., Park, E., Kim, J., Park, S., Park, D., 2005. Anti-inflammatory effects of magnolol and honokiol are mediated through inhibition of the downstream pathway of MEKK-1 in NF-kappaB activation signaling. Planta 71, 338–343.

Matsumura, Y., Ananthaswamy, H.N., 2004. Toxic effect of ultraviolet radiation on the skin. Toxicol. Appl. Pharmacol. 195, 298–308.

Okamoto, T., 2006. NF-κB and rheumatic diseases. Endocr. Metab. Immune. Disord. Drug Targets 6, 359–372.

Okamoto, T., Sakurada, S., Yang, J.P., Merin, J.P., 1997. Regulation of NF-κB and Disease control. Identification of a novel serine kinase and thioredoxin as effectors for signal transduction pathway for NF-κB activation. Curr. Top. Cell. Regul. 35, 149–161.

Park, J., Lee, J., Jung, E., Park, Y., Kim, K., Park, B., Jung, K., Park, E., Kim, J., Park, D., 2004. In vitro antibacterial and anti-inflammatory effects of honokiol and magnolol against Propionibacterium sp. Eur. J. Pharmacol. 496, 189–195.

Pasparakis, M., Courtois, G., Hafner, M., Schmidt-Supprian, M., Nenci, A., Toksoy, A., Krampert, M., Goebeler, M., Gillitzer, R., Israel, A., Krieg, T., Rajewsky, K., Haase, I., 2002. TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. Nature 417, 861–866.

Sabourin, C.L., Kusewitt, D.F., Applegate, L.A., Budge, C.L., Ley, R.D., 1993. Expression of fibroblast growth factors in ultraviolet radiation-induced corneal tumors and corneal tumor cell lines from *Monodelphis domestica*. Mol. Carcinog. 7, 197–205.

Sakurada, S., Kato, T., Okamoto, T., 1996. Induction of cytokines and ICAM-1 by proinflammatory cytokines in primary rheumatoid synovial fibroblasts and inhibition by N-acetyl-L-cysteine and aspirin. Int. Immunol. 8, 1483–1493.

Saliou, C., Kitazawa, M., McLaughlin, L., Yang, J.P., Lodge, J.K., Tetsuka, T., Iwasaki, K., Cillard, J., Okamoto, T., Packer, L., 1999. Antioxidants modulate acute solar ultraviolet radiation-induced NF-kappaB activation in a human keratinocyte cell line. Free Radic. Biol. 26, 174–183.

Sanda, T., Iida, S., Ogura, H., Asamitsu, K., Murata, T., Bacon, K.B., Ueda, R., Okamoto, T., 2005. Growth inhibition of multiple myeloma cells by a novel Iκb kinase inhibitor. Clin. Cancer Res. 11, 1974–1982.

Shishodia, S., Amin, H.M., Lai, R., Aggarwal, B.B., 2005. Curcumin (diferuloylmethane) inhibits constitutive NF-kappaB activation, induces G1/S arrest, suppresses proliferation, and induces apoptosis in mantle cell lymphoma. Biochem. Pharmacol. 70, 700–713.

Sun, H.B., Malacinski, G.M., Yokota, H., 2002. Promoter competition assay for analyzing gene regulation in joint tissue engineering. Front. Biosci. 7, 169–174.

Takao, J., Yudate, T., Das, A., Shikano, S., Bonkobara, M., Ariizumi, K., Cruz, P.D., 2003. Expression of NF-κB in epidermis and the relationship between NF-κB activation and inhibition of keratinocyte growth. Br. J. Dermatol. 148, 680–688.

Tanaka, K., Hasegawa, J., Asamitsu, K., Okamoto, T., 2005. Prevention of the ultraviolet B-mediated skin photoaging by a nuclear factor κB inhibitor parthenolide. J. Pharmacol. Exp. Ther. 315, 624–630.

Tebbe, B., Schwarz, C., Ruderisch, H.S., Treudler, R., Orfanos, C.E., 2001. L-ascorbic acid increase NFkB binding activity in UVA-irradiated HaCaT keratinocyte. J. Invest. Dermatol. 117, 154–156.

Tetsuka, T., Uranishi, H., Imai, H., Ono, T., Sonta, S., Takahashi, N., Asamitsu, K., Okamoto, T., 2000. Inhibition of nuclear factor-κB-mediated transcription by association with the amino-terminal enhancer of split, a Groucho-related protein lacking WD40 repeats. J. Biol. Chem. 275, 4383–4390.

Umezawa, K., Chaicharoenpong, C., 2002. Molecular design and biological activities of NF-kappaB inhibitors. Mol. Cells 14, 163–167.

Uranishi, H., Tetsuka, T., Yamashita, M., Asamitsu, K., Shimizu, M., Itoh, M., Okamoto, T., 2001. Involvement of the pro-oncoprotein TLS (translocated in liposarcoma) in nuclear factor-κB p65-mediated transcription as a coactivator. J. Biol. Chem. 276, 13395–13401.

Victoriano, A.F.B., Asamitsu, K., Hibi, Y., Imai, K., Barzaga, N.G., Okamoto, T., 2006. Inhibition of human immunodeficiency virus Type 1 (HIV-1) replication in latently infected cells by a novel IKK inhibitor, antimicrob. Agents. Chemother. 50, 547–555.

Wakisaka, N., Murono, S., Yoshizaki, T., Furukawa, M., Pagano, J.S., 2002. Epstein-Barr virus latent membrane protein 1 induces and causes release of fibroblast growth factor-2. Cancer Res. 62, 6337–6344.

Wang, J.P., Ho, T.F., Chang, L.C., Chen, C.C., 1995. Anti-inflammatory effect of magnolol, isolated from *Magnolia officinalis*, on A23187-induced pleurisy in mice. J. Pharm. Pharmacol. 47, 857–860.

Yarosh, D., Both, D., Kibitel, J., Anderson, C., Elmets, C., Brash, D., Brown, D., 2000. Regulation of TNFa production and release in human and mouse keratinocytes and mouse skin after UV-B irradiation. Photodermatol. Photoimmunol. Photomed. 16, 263–270.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 12, pp. 7834–7843, March 21, 2008

AKIP1 Enhances NF-κB-dependent Gene Expression by Promoting the Nuclear Retention and Phosphorylation of p65*

Received for publication, December 18, 2007 Published, JBC Papers in Press, January 4, 2008, DOI 10.1074/jbc.M710285200

Nan Gao, Kaori Asamitsu, Yurina Hibi, Takaharu Ueno, and Takashi Okamoto[1]

From the Department of Molecular and Cellular Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan

In this study, we have identified protein kinase A-interacting protein 1 (AKIP1) as a binding partner of NF-κB p65 subunit, and AKIP1 enhances the NF-κB-mediated gene expression. AKIP1 is a nuclear protein and known to interact with the catalytic subunit of PKA (PKAc). We identified AKIP1 by a yeast two-hybrid screen using the N terminus region of p65 as bait. The interaction between AKIP1 and p65 was confirmed by glutathione *S*-transferase pull-down assay *in vitro* and immunoprecipitation-Western blotting assay *in vivo*. We found that the PKAc was present in the AKIP1·p65 complex and enhanced the transcriptional activity of NF-κB by phosphorylating p65. In a transient luciferase assay, AKIP1 cotransfection efficiently increased the transcriptional activity of NF-κB induced by phorbol 12-myristate 13-acetate (PMA). When AKIP1 was knocked down by RNA interference, the PMA-mediated NF-κB-dependent gene expression was abolished, indicating a physiological role of AKIP1. We found that PKAc, which is maintained in an inactive form by binding to IκBα and NF-κB in resting cells, was activated by PMA-induced signaling and could phosphorylate p65. Overexpression of AKIP1 increased the PKAc binding to p65 and enhanced the PKAc-mediated phosphorylation of p65 at Ser-276. Interestingly, this p65 phosphorylation promoted nuclear translocation of p65 and enhanced NF-κB transcription. In fact, we observed that AKIP1 colocalized with p65 within the cells and appeared to retain p65 in nucleus. These findings indicate a positive role of AKIP1 in NF-κB signaling and suggest a novel mechanism by which AKIP1 augments the transcriptional competence of NF-κB.

NF-κB is an inducible transcription factor for the expression of wide variety of genes involved in immunoinflammatory responses, cell proliferation, and survival, thus playing crucial roles in the pathogenesis of many diseases including cancer, leukemia, and autoimmune diseases (1–4). NF-κB exists as either a heterodimer or a homodimer, among which the p65/p50 is the most ubiquitous heterodimer. In resting cells, NF-κB dimers are sequestered in the cytoplasm through association with inhibitory proteins IκBs (5). Upon treatment with NF-κB inducers such as phorbol 12-myristate 13-acetate (PMA)[2] or pro-inflammatory cytokines, IκB is phosphorylated and degraded through the ubiquitin/proteasome pathway, which eventually leads to the nuclear translocation of NF-κB and binding to the κB site of target genes (6, 7).

It has been established that the phosphorylation of p65 is important for the transcriptional activity of NF-κB (8–12). The phosphorylation of p65 by the PKA catalytic subunit dramatically enhances NF-κB transcriptional activity by recruiting histone acetyltransferase CBP/p300 (13–15). PKA, existing predominantly in the cytoplasm as an inactive tetramer holoenzyme in resting cells, is composed of two catalytic subunits and a homodimer of two regulatory subunits that can dissociate upon activation by cAMP (16–20). In resting cells, PKAc is involved in the IκB·NF-κB complex present in the cytoplasm, and IκB keeps PKAc inactivated by masking the catalytic center. In the presence of extracellular stimuli such as PMA or TNFα, IκB is phosphorylated and degraded, thus activating the IκB-sequestered PKAc to phosphorylate p65 on Ser-276. Phosphorylation by PKAc facilitates p65 to assemble with the transcriptional coactivator CBP/p300, and the binding of NF-κB to its target sites on DNA (8, 9, 13).

One of the target genes of NF-κB is IκBα, newly synthesized IκBα proteins can enter into nucleus, bind with p65, and export to the cytoplasm as a complex. This negative feedback system limits the NF-κB transcription response (21–23). It is reported that phosphorylation of p65 by PKAc enhances acetylization of NF-κB by CBP/p300, which prevents p65 binding to the nuclear IκB, and the subsequent nuclear export (24). As discussed later, we consider from our data presented in this paper that the mechanism by which AKIP1 stimulates the NF-κB-dependent transcription involves the competition between AKIP1 and IκB in binding to NF-κB in the nucleus.

AKIP1 was initially reported as a breast cancer-associated protein 3, called BCA3, highly expressed in the breast cancer and prostate cancer cell lines. However, its expression was minimal in normal breast and prostate tissues (25). It was also reported that AKIP1 was a nuclear protein and appeared to facilitate the nuclear translocation of PKAc (26).

In this study, our initial findings with a yeast two-hybrid screen of the molecular interaction between AKIP1 and p65, as

* This work was supported in part by grants-in-aid from the Ministry of Health, Labor and Welfare and the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and the Japanese Health Sciences Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, Aichi 467-8601, Japan. Tel.: 81-52-853-8205; Fax: 81-52-859-1235; E-mail: tokamoto@med.nagoya-cu.ac.jp.

[2] The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; NF-κB, nuclear factor κB; AKIP1, A-kinase-interacting protein 1; PKA, protein kinase A; PKAc, catalytic subunit of protein kinase A; PKI, protein kinase inhibitor; aa, amino acid(s); GST, glutathione *S*-transferase; RNAi, RNA interference; IP, immunoprecipitation; TNF, tumor necrosis factor; CREB, cAMP-response element-binding protein.

well as the fact that NF-κB-mediated transcription is activated by AKIP1, prompted us to investigate the molecular action of AKIP1 in the NF-κB-mediated transcription. Here we provide evidences suggesting that AKIP1 enhances the transcriptional activity by retaining the nuclear localization of p65 and promoting the Ser-276 phosphorylation of p65 by PKAc.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening and Construction of Plasmids—A bait construct, pB27-p65-(1–186), expressing a fusion protein containing the LexA binding domain and the N-terminal region of p65 (aa 1–186), was constructed and used for the yeast two-hybrid screen against a human cDNA library, CEMC7_RP, obtained from a CD4(+) T cell line. A clone encoding AKIP1 obtained from the yeast two-hybrid screening using the N terminus of p65 was cloned into pGEM-T-vector (Promega). Full-length AKIP1 cDNA was amplified by reverse transcriptase-PCR using mRNA purified from 293 cells and oligonucleotide primers, 5′-ACCATGGACAACTG-TTTGGCGGCCGC-3′ and 5′-CACAGGGAAGACCAGGT-CCACGCTTTGC-3′, containing HindIII and BamHI sites, and inserted into pcDNA3.1 vector containing the FLAG immunoepitope tag in-frame, thus creating pcDNA3.1-FLAG-AKIP1. pcDNA3.1-FLAG-AKIP1 (aa 1–72) was generated by amplifying the corresponding AKIP1 fragment by PCR using oligonucleotide primers 5′-CCCAAGCTTGACAACTGTTTGGC-GGCCGCA-3′ and 5′-CGGGATCCTCACTCTCCCGGG-AGAACGCGCTGC-3′ containing HindIII and BamHI, respectively, and inserted into FLAG-pcDNA3.1 vector. pcDNA3.1-FLAG-AKIP1 (aa 73–210) was generated by inserting the HindIII-BamHI fragment containing the AKIP1 cDNA to FLAG-pcDNA3.1 vector by using the oligonucleotide primers 5′-CCCAAGCTTAGAGAAGAGAGACCCCCAACC-3′ and 5′-CGGGATCCCCACAGGGAAGACCAGGTCCAC-3′. pGEXGSTp65N expressing the p65 N-terminal region (aa 1–185), to express the recombinant p65 N-terminal region fused to the GST moiety, was constructed by inserting the cDNA fragment of pMp65, containing the aa 1–185 region of p65 and additional BamHI site in both ends to pGEX4T3 (Promega). The plasmids expressing kinase substrate p65, pGEXGSTp65 (aa 12–317) and pGEXGSTp65 (aa 12–317) S276C, where Ser-276 was substituted by Cys (10), kindly provided by Dr. G. Haegeman.

Cell Culture and Transfection—293, HeLa, and MCF7 cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum, 100 units of penicillin, and 100 μg/ml streptomycin. Cells were transfected using either FuGENE 6 transfection reagent (Roche) or the $CaCl_2$ method (27) according to the manufacturer's recommendations.

AKIP1 Knockdown by RNAi—The RNAi sequence of AKIP1 (UCCUCUUGGCCCUCUCCAGCACUUC) was designed from exon 2 of AKIP1 (aa 155–179), which can knockdown all endogenous AKIP1 and its splicing variants (AKIP1b and AKIP1c), as previously demonstrated (26). Stealth RNAi for AKIP1 was purchased from Invitrogen. Transfection of Stealth RNAi of AKIP1 into MCF7 cells was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's recommendations.

In Vitro Binding Assay—The GST and GST-p65 N protein were expressed in *Escherichia coli* and purified by glutathione-Sepharose beads. [^{35}S]Methionine-labeled AKIP1 proteins, full-length AKIP1, AKIP1 (aa 1–72), and AKIP1 (aa 73–210), were synthesized by the *in vitro* transcription and translation protocol using the TNT-coupled wheat germ extract system (Promega) (28). For *in vitro* protein-protein interaction studies, an equal amount of the *in vitro* translated [^{35}S]methionine-labeled AKIP1 mutants were incubated with 20 μg of purified GST-p65 N-terminal protein (GST-p65 N) or GST alone (as negative control) that were bound to glutathione-Sepharose beads in 1 ml of buffer A (40 mM HEPES, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, and proteasome inhibitors (Roche)) at 4 °C overnight. The beads were then washed by buffer A three times with 500 μl of ice-cold binding buffer A. Bound proteins were eluted with an equal volume of SDS loading buffer, boiled for 3 min, resolved by 15% SDS-PAGE, and visualized by autoradiography.

Immunoprecipitation (IP)-Western Blotting—After transfection of FLAG-AKIP1, HeLa cells were cultured for 24 h and stimulated with PMA (50 ng/ml) for another 24 h. After washing with cold phosphate-buffered saline, cells were lysed in 500 μl of pre-chilled lysis buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 120 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, and protease inhibitors (Roche)) for 30 min. Cell lysates were cleared by centrifugation and cell supernatants were incubated with anti-FLAG M2 beads (Sigma) for 4 h at 4 °C, and beads were washed 3 times with 1 ml of lysis buffer. Bound proteins were eluted with an equal volume of SDS loading buffer at 100 °C for 3 min and fractionated on 10% SDS-PAGE. Western blotting was conducted using anti-NF-κB p65 (C20) or anti-PKAc antibodies (Santa Cruz). Reciprocal experiments were performed as described above using NF-κB p65 (C20) antibody and eluted proteins were immunoblotted by anti-FLAG M2 antibody (Sigma) or anti-PKAc antibody (Santa Cruz). MCF7 cells were treated with PMA (50 ng/ml) with or without transfection with RNAi-AKIP1. IP-Western blotting assay was then performed similarly using NF-κB p65 (C20) antibody (Santa Cruz) or AKIP1 polyclonal antibody (kindly provided by Dr. S. Taylor).

Immunofluorescence—HeLa cells were cultured in 4-well chamber slides and transfected with pcDNA3.1-FLAG-AKIP1 expressing FLAG-AKIP1 using FuGENE 6. After 24 h, cells were untreated or treated with 10 ng/ml PMA for 30 min, 1 h, 2 h, 3 h, and 6 h, respectively. Cells were fixed in 4% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 15 min, and reacted with 10% goat serum for 30 min at room temperature. Immunostaining was carried out with two primary antibodies including mouse monoclonal anti-FLAG M2 antibody (Sigma) and rabbit polyclonal anti-NF-κB p65 (C20) antibody (Santa Cruz), and two secondary antibodies, rhodamine- and fluorescein 5-isothiocyanate-conjugated antibodies against mouse and rabbit IgGs (Calbiochem), respectively. Cells were then incubated with 4′,6-diamidino-2-phenylindole (Sigma) for 10 min at room temperature to depict the nuclear morphol-

ogy. The intracellular localizations of p65 and AKIP1 were examined by fluorescence microscopy.

In Vitro Kinase Assay—293 cells were transfected with FLAG-AKIP1 with or without PKAc and immunoprecipitation was performed with beads containing anti-FLAG M2 antibody (Sigma) for 24 h after the transfection. The cellular proteins immunoprecipitated by binding to the FLAG M2 beads and 100 ng purified substrate protein GSTp65(12–317) were mixed and incubated in various kinase buffers (buffer A: 20 mM HEPES-KOH, pH 7.8, 10 mM $MgCl_2$, 0.1 mM EGTA, 10 mM NaF, 5 mM dithiothreitol, 0.2 mM ATP, 10 μCi of [γ-^{32}P]ATP; buffer B: 20 mM HEPES-KOH, pH 7.8, 10 mM $MnCl_2$, 0.1 mM EGTA, 10 mM NaF, 5 mM dithiothreitol, 0.2 mM ATP, 10 μCi [γ-^{32}P]ATP; buffer C: 20 mM HEPES-KOH, pH 7.8, 10 mM $CaCl_2$, 10 mM NaF, 5 mM dithiothreitol, 0.2 mM ATP, 10 μCi of [γ-^{32}P]ATP) as previously described (8) as well as in the original PKA buffer (40 mM Tris-HCl, pH 7.4, 20 mM magnesium acetate, 0.2 mM ATP, 10 μCi of [γ-^{32}P]ATP) (29). The *in vitro* kinase assay was performed at 30 °C for 20 min. Other protein substrates, GSTp65(12–317) S276C and GST, were used as negative controls. 50 or 100 nM PKI_{6-22} (Sigma), a peptide inhibitor specific for PKAc (29–33), was supplemented in the *in vitro* kinase assay to block the kinase activity of PKAc. The dose-dependent inhibitory action of PKI_{6-22} was assessed on the p65 phosphorylation by PKAc in the aforementioned conditions. Phosphorylated proteins were subjected to SDS-PAGE, and phosphate incorporation was visualized by PhosphorImager analysis using BAS-1800 II (Fuji Film, Tokyo).

Transient Luciferase Assay—293 cells were cultured in 24-well plates and transfections were conducted with FuGENE 6 transfection reagent (Roche). For each transfection, FLAG-AKIP1 or its mutant plasmids together with 15 ng of pGL-3κB-luc, a κB-dependent reporter plasmid, and 5 ng of pRL-TK, expressing *Renilla* luciferase as an internal control, were used. The empty vector pcDNA3.1 was used to adjust the total amount of transfected DNA to 50 ng. Twenty-four h after transfection, cells were stimulated with 10 ng/ml PMA for 24 h, with or without PKAc inhibitor PKI_{6-22} (10 nM), and harvested for luciferase assay. The luciferase activity was normalized by the *Renilla* luciferase activity of each transfection to normalize the transfection efficiency. Luciferase activities of CREB-luc or NFAT-luc reporter genes were also examined as controls. MCF7 cells were treated with PMA (10 ng/ml) with or without transfection with RNAi-AKIP1 (3, 10, and 20 nM). Cell lysates were then subjected to the luciferase assay. HIV-1-LTR-luc reporter plasmid with or without authentic κB sites (34) was also used for testing NF-κB-dependent transcriptional activity.

RESULTS

Interaction between p65 and AKIP1—By virtue of yeast two-hybrid screening using the N terminus of p65 (aa 1–186) as a bait against human cDNA library obtained from CEM cells with random priming, CEMC7_RP, we identified AKIP1 interacting with the p65 subunit of NF-κB. By screening ~8.2×10^7 yeast transformants, 260 clones grew on selective medium and they were confirmed by β-galactosidase assay. Fig. 1*A* depicts a diagram of three independent clones of the same gene including Clone 107 (aa 36–210), Clones 41 and 139 (aa 46–210), which

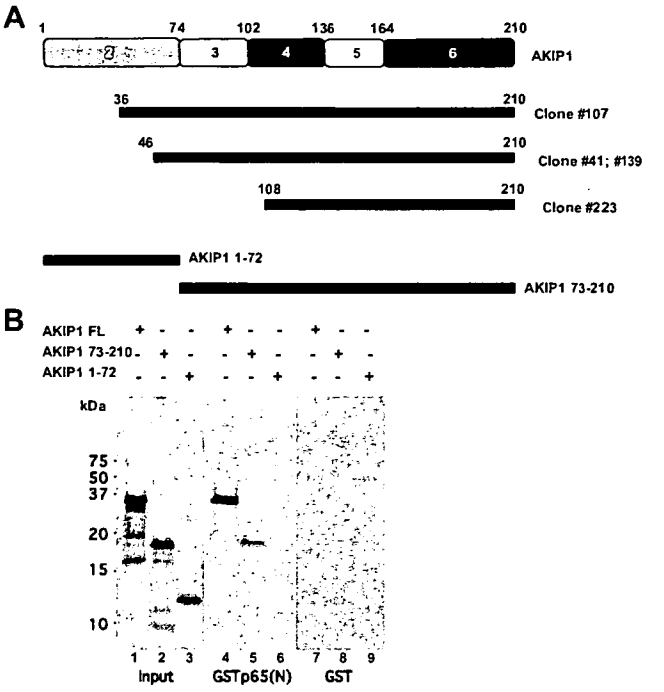

FIGURE 1. **Detection of AKIP1 as an interacting protein with p65.** *A*, results of the yeast two-hybrid screen with p65 as a bait. The N-terminal region (aa 1–186) of p65 was used as a bait and screened against a cDNA expression library obtained from human lung. Four clones containing the three different regions and sharing a common C terminus of AKIP1 (aa 73–210), 107, 41, 139, 223, were found to interact with p65 in the yeast two-hybrid screening assay. The *lower two bars* indicate the mutant AKIP1 constructs, AKIP1 (aa 1–72) and AKIP1 (aa 73–210), for the *in vitro* pull-down assay. *B*, binding of AKIP1 and p65 *in vitro*. *In vitro* GST pull-down assay was performed with full-length AKIP1 (*AKIP1 FL*), and its truncated mutants, AKIP1 (aa 73–210) and AKIP1 (aa 1–72) (*AKIP1 73–210* and *AKIP1 1–72*). The protein-protein interaction assay was performed as described under "Experimental Procedures." The ^{35}S-labeled AKIP1 proteins were incubated with GSTp65 (N) or GST alone and extensively washed. The bound proteins were eluted by SDS-loading buffer and resolved on a 15% SDS-PAGE. Proteins loaded on each column containing GSTp65 (*N*) (*lanes 4–6*) and GST alone (*lanes 7–9*) were: *lanes 1–3*, 1/10 input; *lanes 4 and 7*, AKIP1 FL; *lanes 5* and *8*, AKIP1-(73–210); *lanes 6* and *9*, AKIP1-(1–72). Note that the full-length AKIP1 and the C terminus of AKIP1 (aa 73–210), but not the N terminus of AKIP1 (aa 1–72), bind to GSTp65 (N).

were identical, and Clone 223 (aa 108–210) containing various portions of C termini of AKIP1, which interacted with p65.

To confirm the direct interaction between p65 and AKIP1, we performed *in vitro* GST pull-down assay between GSTp65 N proteins, containing the N-terminal (aa 1–185) region of p65, and various truncated proteins of AKIP1, AKIP1 (aa 1–72), AKIP1 (aa 73–210), and full-length AKIP1. As shown in Fig. 1*B*, full-length AKIP1 (*lane 4*) and C-terminal AKIP1 (aa 73–210) (*lane 5*), but not N-terminal AKIP1 (aa 1–72) (*lane 6*), interacted strongly with GST-p65 N. These results confirmed the molecular interaction between p65 and AKIP1 *in vitro*, and suggested that the N-terminal of p65 specifically binds the C-terminal of AKIP1 (aa 73–210).

To examine the protein-protein interaction between AKIP1 and p65 in intact cells, we carried out immunoprecipitation followed by Western blotting assay using antibodies to FLAG epitope and to p65, respectively. HeLa cells were transiently transfected with a plasmid expressing the full-length AKIP1 tagged with the FLAG epitope, incubated for 24 h, and then treated with 50 ng/ml PMA for an additional 24 h. Cell extracts

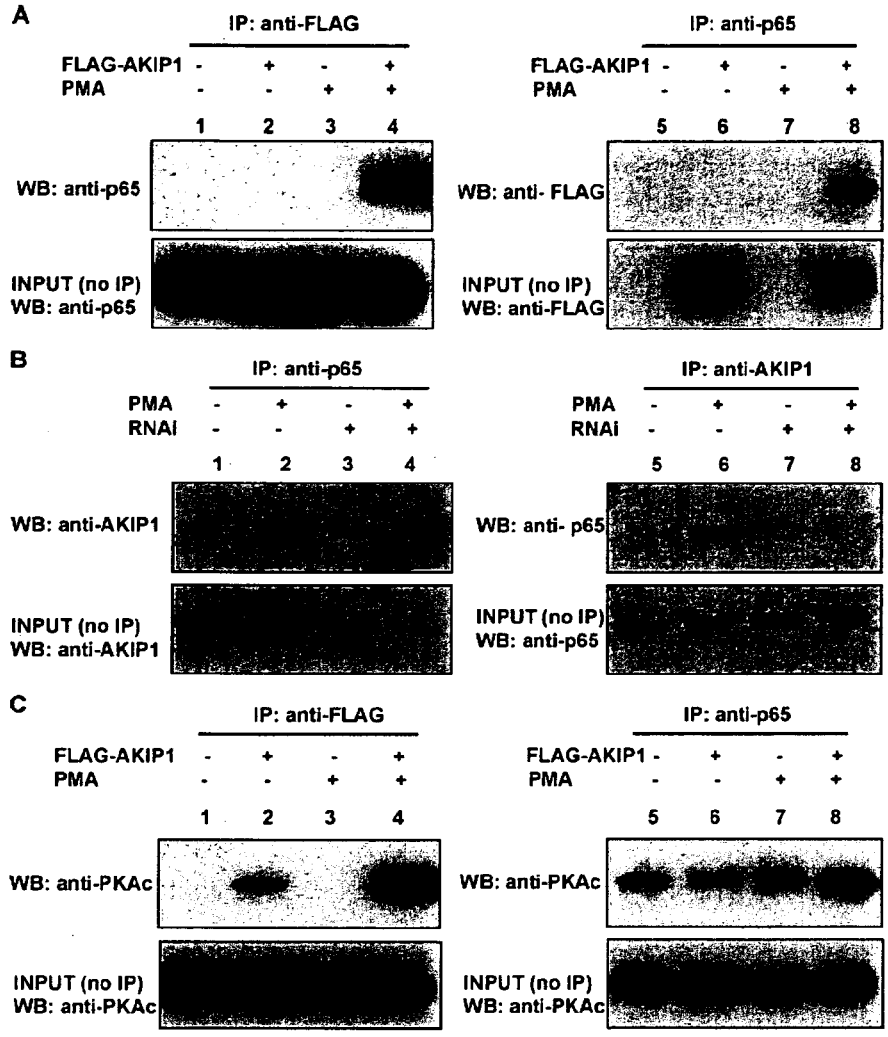

FIGURE 2. **Interaction of AKIP1 and PKAc with p65 *in vivo*.** HeLa cells transfected with FLAG-AKIP1, and MCF7 cells transfected with RNAi-AKIP1 were treated with 50 ng/ml PMA for 24 h, then IP-Western blot assays were performed to examine whether the immune complex of FLAG-AKIP1 contains p65 and PKAc. *A*, the whole cell lysate of transfected HeLa cells was immunoprecipitated with anti-FLAG M2 beads or anti-p65 antibody, then immunoblotted with anti-p65 or anti-FLAG antibodies. *B*, MCF7 cells transfected with RNAi were treated with 50 ng/ml PMA for 24 h, then the whole cell lysate was immunoprecipitated with anti-AKIP1 or anti-p65 antibody followed by Western blotting (*WB*) assay with anti-p65 or anti-AKIP1 antibody. *C*, the immunoblot from the aforementioned HeLa cells was reprobed with antibody to PKAc. Note that p65 was detected in the AKIP1 immune complex and appeared to interact with AKIP1 when cells were stimulated with PMA (*A*) and that PKAc bound to AKIP1 irrespective of the PMA treatment but PMA stimulation could augment the interaction between PKAc and AKIP1 (*C*).

were prepared and after immunoprecipitation with anti-FLAG M2 antibody, the immune complex was fractionated by SDS-PAGE and subsequently immunoblotted with anti-NF-κB p65 (C20) antibody. As shown in Fig. 2*A* (*left panel*), after immunoprecipitation with anti-FLAG M2 beads, the 65-kDa band recognized by anti-p65 antibody, was detected. When cells were overexpressing FLAG-AKIP1 and treated with PMA (Fig. 2*A*, *left panel, lane 4*), the interaction between p65 and AKIP1 was demonstrated *in vivo*. However, without PMA treatment, the binding between p65 and FLAG-AKIP1 was not detected (Fig. 2*A*, *left panel, lane 2*), suggesting that the association of AKIP1 and p65 requires the signal-activated NF-κB. The reciprocal experiment using anti-p65 antibody to immunoprecipitate the NF-κB complex followed by immunoblotting with anti-FLAG antibody was carried out and similar results were obtained (Fig. 2*A*, *right panel*). These findings demonstrate that the binding between p65 and AKIP1 occurs in the cells where NF-κB is activated. Similar experiments using anti-FLAG M2 beads for immunoprecipitation and anti-p50 antibody for Western blotting were performed, but no p50 binding to AKIP1 was detected (data not shown).

To further confirm the interaction between lines AKIP1 and p65 under the physiological conditions, we performed the IP-Western blotting assay in MCF7 cells expressing high levels of endogenous AKIP1 (25, 26). After cells were treated with 50 ng/ml PMA, cell lysates were subjected to the immunoprecipitation assay by anti-p65 antibody, then the p65-bound AKIP1 was detected by Western blotting using polyclonal antibody against AKIP1 (Fig. 2*B*, *left panel, lanes 1* and *2*). Treatment of PMA enhanced this interaction between p65 and endogenous AKIP1 (Fig. 2*B*, *left panel, lane 2*; compare with *lane 1*). AKIP1 knockdown with 10 nM RNAi-AKIP1 remarkably inhibited the binding of AKIP1 to p65 (Fig. 2*B*, *left panel, lanes 3* and *4*). Reciprocal experiments using anti-AKIP1 for the immunoprecipitation and anti-p65 for the immunoblotting were performed and consistent results were obtained (Fig. 2*B*, *right panel, lanes 5–8*). The control RNAi, nonspecific for AKIP1, did not affect the p65-AKIP1 binding (data not shown). These results suggested that AKIP1 interacts with p65 in physiological conditions and this interaction can be enhanced by PMA treatment.

Involvement of PKAc in the AKIP1·p65 Complex—Because it is previously known that AKIP1 interacts with PKAc (26), we next examined whether PKAc is contained in the AKIP1·p65 complex. In the immunoprecipitation-Western blotting assay, PKAc was coprecipitated with FLAG-AKIP1 (Fig. 2*C*, *left panel*). Without any stimulation, there was a small amount of PKAc bound to FLAG-AKIP1 (*lane 2*), whereas upon treatment with PMA, a larger amount of PKAc was detected bound to FLAG-AKIP1 (*lane 4*) confirming the previous finding that the degradation of IκB activates the PKAc (9). Fig. 2*C* (right panel) shows that PKAc was also found interacting with p65 in addition to AKIP1 *in vivo* especially when cells were stimulated with

PMA (compare *lanes 5* and *6* with *lanes 7* and *8*). Without PMA stimulation, overexpression of FLAG-AKIP1 did not significantly change the binding between PKAc and p65 (Fig. 2*C*, *right panel*, compare *lanes 5* and *6*), presumably because both PKAc and p65 were sequestered by the IκB protein in the resting cells. However, in the presence of PMA treatment (compare *lanes 7* and *8*), overexpression of FLAG-AKIP1 slightly increased the interaction between PKAc and p65. These results suggest that NF-κB activation, and IκB degradation, promotes the binding of AKIP1 to the PKAc·p65 complex. It appears that AKIP1 consolidates the PKAc·p65 complex formation by an un-identified mechanism.

AKIP1 Colocalizes with p65 in the Nucleus and Promotes Its Nuclear Retention—To analyze the subcellular distribution of AKIP1 protein, we transfected FLAG-tagged AKIP1 into HeLa cells. These cells were then treated with PMA for different time periods and the intracellular localization of p65 and AKIP1 was examined. After 30 min and 1 h treatment of PMA, ~79 and 91% of the FLAG-AKIP1-transfected cells showed the nuclear distribution of p65 in contrast to 63 and 77% of the nontransfected cells. As shown in Fig. 3, AKIP1 appears to promote the nuclear retention of p65 (compare Fig. 3, *A* and *B*, and see the comparison graph in Fig. 3*C*). After 2 h treatment with PMA, majorities of p65 started to move from the nucleus to the cytoplasm. In ~68% of cells p65 was predominantly found in the nucleus, whereas significantly higher numbers of cells (81%) to which FLAG-AKIP1 was transfected showed p65 retained in the nucleus. This effect of AKIP1 was observed at least for 6 h. However, overexpression of FLAG-AKIP1 did not change the intracellular distribution of p65 without PMA stimulation (Fig. 3), indicating that nuclear protein AKIP1 promotes the nuclear translocation of p65 only upon stimulation with PMA, which may liberate and activate the IκB-sequestered PKAc. Inhibition of PKAc by H89 blocked the PMA-induced nuclear translocation of p65 irrespective of the presence of AKIP1 (data not shown). These findings collectively indicate that overexpression of AKIP1 facilitates the nuclear translocation and retention of p65 and that the intracellular signaling, such as the PMA signaling, is required.

AKIP1 Enhances Phosphorylation of p65 by Recruiting PKAc—As AKIP1 interacts with both PKAc and p65 at the same time (Fig. 2) and phosphorylation of p65 by PKAc is considered to be critical for the NF-κB-mediated gene expression (8, 9), we examined whether p65 phosphorylation by PKAc is up-regulated by AKIP1 by the *in vitro* kinase assay. As shown in Fig. 4*A*, *left panel*, we found that co-transfection of PKAc could augment the amount of PKAc that is co-immunoprecipitated with AKIP1 (compare *lanes 2* and *3*), as expected. The involvement of other endogenous kinases such as PKC that might be co-precipitated with FLAG-AKIP1 was excluded because the maximum performance was observed with the optimal reaction condition for PKA (Fig. 4*A*, *right panel*). Regarding the requirement of a divalent cation, we found that either Mg^{2+} or Mn^{2+} was required for the kinase activity and the best performance obtained with the original PKA buffer containing magnesium acetate (29) is involved in the AKIP1-augmented p65 phosphorylation. Importantly, Ca^{2+} did not support but blocked the kinase activity, excluding a possibility that PKC, which is known to be dependent on Ca^{2+}, is involved.

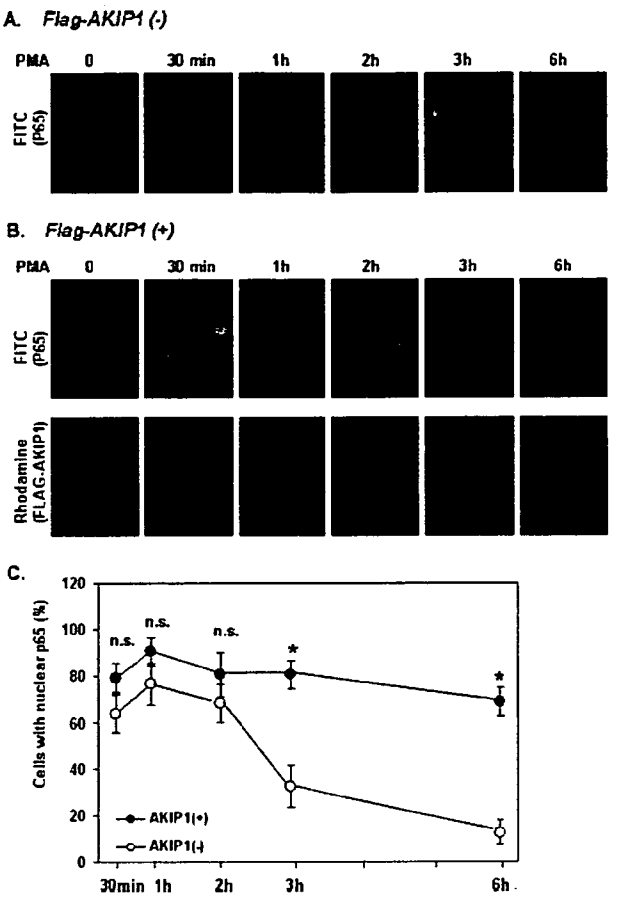

FIGURE 3. **Induction of p65 nuclear retention by AKIP1 and its colocalization with AKIP1 in the nucleus.** *A* and *B*, temporal profiles of intracellular localization of p65. HeLa cells were transfected with or without FLAG-AKIP1 for 24 h, after which cells were treated with or without 10 ng/ml PMA for the indicated time periods (30 min, 1 h, 2 h, 3 h, and 6 h). Cells were then fixed and subjected to immunofluorescence with anti-p65 antibody. *B*, colocalization of AKIP1 and p65. *C*, nuclear retention of p65 in the presence of AKIP1. Numbers of cells in which p65 localized in the nucleus were counted with at least 250 cells in four different fields of each cell culture. Immunofluorescence microscopic examinations were carried out with rhodamine anti-FLAG antibody and fluoresein-5-isothiocyanate (*FITC*) anti-p65 antibody. Cells were incubated with 4′,6-diamidino-2-phenylindole to stain the nuclear morphology (data not shown). The average numbers and standard deviation values of cells in which p65 predominantly located in the nucleus were plotted in this figure. The statistical analyses were performed by Student's *t* test to evaluate the difference of the mean number of cells with p65 in the nucleus. *n.s.*, not significant; *, $p < 0.01$.

We then further examined the effects of PKAc and AKIP1 on the p65 phosphorylation (Fig. 4*B*). Cells were transfected with various amounts of PKAc and the fixed amount of AKIP1. The cell extracts were prepared, reacted with anti-PKAc antibody, immunoprecipitated with Protein A-Sepharose beads, and subjected to *in vitro* kinase assay with wild type GSTp65 (aa 12–317) ("wt") protein as a substrate. PKAc dose-dependently enhanced the phosphorylation of p65 (Fig. 4*B*, *lanes 3*, *5*, and *7*), and overexpression of AKIP1 enhanced the PKAc-mediated p65 phosphorylation (Fig. 4*B*, compare *lanes 3* and *4*, *5* and *6*, and *7* and *8*). However, no phosphorylation was found by AKIP1 alone (*lane 2*). There was a clear dose-dependent aug-

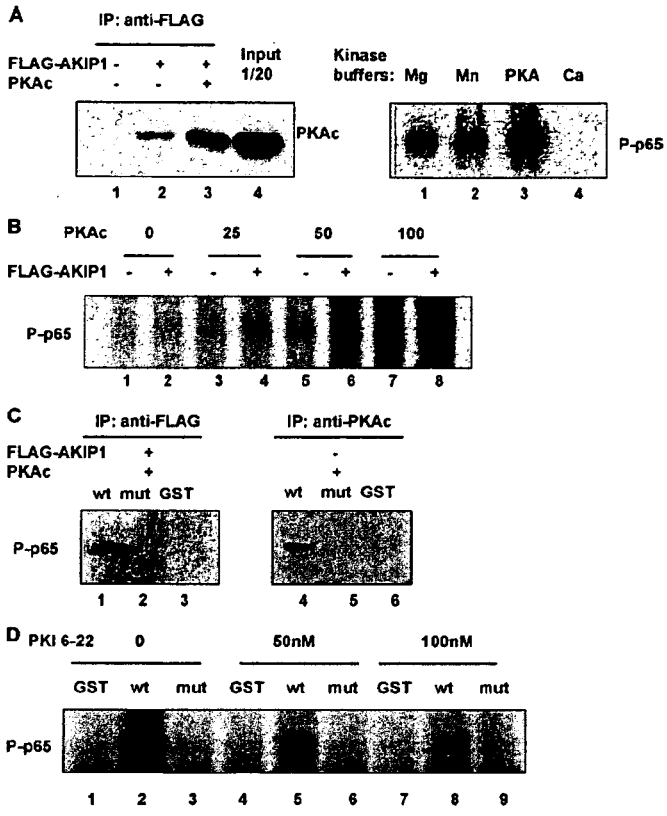

FIGURE 4. **Enhancement of PKAc-dependent p65 phosphorylation by AKIP1.** *A*, requirement of divalent cation for the p65 phosphorylation activity. 293 cells were transfected with FLAG-AKIP1 and PKAc, after 24 h, cell extracts were prepared and precipitated by anti-FLAG antibody. The precipitated proteins were immunoblotted with anti-PKAc antibody (*left panel*). In *lane 4*, 1/20 of the protein lysate obtained from cells transfected with FLAG-AKIP1 and PKAc was loaded as a positive control. The protein band in *lane 2* indicates the endogenous PKAc. These proteins were incubated with wild type GSTp65-(12–317) fusion protein in different kinase buffers containing $MgCl_2$, $MnCl_2$, $CaCl_2$, or in PKA buffer containing magnesium acetate in the presence of 10 μCi of [γ-^{32}P]ATP and processed for *in vitro* kinase assay (*right panel*). *B*, synergistic effects of PKAc and AKIP1 on the phosphorylation of p65. The different amounts of PKAc were transfected with or without 100 ng of FLAG-AKIP1. Cell lysates were immunoprecipitated using anti-PKAc antibody and then subjected to *in vitro* kinase assay with wild type GSTp65(12–317) as substrate. *C*, substrate specificity of AKIP1·PKAc complex. The FLAG-AKIP1·PKAc protein complex was isolated from FLAG-AKIP1-PKAc-cotransfected cells with anti-FLAG beads (the protein complex of the *lane 3* in *A* (*left*)) or anti-PKAc antibody. These protein complexes were incubated in PKA buffer with wild type GSTp65(12–317) (wt), mutant GSTp65(12–317) S276C (mut), and GST protein (*GST*) as a negative control. *D*, inhibition of the p65 phosphorylation by a PKA-specific peptide inhibitor PKI_{6-22}. *In vitro* kinase assay was similarly performed with Fig. 4*C* supplemented with 50 or 100 nM PKI_{6-22}.

mentation of the PKAc-mediated p65 phosphorylation by AKIP1 (data not shown).

Because it is known that Ser-276 of p65 plays a crucial role in the phosphorylation by PKAc (9), we used wild type GSTp65 (aa 12–317) ("wt") or S276C-mutated GSTp65 (aa 12–317) ("mut") as substrates for the *in vitro* kinase assay to examine whether the target phosphorylation site on p65 was Ser-276 with or without the overexpression of AKIP1. In Fig. 4*C*, the substrate specificity was tested by incubating the immunoprecipitated AKIP1 in the presence of overexpressed PKAc with various substrates including GSTp65 (aa 12–317) ("wt"), GSTp65 (aa 12–317) S276C ("mut"), and GST alone ("GST"). As shown here, although AKIP1 enhanced the phosphorylation of wild type p65 by PKAc, it did not change the substrate specificity and it also confirmed that PKAc-mediated phosphorylation depended on the presence of Ser-276 because no phosphorylation was observed on p65 mut (Fig. 4*C*, compare *lanes 1 versus 2*, and *lanes 4 versus 5*).

In Fig. 4*D*, we examined whether the PKAc-mediated p65 phosphorylation at Ser-276 could be inhibited by the PKAc-specific inhibitory peptide PKI_{6-22} (30, 31). The FLAG·AKIP1 complex immunoprecipitated from cells overexpressing PKAc was incubated with 50 or 100 nM PKI_{6-22} prior to the *in vitro* kinase assay. PKI_{6-22} inhibited the p65 phosphorylation by PKAc in a dose-dependent manner (Fig. 4*D*, *lane 5* and *8*). The results demonstrated in Fig. 4 collectively indicate that AKIP1 could enhance the phosphorylation of p65 by recruiting PKAc without changing the substrate specificity. Therefore, AKIP1 is considered as a regulator of NF-κB activation by augmenting the p65 phosphorylation.

AKIP1 Augments NF-κB-dependent Gene Expression—Because AKIP1 interacts with the p65 subunit of NF-κB and retains p65 in the nucleus, thus promoting PKAc to phosphorylate p65, we examined whether AKIP1 stimulates transcription of the NF-κB-dependent gene (Fig. 5). The luciferase reporter plasmid (3κB-luc) containing three tandem copies of the κB sequence was cotransfected with increasing amounts of the FLAG-AKIP1-expressing plasmid into 293 cells. Twenty-four h after transfection, cells were treated with or without PMA for an additional 24 h, and then luciferase activities were examined. As shown in Fig. 5*A* (*lanes 1–3*), AKIP1 stimulated NF-κB-dependent gene expression by 3.6-fold in a dose-dependent manner. In the presence of PMA-induced signaling, by which IκBα is degraded and NF-κB is activated, AKIP1 stimulated the NF-κB-dependent gene expression much further (6.2-fold) (Fig. 5*A*, *lanes 4–6*). These observations coincided with the dose-dependent enhancement of the PKAc-mediated p65 phosphorylation by AKIP1 (Fig. 4). The extent of control gene expression, *Renilla* luciferase expression under the herpes simplex virus thymidine kinase promoter, was not significantly changed either by PKAc or even with AKIP1 (data not shown). Moreover, AKIP1 did not have any effect on the NFAT-dependent gene expression even in the presence of TNFα (Fig. 5*B*, *left panel*). These findings indicate the specificity of AKIP1 and suggest that augmentation of the NF-κB-dependent gene expression by AKIP1 might be through the phosphorylation of p65. We also examined the effect of AKIP1 on the CREB-mediated gene expression as it is known that PKA stimulates the CREB-dependent gene expression. As shown in Fig. 5*B* (*right panel*), although AKIP1 further stimulates the PMA-induced CREB-dependent gene expression, the extent by which gene expression was augmented by AKIP1 was not so dramatic as in the case of NF-κB-dependent gene expression (Fig. 5*B*, *right panel*, *lanes 10–12*).

Because the interaction between p65 and AKIP1 required the C terminus of AKIP1 (aa 73–210) (Fig. 1*B*), we examined the effect of the different regions of AKIP1 on NF-κB-dependent transcription (Fig. 5*C*). We transfected 293 cells with plasmids expressing N terminus (aa 1–72), C terminus (aa 73–210), or full-length AKIP1 together with the NF-κB-dependent reporter construct (3κB-luc). These cells were then treated

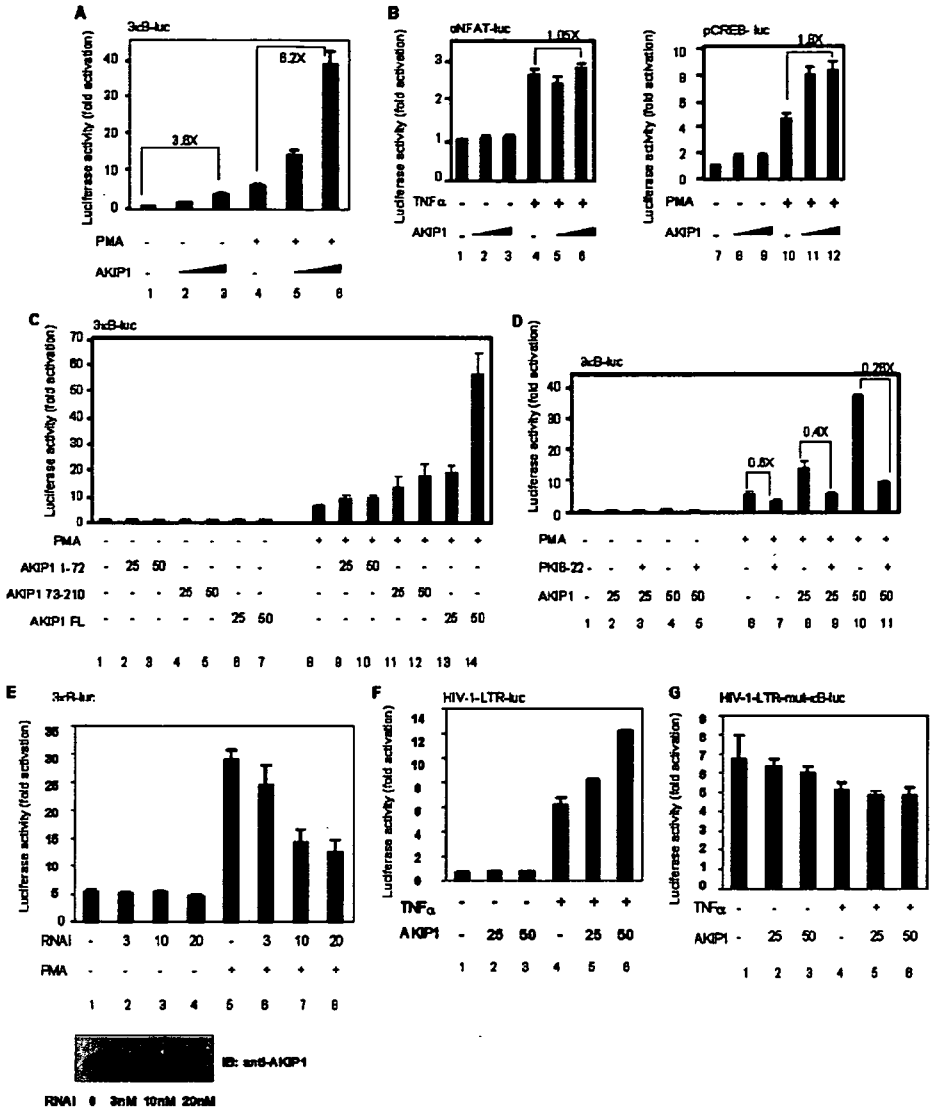

FIGURE 5. **Augmentation of the NF-κB-dependent gene expression by AKIP1.** *A*, dose-dependent augmentation of the PMA-induced NF-κB-dependent transcription by AKIP1. 293 cells were transfected with 3κB-luc (15 ng), full-length FLAG-AKIP1 (25 or 50 ng), with or without 10 ng/ml PMA. Twenty-four h post-transfection, cells were lysed and subjected to luciferase assay and normalized with the *Renilla* luciferase activity that was cotransfected as an internal control. The *vertical axis* indicates the luciferase activity in -fold activation. Values are representative of triplicate experiments (mean ± S.D.). *B*, effects of AKIP1 on NFAT- and CREB-dependent gene expression. FLAG-AKIP1 (25 and 50 ng) and pNFAT-luc (15 ng) or pCREB-luc (15 ng) expression plasmids were transfected into 293 cells, treated with 2 ng/ml TNFα or 10 ng/ml PMA for 24 h, and luciferase activities were determined. *C*, domain mapping study of AKIP1 in augmenting the NF-κB-dependent gene expression. Plasmids encoding FLAG-AKIP1 (aa 1–72), FLAG-AKIP1 (aa 73–210), or full-length FLAG-AKIP1 (*AKIP1 1–72*, *AKIP1 73–210*, and *AKIP1 FL*) were transfected with or without treatment with PMA (10 ng/ml) for 24 h and luciferase assay was carried out. *D*, effects of AKIP1 on the NF-κB-dependent gene expression depend on the kinase activity of PKAc. Effect of the catalytic action of PKAc on the NF-κB-dependent gene expression was examined with 3κB-luc reporter gene in the presence of absence of 10 nM PKAc inhibitor, PKI_{6-22}. The *numbers*, in -fold activation (suppression), *above bars* of *lanes 6–11* indicate the extents of suppression by PKI_{6-22}. *E*, knockdown of AKIP1. When endogenous AKIP1 in MCF7 cells was knocked down by RNAi, the NF-κB-dependent transcriptional activity was repressed. MCF7 cells were transfected with RNAi-AKIP1 (3, 10, and 20 nM), treated with 10 ng/ml PMA, and the cell lysate was subjected to luciferase assay. The expression of endogenous AKIP1 was detected by immunoblotting with antibody against AKIP1. *F* and *G*, effects of AKIP1 on HIV-1 LTR gene expression. 293 cells were transfected with HIV-1-LTR-luc, containing two κB sequences (15 ng) (*F*) or HIV-1-LTR-mutant-κB-luc (15 ng) (*G*), together with FLAG-AKIP1, stimulated with 1 ng/ml TNFα, and 24 h later the cell lysate was obtained for the luciferase assay.

with or without PMA, and luciferase activity was determined. Although the full-length AKIP1 greatly augmented the transcriptional activity of NF-κB (compare *lanes 13* and *14*) (3.0-fold), there was no significant effect with the N-terminal region of AKIP1 (compare *lanes 9* and *10*). The C-terminal region of AKIP1 showed a marginal effect (1.3-fold), suggesting that the entire AKIP1 molecule is necessary for the full effect of AKIP1 on NF-κB transcription although the AKIP1 C-terminal region is primarily responsible. It is also noted that neither the N-terminal nor the C-terminal region of AKIP1 acted as a dominant negative mutant. The immunoblotting with anti-AKIP1 antibody showed lower expression levels of AKIP1 (aa 1–72) as compared from other AKIP1 proteins, which may also explain the inefficiency of AKIP1 (aa 1–72) on NF-κB-dependent gene expression (data not shown).

Because we observed the inhibitory effect of peptide PKI_{6-22} amide on p65 phosphorylation (Fig. 4), we investigated whether the cell-permeable PKI_{6-22} could inhibit the AKIP1-regulated NF-κB-dependent transcription. In Fig. 5*D*, PKI_{6-22} was added to the cells 24 h after transfection with FLAG-AKIP1 and 3κB-luc by changing the medium with fresh medium containing PKI_{6-22} (10 nM) together with PMA, and further incubated for an additional 24 h. As shown here, although there was no obvious suppression by PKI_{6-22} in the absence of PMA, PKI_{6-22} inhibited the PMA-induced NF-κB-dependent gene expression in the presence or absence of AKIP1 overexpression (Fig. 5*C*, compare *lanes 6* and *7*, *8* and *9*, and *10* and *11*), indicating that the kinase activity of PKAc is involved. It is noted that AKIP1 overexpression does not appear to abolish the inhibitory effects of PKI_{6-22}. It rather appeared that the inhibitory effect of PKI_{6-22} was enhanced by the presence of AKIP1. In addition, inhibition of such effects of AKIP1 was also observed with another PKAc inhibitor H89 (data not shown).

It is reported that AKIP1 is highly expressed in human breast cancer cells (25, 26). Thus, we used MCF7 cells to examine the effect of endogenous AKIP1 in regulating NF-κB-dependent transcriptional activity. Consistent with previous results, 10 ng/ml PMA induced NF-κB activation

for 5.3-fold (Fig. 5*E*, *upper panel*, *lane 5*). Degradation of AKIP1 protein with RNAi reduced the expression of endogenous AKIP1 (Fig. 5*C*, *lower panel*) and inhibited NF-κB activation in a dose-dependent manner (Fig. 5*E*, *upper panel*, *lanes 6–8*). These observations indicate that NF-κB-dependent gene expression normally requires AKIP1 under physiological condition. The control RNAi did not affect NF-κB-dependent gene expression (data not shown).

To test the effect of AKIP1 on natural promoter, we transfected 293 cells with the HIV-1-LTR-luc reporter gene, containing 2 κB sites (34). Whereas TNFα stimulation induced NF-κB activation by 8.6-fold, co-transfection of AKIP1 further enhanced the transcriptional activity by 1.9-fold (Fig. 5*F*). When we transfected mutant HIV-1-LTR-luc reporter, lacking the κB sites, neither TNFα treatment nor AKIP1 overexpression could enhance NF-κB activation (Fig. 5*G*). We tested the p53-dependent luc reporter gene and found that AKIP1 did not show any effect (data not shown). These results suggest that AKIP1 up-regulates NF-κB-dependent transcriptional activity under physiological conditions.

DISCUSSION

Because biological effects and regulation are mediated through protein-protein interactions in general, we attempted to identify proteins interacting with the transcription-competent subunit of NF-κB to find upstream or downstream proteins that are located within the functional cascade of NF-κB. In the last nine years, we have reported 6 novel proteins identified by the yeast two-hybrid system and characterized their biological and biochemical actions including the RelA-associated inhibitor that inhibits the NF-κB DNA binding (28, 35), proapoptotic protein 53BP2 (36), AES/TLE corepressor (37), FUS/TLS coactivator (38), AO7 coactivator (39), and RNA helicase A that is involved in the transcriptional machinery of RNA polymerase II (40). Identification of these proteins has clarified the protein-protein interaction network that determines the transcriptional activity of NF-κB and its biological action. In this study, we have utilized the N-terminal domain of p65 that constitutes an independent three-dimensional structure as bait in the "two-hybrid" screening of the human cDNA expression library. We have demonstrated that AKIP1 is one of the p65-interacting proteins and revealed interesting biological and biochemical actions of AKIP1 including the enhancement of NF-κB (p65) nuclear retention and the up-regulation of the p65 phosphorylation by PKAc.

It has been reported that the inactive form of the NF-κB complex contains the HDAC1 corepressor protein, and that phosphorylation of p65 by PKAc can release HDAC1 and recruit CBP/p300 coactivators, thus leading to the activation of target genes (8, 9, 13, 14). Our results showed that AKIP1 enhanced PKAc binding to p65 (Fig. 2*C*), and increased the phosphorylation of p65 by PKAc (Fig. 4*B*). Thus, it is likely that AKIP1 stimulates the Ser-276 phosphorylation mediated by PKAc (Fig. 4*C*), thus inducing the conformational change of p65. As suggested in Zhong *et al.* (13), the phosphorylated p65 could recruit the transcriptional coactivators such as p300 and CBP to the NF-κB-bound promoter and facilitate the DNA binding ability of p65 (24). We treated cells with PMA to stimulate PKCα and induce the NF-κB pathway through activation of IKKβ that phosphorylates IκB and leads to its degradation (6, 41). We speculate that the IκB degradation by PMA might be required for PKAc to catalyze the phosphorylation of p65 at Ser-276 (Fig. 6). There have been accumulating reports with regard to the actions of PKA in the regulation of NF-κB activity, which appeared to be controversial. Some claimed that cAMP-dependent PKA activation down-regulated NF-κB-dependent transcription by changing its DNA-binding ability (42, 43), modifying the transactivation domain of p65 (45), or blocking the degradation of IκB proteins (44, 46). Others presented evidence that lead to opposite conclusions such as that the cAMP-independent PKA activation up-regulated NF-κB-dependent transcription by phosphorylating p65 (8, 9). However, in view of the actions of AKIP1, these observations may possibly be because of the different expression levels of endogenous AKIP1

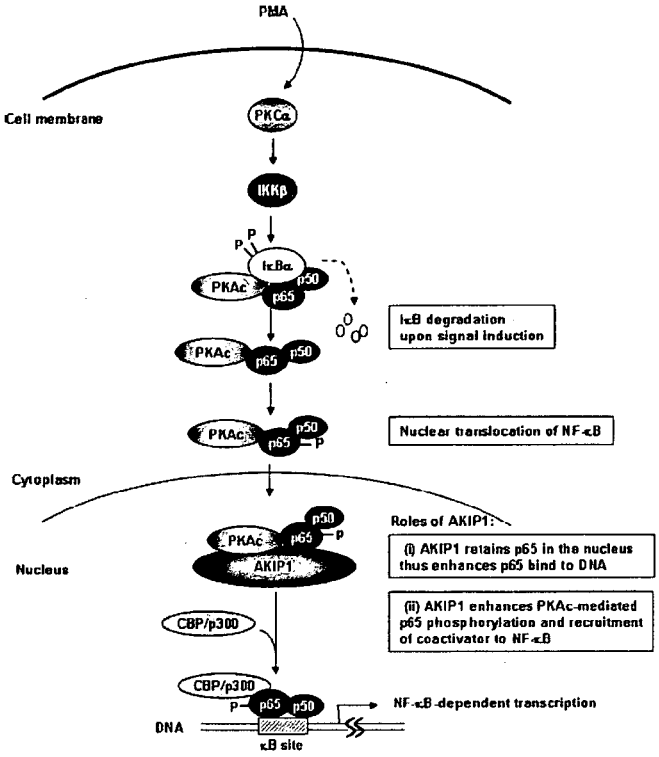

FIGURE 6. **A schematic representative of possible molecular mechanisms by which AKIP1 augments the NF-κB-dependent gene expression.** PMA-induced signal transduction pathway that leads to NF-κB activation is described. PMA stimulates PKCα activity followed by IKKβ activation and IκBα phosphorylation by IKKβ (6, 41). The phosphorylated IκBα is subjected to ubiquitination and degradation by 26 S proteasome. NF-κB resides in the cytoplasm in a complex with IκBα and PKAc (8, 9). After the degradation of IκBα, the recruitment of PKAc into the NF-κB complex is facilitated and PKAc phosphorylates the Ser-276 on p65, although it is not known whether this phosphorylation occurs in the cytoplasm or in the nucleus or during its nuclear transportation. The phosphorylated p65 recruits coactivator CBP/p300 in the nucleus (13) and stimulates transcription of the NF-κB-dependent genes. The involvement of AKIP1 in this cascade is diagrammatically depicted. Because AKIP1 is constitutively located within the nucleus, the interaction between p65 and AKIP1 is considered to occur in the nucleus. AKIP1 appears to stimulate the transcriptional activity of p65 (NF-κB) via two mechanisms: (i) by retaining the p65 in the nucleus through direct binding, and (ii) by enhancing the PKAc-mediated phosphorylation of p65. These actions of AKIP1 are depicted in this figure based on the findings described in this article. Also, in this diagram, each component of the NF-κB activation pathway has been simplified and only the "classical" pathway is shown.

in different cell types. For example, in HeLa or 293 cells, where endogenous AKIP1 expression is minimal, the action of the catalytic subunit of PKA was limited and was enhanced by AKIP1 overexpression (26). It is possible that, in cell lines with high AKIP1 expression, PKAc-mediated p65 phosphorylation can be enhanced by AKIP1, eventually leading to the augmentation of the NF-κB-dependent transcription. Whereas in cell lines with minimal AKIP1 expression, PKAc may go through the NF-κB inhibition pathway in which the signal-induced phosphorylation of the p65 C-terminal domain is involved (44, 46). However, currently, we do not know what signaling modality is involved in this switch in the target amino acid selection by PKAc. It is possible that prolonged nuclear retention of p65 happens upon induction of IκB degradation, which exposes the PKAc phosphorylation site on p65 (Ser-276).

Our results also provide evidence for another previously uncovered effect of AKIP1 on regulating NF-κB activity. Experiments described in Fig. 3 demonstrate that AKIP1 colocalizes with p65 in the nucleus upon treatment with PMA and retains p65 in the nucleus, thus enhancing the DNA binding of p65 to stimulate NF-κB-dependent transcription. This effect of AKIP1 appears to require the simultaneous NF-κB activation because without PMA treatment neither the AKIP1-p65 interaction (Fig. 2*A*) nor the AKIP1-mediated p65 nuclear retention (Fig. 3) was observed. It is likely that the PMA-induced IκB degradation is necessary for AKIP1 to translocate the NF-κB (p65) into the nucleus.

Thus, we have entertained two mechanisms of AKIP1 in upregulating NF-κB-dependent gene expression: (i) AKIP1 promotes the nuclear retention of p65 and (ii) AKIP1 enhances the phosphorylation of p65 by PKAc. These activities of AKIP1 could not be attributed to separate domains of AKIP1 protein because neither the full-length, N terminus, nor C terminus mutants exhibited dominant-negative phenotypes. Either of these two mechanisms is required for the full activity of AKIP1 in augmenting the NF-κB-dependent transcription (Fig. 5*C*). Domain mapping studies of AKIP1 indicate that the AKIP1 C-terminal region binds to PKAc (26) and p65 (Figs. 1 and 2) and these interacting regions on AKIP1 molecule are likely to be distinct because AKIP1 protein simultaneously binds both PKAc and p65 (Fig. 2), and enhances the PKAc-mediated phosphorylation of p65. The AKIP1 N terminus, containing the nuclear localizing sequence (aa 15–21) that accounts for its nuclear localization (26), is responsible for the retention of p65 in the nucleus. In Fig. 5*C* (compare *lanes 8* and *11–12*), AKIP1 C terminus (aa 73–210), interacting with p65 *in vitro* (Fig. 1*B*) but lacking the N-terminal nuclear localization site, is considered to be a dominant-negative mutant, but it did not suppress the NF-κB-dependent transcription. It is thus assumed that although the AKIP1 C terminus itself is not responsible for the promotion of p65 nuclear retention, it is still competent for enhancing the p65-PKAc association and subsequent phosphorylation of p65 by PKAc and augmenting gene expression by binding to both proteins. In addition, results shown in Fig. 5*C* (compare the extents of stimulation in *lanes 11* and *12* and *13* and *14* with those of *lane 8*) suggest that the AKIP1 mutant lacking the N terminus lost a great deal of enhancing activity presumably due to the lack of nuclear localization site and that the nuclear retention of p65 might be more important than its association and promotion of the PKAc-mediated Ser-276 phosphorylation. It is also noted that overexpression of AKIP1 itself can slightly enhance the luciferase activity without PMA treatment (Fig. 5*A*, *lane 1-3*). Therefore, it appears that nuclear retention of p65 might be the primary role of AKIP1.

AKIP1 was initially reported as a cancer-related protein and is abundantly present in cell lines obtained from breast and prostate cancer tissues, whereas its expression is very low in normal tissues (25). In addition, because NF-κB is known to be actively involved in carcinogenesis and its progression (47), it is possible that AKIP1 may promote the growth of cancer cells through enhancing the NF-κB pathway. Recently, it has been reported that upon high concentration (20 ng/ml) of TNFα treatment, the overexpressed AKIP1 was modified by an ubiquitin-like protein NEDD8, called "neddylation," to form multiple ladder bands of AKIP1 proteins upon SDS-PAGE (48). Gao *et al.* (48) observed that the NF-κB-dependent gene expression was consequently inhibited. However, AKIP1 dose-dependently enhanced NF-κB activation upon stimulation within the physiological concentration of TNF (less than 2 ng/ml) or treatment with PMA. Consistently, when a higher TNFα dose (20 ng/ml) was applied, we observed the down-regulation of NF-κB-dependent gene expression as observed by Gao *et al.* (48).[3] Although it is still not conclusive whether AKIP1 is involved in carcinogenesis, it is possible to assume AKIP1 as a self-defense factor of carcinogenesis and its progression by shifting the PKA signaling in favor of cell proliferation and anti-apoptosis. These findings support an idea that AKIP1 inhibitors should block the PKAc-mediated NF-κB activation specifically in some cancer cells and tissues.

Acknowledgments—We thank Dr. S. Taylor and G. Haegeman for a generous gift of anti-AKIP1 antibody and various GST-p65 plasmids, respectively.

REFERENCES

1. Baeuerle, P. A., and Baichwal, V. R. (1997) *Adv. Immunol.* **65,** 111–137
2. Baldwin, A. S., Jr. (1996) *Annu. Rev. Immunol.* **14,** 649–683
3. Okamoto, T., Sakurada, S., Yang, J-P., and Merin, J. P. (1997) *Curr. Top. Cell Regul.* **35,** 149–161
4. Moynagh, P. N. (2005) *J. Cell Sci.* **118,** 4389–4392
5. Sun, S. C., Ganchi, P. A., Beraud, C., and Ballard, D. W. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 1346–1350
6. Lallena, M. J., Diaz-Meci, M. T., Bren, G., Paya, C. V., and Moscat, J. (1999) *Mol. Cell. Biol.* **19,** 2180–2188
7. Didonato, J., Mercurio, F., Rosette, C., Li, J. W., Yang, H. S., Ghosh, S., and Karin, M. (1996) *Mol. Cell. Biol.* **16,** 1295–1304
8. Hayashi, T., Sekine, T., and Okamoto, T. (1993) *J. Biol. Chem.* **268,** 26790–26795
9. Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997) *Cell* **89,** 413–424
10. Vermeulen, L., Wilde, G. D., Damme, P. V., Berghe, W. V., and Haegeman, G. (2003) *EMBO J.* **22,** 1313–1324
11. Sakurai, H., Suzuki, S., Kawasaki, N., Nakano, H., Okazaki, T., Chino, A., Doi, T., and Saiki, I. (2003) *J. Biol. Chem.* **278,** 36916–36923
12. Wang, D., Westerheide, S. D., Hanson, J. L., and Baldwin, A. S., Jr. (2000) *J. Biol. Chem.* **275,** 32592–32597

[3] N. Gao and T. Okamoto, unpublished data.

13. Zhong, H., Voll, R. E., and Ghosh, S. (1998) *Mol. Cell* **1,** 1661–1671
14. Zhong, H., May, M. J., Jimi, E., and Ghosh, S. (2002) *Mol. Cell* **9,** 625–636
15. Hoberg, J. E., Popko, A. E., Ramsey, C. S., and Mayo, M. W. (2006) *Mol. Cell. Biol.* **26,** 457–471
16. Carnegie, G. K., and Scott, J. D. (2003) *Genes Dev.* **17,** 1557–1568
17. Taylor, S. S., Buechler, J. A., and Yonemoto, W. (1990) *Annu. Rev. Biochem.* **59,** 971–1005
18. Scott, J. D. (1991) *Pharmacol. Ther.* **50,** 123–145
19. Francis, S. H., and Corbin, J. D. (1994) *Annu. Rev. Physiol.* **56,** 237–272
20. Walsh, D. A., and Van Patten, S. M. (1994) *FASEB J.* **8,** 1227–1236
21. Le Bail, O., Schmidt-Ullrich, R., and Israël, A. (1993) *EMBO J.* **12,** 5043–5049
22. Zabel, U., and Baeuerle, P. A. (1990) *Cell* **61,** 255–265
23. Johnson, C., Van Antwerp, D., and Hope, T. J. (1999) *EMBO J.* **18,** 6682–6693
24. Chen, L. F., Fischle, W., Verdin, E., and Greene, W. C. (2001) *Science* **293,** 1653–1657
25. Kitching, R., Li, H., Wong, M. J., Kanaganayakam, S., Kahn, H., and Seth, A. (2003) *Biochim. Biophys. Acta* **1625,** 116–121
26. Sastri, M., Barradough, D. M., Carmichae, P. T., and Taylor, S. S. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 349–354
27. Blochlinger, K., and Diggelmann, H. (1984) *Mol. Cell. Biol.* **4,** 2929–2931
28. Yang, J. P., Hori, M., Sanda, T., and Okamoto, T. (1999) *J. Biol. Chem.* **274,** 15662–15670
29. Cobb, C. E., and Corbin, J. D. (1988) *Methods Enzymol.* **159,** 202–208
30. Fantozzi, D. A., Taylor, S. S., Howard, P. W., Maurer, R. A., Feramisco, J. R., and Meinkoth, J. L. (1992) *J. Biol. Chem.* **267,** 16824–16828
31. Wen, W., Harootunian, A. T., Adams, S. R., Feramisco, J., Tsien, R. Y., Meinkoth, J. L., and Taylor, S. S. (1994) *J. Biol. Chem.* **269,** 32214–32220
32. Wen, W., Meinkoth, J. L., Tsien, R. Y., and Taylor, S. S. (1995) *Cell.* **82,** 463–473
33. Wiley, J. C., Wailes, L. A., Idzerda, R. L., and McKnight, G. S. (1999) *J. Biol. Chem.* **274,** 6381–6387
34. Victoriano, A. F. B., Asamitsu, K., Hibi, Y., Imai, K., Barzaga, N. G., and Okamoto, T. (2006) *Antimicrob. Agents Chemother.* **50,** 547–555
35. Yang, J., Bardes, E. S., Moore, J. D., Brennan, J., Powers, M. A., and Kornbluth, S. (1998) *Genes Dev.* **12,** 2131–2143
36. Takada, N., Sanda, T., Okamoto, H., Yang, J. P., Asamitsu, K., Sarol, L., Kimura, G., Uranishi, H., Tetsuka, T., and Okamoto, T. (2002) *J. Virol.* **76,** 8019–8030
37. Yang, J. P., Hori, M., Takahashi, N., Kawabe, T., Kato, T., and Okamoto, T. (1999) *Oncogene* **18,** 5177–5186
38. Tetsuka, T., Uranishi, H., Imai, H., Ono, T., Sonta, S., Takahashi, N., Asamitsu, K., and Okamoto, T. (2000) *J. Biol. Chem.* **275,** 4383–4390
39. Uranishi, H., Tetsuka, T., Yamashita, M., Asamitsu, K., Shimizu, M., Itoh, M., and Okamoto, T. (2001) *J. Biol. Chem.* **276,** 13395–13401
40. Asamitsu, K., Tetsuka, T., Kanazawa, S., and Okamoto, T. (2003) *J. Biol. Chem.* **278,** 26879–26887
41. Tetsuka, T., Uranishi, H., Sanda, T., Asamitsu, K., Yang, J. P., Wong-Staal, F., and Okamoto, T. (2004) *Eur. J. Biochem.* **271,** 3741–3751
42. Kim, Y. S., Cho, K., Lee, H. J., Kim, S. Y., Sato, Y., and Cho, Y. (2006) *J. Biol. Chem.* **281,** 39051–39061
43. Neumann, M., Grieshammer, T., Chuvpilo, S., Kneitz, B., Lohoff, M., Schimpl, A., Franza, B. R., Jr., and Serfling, E. (1995) *EMBO J.* **14,** 1991–2004
44. Majumdar, S., and Aggarwal, B. B. (2003) *Oncogene* **22,** 1206–1218
45. Takahashi, N., Tetsuka, T., Uranishi, H., and Okamoto, T. (2002) *Eur. J. Biochem.* **269,** 4559–4565
46. Minguet, S., Huber, M., Rosenkranz, L., Schamel, W. W. A., Reth, M., and Brummer, T. (2005) *Eur. J. Immunol.* **35,** 31–41
47. Okamoto, T., Sanda, T., and Asamitsu, K. (2007) *Curr. Pharm. Des.* **13,** 447–462
48. Gao, F., Cheng, J., Shi, T., and Yeh, E. T. (2006) *Nat. Cell Biol.* **8,** 1171–1177