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

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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 screening 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 a 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) 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 acetylation 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

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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 evidence 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′-ACCATGGCAACTGT-3′ and 5′-CACAGGGGAAGACAGGCACC-3′, containing HindIII and BamHI sites, and inserted into pcDNA3.1 vector containing the FLAG epitope 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′-CCCAAGCTTGACAACTGTTTGCGG-3′ and 5′-CGGGATCCACTTCCTCCCGGGAGAAGCCGCTG-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′-CCCAAGCTTGACAACTGTTTGCGG-3′ and 5′-CGGGATCCACTTCCTCCCGGGAGAAGCCGCTG-3′. pGEXGSTp65N expressing the p65 N-terminal region (aa 1–185), to express the recombinant p65 N-terminal region (aa 1–186), was constructed and used for protein containing the LexA binding domain and the N-terminal region of p65.

In Vitro Binding Assay—The GST and GST-p65 N protein were expressed in Escherichia coli and purified by glutathione-Sepharose beads. [35S]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 T7-T coupled wheat germ extract system (Promega) (28). For in vitro protein-protein interaction studies, an equal amount of the in vitro translated [35S]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 MgCl2, 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 5 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-
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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 MgCl₂, 0.1 mM EGTA, 10 mM NaF, 5 mM dithiothreitol, 0.2 mM ATP, 10 μCi of [γ-32P]ATP; buffer B: 20 mM HEPES-KOH, pH 7.8, 10 mM MnCl₂, 0.1 mM EGTA, 10 mM NaF, 5 mM dithiothreitol, 0.2 mM ATP, 10 μCi of [γ-32P]ATP; buffer C: 20 mM HEPES-KOH, pH 7.8, 10 mM CaCl₂, 10 mM NaF, 5 mM dithiothreitol, 0.2 mM ATP, 10 μCi of [γ-32P]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-32P]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 PKI6–22 (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 PKI6–22 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-3-B-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 an additional 24 h. Cell extracts were subjected to the luciferase assay. HIV-1-LTR-luc, a κB-dependent reporter plasmid, and 5 ng of pRL-TK, expressing Renilla luciferase as an internal control, were used.

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 × 10⁷ yeast transformants, 260 clones grew on selective medium and they were confirmed by β-galactosidase assay. Fig. 1A 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 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(1–210), AKIP1(1–72), and its truncated mutants, AKIP1(1–210) and AKIP1(1–72). The protein-protein interaction assay was performed as described under “Experimental Procedures.” The 35S-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 1–6) and GST alone (lanes 7–9) were: lanes 1–3, 1/10 input; lanes 4 and 7, AKIP1 FL; lanes 5 and 8, AKIP1(1–210); 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. We therefore adopted Clone 107 as the bait.

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 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 1–210) and AKIP1 (aa 1–72). The protein-protein interaction assay was performed as described under “Experimental Procedures.” The 35S-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(1–210); 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.
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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, followed by Western blotting with anti-p65 antibody. 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. 2A (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. 2A, 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. 2A, 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 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. 2C, 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. 2C (right panel) shows that PKAc was also found interacting with p65 in addition to AKIP1 in vivo especially when cells were stimulated with
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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. 2C, 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 unidentified 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. 3C). 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. 4A, 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. 4A, right panel). Regarding the requirement of a divalent cation, we found that either Mg2+ or Mn2+ 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, Ca2+ did not support but blocked the kinase activity, excluding a possibility that PKC, which is known to be dependent on Ca2+, is involved.

We then further examined the effects of PKAc and AKIP1 on the p65 phosphorylation (Fig. 4B). 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. 4B, lanes 3, 5, and 7), and overexpression of AKIP1 enhanced the PKAc-mediated p65 phosphorylation (Fig. 4B, 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 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 fluorescein-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.
As shown here, although AKIP1 enhanced the phosphorylation of various substrates including GSTp65 (aa 12–317) (“wt”), the phosphorylation by PKAc in the presence of overexpressed PKAc with substrate specificity was tested by incubating the immunoprecipitated AKIP1 in different kinase buffers containing MgCl₂, MnCl₂, or in PKA buffer containing magnesium acetate in the presence of 10 μCi of [γ-³²P]ATP and processed for in vitro kinase assay (right panel; B, C), substrate specificity of AKIP1 and PKAc on the phosphorylation of p65. The different amounts of PKAc were transfected with or without 100 ng of FLG-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 containing 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 PKI₆₋₂₂ in vitro kinase assay was similarly performed with Fig. 4C supplemented with 50 or 100 nM PKI₆₋₂₂.

**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 MgCl₂, MnCl₂, or in PKA buffer containing magnesium acetate in the presence of 10 μCi of [γ-³²P]ATP and processed for in vitro kinase assay (right panel; B, C), synergistic effects of PKAc and AKIP1 on the phosphorylation of p65. The different amounts of PKAc were transfected with or without 100 ng of FLG-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 containing 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 PKI₆₋₂₂ in vitro kinase assay was similarly performed with Fig. 4C supplemented with 50 or 100 nM PKI₆₋₂₂.

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. 4C, 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. 4C, compare lanes 1 versus 2, and lanes 4 versus 5).

In Fig. 4D, we examined whether the PKAc-mediated p65 phosphorylation at Ser-276 could be inhibited by the PKAc-specific inhibitory peptide PKI₆₋₂₂ (30, 31). The FLAG-AKIP1 complex immunoprecipitated from cells overexpressing PKAc was incubated with 50 or 100 nM PKI₆₋₂₂ prior to the in vitro kinase assay. PKI₆₋₂₂ inhibited the p65 phosphorylation by PKAc in a dose-dependent manner (Fig. 4D, 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 Activates 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 (3xκB-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. 5A (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. 5A, 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. 5B, left panel). These observations coincided with 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. 5B (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. 5B, right panel, lanes 10–12).

Because the interaction between p65 and AKIP1 required the C terminus of AKIP1 (aa 73–210) (Fig. 1B), we examined the effect of the different regions of AKIP1 on NF-κB-dependent transcription (Fig. 5C). 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 (3xκB-luc). These cells were then treated
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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 3xB-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 cotransfected as an internal control. The horizontal axis indicates the luciferase activity in -fold activation (suppression), and the vertical axis indicates the extents of suppression by PKI6–22. The horizontal bars indicate the luciferase activity in -fold activation. Values are representative of triplicate experiments (mean ± S.D.). 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 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. The effect of the catalytic action of PKAc on the NF-κB-dependent gene expression was examined with 3xB-luc reporter gene in the presence of absence of 10 nM PKAc inhibitor, PKI6–22. The numbers, in fold activation (suppression), above bars of lanes 6–11 indicate the extents of suppression by PKI6–22. E, knockdown of AKIP1. When endogenous AKIP1 in MCF7 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 xB 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 PKI6–22 amide on p65 phosphorylation (Fig. 4), we investigated whether the cell-permeable PKI6–22 could inhibit the AKIP1-regulated NF-κB-dependent transcription. In Fig. 5D, PKI6–22 was added to the cells 24 h after transfection with FLAG-AKIP1 and 3xB-luc by changing the medium with fresh medium containing PKI6–22 (10 nM) together with PMA, and further incubated for an additional 24 h. As shown here, although there was no obvious suppression by PKI6–22 in the absence of PMA, PKI6–22 inhibited the PMA-induced NF-κB-dependent transcription in the presence or absence of AKIP1 overexpression (Fig. 5C, 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 PKI6–22. Rather it appeared that the inhibitory effect of PKI6–22 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. 5E, upper panel, lane 5). Degradation of AKIP1 protein with RNAi reduced the expression of endogenous AKIP1 (Fig. 5C, lower panel) and inhibited NF-κB activation in a dose-dependent manner (Fig. 5E, 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. 5F). 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. 5G). 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), A07 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. 2C), and increased the phosphorylation of p65 by PKAc (Fig. 4B). Thus, it is likely that AKIP1 stimulates the Ser-276 phosphorylation mediated by PKAc (Fig. 4C), 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 PKAc 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 PKAc activity followed by IKKβ 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.
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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 1kB 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. 24) nor the AKIP1-mediated p65 nuclear retention (Fig. 3) was observed. It is likely that the PMA-induced 1kB degradation is necessary for AKIP1 to translocate the NF-κB (p65) into the nucleus.

Thus, we have entertained two mechanisms of AKIP1 in up-regulating 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. 5C). 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. 5C (compare lanes 8 and 11–12), AKIP1 C terminus (aa 73–210), interacting with p65 in vitro (Fig. 1B) 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. 5C (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. 5A, 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). 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.

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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. Fantozzi, D. A., Taylor, S. S., Howard, P. W., and Meinkoth, J. L. (1992) J. Biol. Chem. 267, 16824–16828
26. Wen, W., Meinkoth, J. L., Tsien, R. Y., and Taylor, S. S. (1995) Cell 82, 463–473
27. Wiley, J. C., Wailes, L. A., Idzerda, R. L., and McKnight, G. S. (1999) J. Biol. Chem. 274, 15662–15670
28. Cobb, C. E., and Corbin, J. D. (1988) Methods Enzymol. 159, 202–208
29. 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
30. Wen, W., Harooutunian, A. T., Adams, S. R., Feramisco, J., Tsien, R. Y., Meinkoth, J. L., and Taylor, S. S. (1994) J. Biol. Chem. 269, 32214–32220
31. Wen, W., Meinkoth, J. L., Tsien, R. Y., and Taylor, S. S. (1995) Cell 82, 463–473
32. Wiley, J. C., Wailes, L. A., Idzerda, R. L., and McKnight, G. S. (1999) J. Biol. Chem. 274, 6381–6387
33. Victoriano, A. F. B., Asamitsu, K., Hibi, Y., Imai, K., Barzaga, N. G., and Okamoto, T. (2006) Antimicrob. Agents Chemother. 50, 547–555
34. Yang, J., Hoberg, J. E., Moore, J. D., Brennan, J., Powers, M. A., and Kornbluth, S. (1998) Genes Dev. 12, 2131–2143
35. Takada, N., Sonta, S., Okamoto, H., Yang, J. P., Asamitsu, K., Sarol, L., Kimura, G., Uranishi, H., Tetsuka, T., and Okamoto, T. (2002) J. Virol. 76, 8019–8030
36. Yang, J. P., Hori, M., Takahashi, N., Kawabe, T., Kato, T., and Okamoto, T. (1999) Oncogene 18, 5177–5186
37. Tetsuka, T., Uranishi, H., Imai, H., Ono, T., Sonta, S., Takahashi, N., Asamitsu, K., and Okamoto, T. (2000) J. Biol. Chem. 275, 4383–4390
38. Uranishi, H., Tetsuka, T., Yamashita, M., Asamitsu, K., Shimizu, M., Itoh, M., and Okamoto, T. (2001) J. Biol. Chem. 276, 13395–13401
39. Asamitsu, K., Tetsuka, T., Kanazawa, S., and Okamoto, T. (2003) J. Biol. Chem. 278, 26879–26887
40. Majumdar, S., and Aggarwal, B. B. (2003) Oncogene 22, 1206–1218
41. Takahashi, N., Tetsuka, T., Uranishi, H., and Okamoto, T. (2004) Eur. J. Biochem. 271, 3741–3751
42. Neumann, M., Grieshammer, T., Chuvpilo, S., Kneitz, B., Lohoff, M., Schimpl, A., Franz, B. R., Jr., and Serfling, E. (1995) EMBO J. 14, 1991–2004
43. Majumdar, S., and Aggarwal, B. B. (2003) Oncogene 22, 1206–1218
44. Takahashi, N., Tetsuka, T., Uranishi, H., and Okamoto, T. (2002) Eur. J. Biochem. 269, 4559–4565
45. Minguet, S., Huber, M., Rosenkranz, L., Schamel, W. W. A., Reth, M., and Brummer, T. (2005) Eur. J. Immunol. 35, 31–41
46. Okamoto, T., Sonta, S., and Asamitsu, K. (2007) Curr. Pharm. Des. 13, 447–462
47. Gao, F., Cheng, J., Shi, T., and Yeh, E. T. (2006) Nat. Cell Biol. 8, 1171–1177