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RING Finger Protein AO7 Supports NF-κB-mediated Transcription by Interacting with the Transactivation Domain of the p65 Subunit*

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In this study, a novel interactor of the p65 subunit (RelA) of NF-κB has been explored by performing yeast two-hybrid screen using the transactivation domain (TAD) of p65 located in the C terminus as bait. We have isolated a RING finger motif-containing protein, AO7, previously identified as an interacting protein with a ubiquitin-conjugating enzyme, Ubc5B. We confirmed the protein-protein interaction between p65 and AO7 *in vitro* and *in vivo* and found that the C-terminal region of AO7 is responsible for the interaction with p65 TAD. AO7 was predominantly localized in the nucleus and activated the NF-κB-dependent gene expression upon stimulation with IL-1β or TNF or overexpression of NF-κB-inducing kinase. We found that both the RING finger and the C-terminal regions of AO7 were necessary for the transcriptional activation. When cotransfected with plasmids expressing Gal4-p65 fusion proteins containing various functional domains of p65, we found that p65 TAD was essential for the transcriptional activation mediated by AO7. Furthermore, the p65-mediated transactivation was suppressed by a ubiquitination-defective AO7 mutant in which the essential Cys residue within the RING finger motif was substituted by Ser. These data suggest that AO7 interacts with the p65 TAD and modulates its transcriptional activity.

NF-κB plays a crucial role in many cellular events such as inflammation, host defense, cell survival, and proliferation (1–4). The members of the NF-κB family in mammalian cells include the proto-oncogene c-Rel, RelA (p65), RelB, NFkB1 (p50/105), and NFkB2 (p52/p100). Rel family members form hetero- and homodimers with distinct specificities in various combinations and the combination of p65 and p50 represents a major form of NF-κB (1–4). NF-κB exists in the cytoplasm in an inactive form associated with IκB inhibitor proteins (1, 2) and stimulation of cells with inducers such as phorbol esters, interleukin-1β (IL-1β),[1] and tumor necrosis factor (TNF) leads to the activation of extracellular signal-regulated kinase kinase kinase 1/3, NF-κB-inducing kinase (NIK), and IκB kinase (IKK) complex containing IKKα and IKKβ. Once phosphorylated by IKK complex, IκBs are ubiquitinated and degraded by 26 S proteasome, resulting in nuclear translocation of NF-κB (1–4).

The protein region responsible for the transcriptional activation (called the "transactivation domain" (TAD)) of p65 has been mapped in their unique C-terminal region containing at least two TADs within its C-terminal 120 amino acids, termed TA1 and TA2 (5–7). It has been revealed that the p65 TAD interacts with transcriptional coactivators, such as p300/CREB-binding protein (8, 9) and FUS/TLS (10), and general transcription factors, including TBP (11) and TFIIB (7, 12). Interaction of p65 with these factors stimulates transcription by initiating chromatin remodeling or by recruiting RNA polymerase II. In an unexpected scenario, the involvement of ubiquitination has recently been implicated in the regulation of TADs of some transcriptional activators such as VP16 (13, 14), Myc (13), and nuclear receptors (15–17), either directly or indirectly (for an excellent review, see Conaway *et al.* (18)).

In this context, we became interested in AO7, which we have identified as one of the interacting proteins with the p65 TAD in the CytoTrap™ yeast two-hybrid screen. We have adopted this alternative screening method instead of the commonly used method utilizing the Gal4 transcription system, because the p65 TAD is functional in the yeast (10, 19). AO7 encodes a protein containing a RING finger domain and is ubiquitously expressed in various tissues (20). AO7 was initially identified in the yeast two-hybrid screen of a murine T cell library by using UbcH5b, an E2 enzyme, as bait (20). Although the target protein for the uniquitination complex involving AO7 and UbcH5b still remains to be determined, AO7 has been shown to act as a putative E3 ligase at least *in vitro* (20). We found that AO7 acts as a mediator of p65 transactivation. A possible mechanism of its action is discussed.

EXPERIMENTAL PROCEDURES

Plasmids—pSos-p65-(262–551), containing the C-terminal TAD of p65, a bait plasmid for the Cytotrap yeast two-hybrid screen, was generated by inserting the p65 C-terminal region corresponding to amino acids 262–551 into pSos (Stratagene). pCMV-p65, pGal4-p65, and its mutants, pGal4-p65-(1–551), pGal4-p65N (containing amino acid positions 1–286 of p65), pGal4-p65C (containing amino acid positions 286–551 of p65), pGal4-p65C1 (containing amino acid positions 286–520 of p65), and pGal4-p65C3 (containing amino acid positions 521–551 of p65) were described previously (10, 19). pGal4-p65C2, containing amino acid positions 428–551 of p65, was constructed by PCR using 5′ and 3′ oligonucleotide primers containing an *Eco*RI site and *Hin*dIII site, respectively. pCMV-NIK (21) is a generous gift from D. Wallach (Weitzmann Institute of Science, Rehovot, Israel). To generate pcDNA-AO7-(1–459), pcDNA-AO7-(1–219), and pcDNA-AO7-(212–459), each containing a FLAG epitope tag in the N terminus, the various portions of AO7 cDNA were amplified by PCR using pGEMAO7, containing the full-length AO7 cDNA, as a template with 5′ and 3′ oligonucleotide primers containing an *Eco*RI site and *Hin*dIII site, respectively. These

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[1] The abbreviations used are: IL, interleukin; TAD, transcriptional activation domain; NIK, NF-κB-inducing kinase; TNF, tumor necrosis factor; IKK, IκB kinase; GST, glutathione *S*-transferase; Gal4BD, Gal4 DNA binding domain; GFP, green fluorescence protein; luc, luciferase; CREB, cAMP-response element-binding protein; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; hSos, human Sos.

products were digested with *Eco*RI and *Hin*dIII and subcloned in frame into pcDNA 3.1 vector (Invitrogen) at the *Eco*RI-*Hin*dIII site. Two RING finger mutants, pcDNA-AO7(C159S) and pcDNA-AO7(C161S), were created using a commercial site-directed mutagenesis kit (QuikChange site-directed mutagenesis kit; Stratagene). pEGFP-AO7 was constructed by PCR using pGEMAO7 as a template with oligonucleotides containing *Sac*I and *Hin*dIII sites and subcloned in frame into pGFPN1 vector (Clontech). To generate pGEX-AO7-(1–459), pGEX-AO7-(1–219), pGEX-AO7-(212–459), and pGEX-AO7-(341–459), which express glutathione *S*-transferase (GST) fusion proteins, GST-AO7-(1–459), GST-AO7-(1–219), GST-AO7-(212–459), and GST-AO7-(341–459), respectively, the AO7 cDNA was amplified by PCR using pGEMAO7 as a template with the 5′ and 3′ oligonucleotide primers containing *Eco*RI site and *Sal*I site, respectively. These products were digested with *Eco*RI-*Sal*I and subcloned in frame into pGEX-4X-1 vector (Amersham Biosciences) at the *Eco*RI-*Sal*I site. pGEX-65-(441–521) was constructed by PCR with 5′ and 3′ oligonucleotide primers containing *Bam*HI and *Xho*I sites, respectively. To construct pGal4-AO7, expressing a fusion protein of AO7 and Gal4 DNA binding domain (Gal4BD), the AO7 cDNA fragment was obtained from pGST-AO7-(1–459) by digestion with *Eco*RI and *Sal*I and was ligated in frame into the *Eco*RI-*Sal*I site of Gal4 (Clontech), containing Gal4BD. pSport-p65 was constructed by amplifying the full-length p65 cDNA into pSV-Sport plasmid (Invitrogen). Construction of pVP16 and pVP16-p65 were described previously (22). Construction of luciferase (luc) reporter plasmids, 4κBw-luc, 4κBm-luc, ICAM-1-luc, and pFR-luc have been described previously (10, 23).

Yeast Two-hybrid Screening—The CytoTrap™ (Stratagene) yeast screening was performed with human lung cDNA library (Stratagene) and pSos-(262–551) as a bait according to the manufacturer's instructions and the method previously described (24). *Saccharomyces cerevisiae* strain cdc25H was transformed sequentially with pSos-p65-(262–551) and human lung cDNA library fused to the pMyr plasmid containing the myristylation sequence of v-Src. Since the cdc25H yeast cell contains a temperature-sensitive mutant of the yeast homologue (*cdc25H*) of human hSos, it cannot grow at 37 °C. Positive clones were selected by the growth ability of cdc25H cells on galactose plate at 37 °C, in which the hSos-p65-(262–551) protein is recruited to the plasma membrane because of the interaction with the myristoylated protein encoded by a pMyr clone selected from the target library, thereby complementing the cdc25 defect and allowing the growth of the cdc25H yeast clone at 37 °C due to activation of the Ras-signaling pathway. The pMyr plasmids were rescued from positive colonies and identified by nucleotide sequencing.

Isolation of Full-length Human AO7—Human full-length AO7 cDNA was obtained by PCR using oligonucleotide primers, 5′-atggcggcgtctgcgtctgcagc-3′ (forward) and 5′-aaggccaaatatctttattgcctccc-3′ (reverse), and a full-length human cDNA library prepared from neuroblastoma cell line SK-N-MC (25) (a generous gift from S. Sugano, University of Tokyo) as a template. The nucleotide sequences of oligonucleotide primers for full-length AO7 cDNA were designed based on the identity of the prey plasmid with human AO7 homologue, RNF25 (ring finger protein 25). PCR was performed using Expand High Fidelity™ system (Roche Applied Science), and the PCR products were cloned into pGEM vector (Promega). Nucleotide sequencing was performed by the ABI PRISM dye terminator cycle sequencing ready reaction kit with an Applied Biosystems 313 automated DNA sequencer (ABI). Nucleotide sequence of human AO7 was determined using forward and reverse M13 primers and five internal sequencing primers: 5′-ggtgtgcagtgtccagtgtg-3′ (forward 1), 5′-caaagggaggggagtgccacg-3′ (forward 2), 5-tgttatctgtgagaatttcc-3′ (reverse 1), 5′-gcgcaagctctctgcactgg-3′ (reverse 2), and 5′-cgtggcactcccctcccctttg-3′ (reverse 3), corresponding to the various portions of human AO7 cDNA.

Cell Culture and Transfection—293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected with various plasmids using Fugene-6™ transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. At 24 h post-transfection, the cells were harvested, and the whole cell extracts were prepared for the luciferase assay. The luciferase activity was measured by the luciferase assay system (Promega) as previously described (10, 19). The luciferase enzyme activity was normalized based on the protein concentration of whole cell extracts determined by using the Protein Assay kit (Bio-Rad). The data are presented as the -fold increase in luciferase activities (means ± S.D.) relative to control of three independent transfections. Recombinant human IL-1β and TNF were purchased from Roche Applied Science.

Immunofluorescence—Cells were immunostained with rabbit polyclonal anti-p65 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (primary antibody) and fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Cappel) (secondary antibody) to demonstrate the localization of p65 as described previously (26). In order to examine the subcellular localization of AO7, pEGFP-AO7 was transfected into 293 cells, and the intracellular localization of green fluorescence protein (GFP) (excitation at 490 nm and emission at 520 nm) was examined.

In Vitro Binding Assay—pGEX-AO7, its derivatives, and pGEX-p65-(441–521) were transformed in *Escherichia coli* strain DH5 following induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 25 °C for 6 h. Recombinant GST proteins were purified by affinity chromatography on glutathione-agarose beads as described previously (27). *In vitro* protein-protein interaction assays were performed as reported (27). Briefly, the [^{35}S]methionine-labeled p65 and AO7 proteins were incubated with immobilized GST fusion proteins overnight at 4 °C in 1 ml of modified HEMNK buffer (40 mM HEPES-KOH (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1× Complete™ protease inhibitors (Roche Applied Science)). After five washes with 1 ml of HEMNK buffer, bound radiolabeled proteins were eluted with 20 μl of Laemmli sample buffer, boiled for 3 min, and resolved by 10% SDS-PAGE.

Coimmunoprecipitation and Western Blot Assays—In order to examine the protein-protein interaction in cultured 293 cells, pcDNA-AO7-(1–459) was transfected, and cells were cultured for 48 h with or without 20 ng/ml of TNF stimulation for 30 min before the harvest. The cell extract was prepared by treatment with the lysis buffer (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, and 1× Complete™ protease inhibitors) containing 120 mM NaCl (low salt) or 300 mM (high salt). The cell lysate was incubated in the same buffer with 20 μl of anti-FLAG M2 Affinity Gel (Sigma) at 4 °C for 1 h with continuous rotation at 10 rpm. The beads were washed three times with 1 ml of lysis buffer. The antibody-bound complex was eluted by boiling in 1× Laemmli sample buffer. The immunoprecipitated proteins were resolved by 8% SDS-PAGE and transferred on nitrocellulose membrane (Hybond-C; Amersham Biosciences). The membrane was incubated with rabbit polyclonal anti-p65 (C-terminal) antibody (Santa Cruz Biotechnology), and immunoreactive proteins were visualized by enhanced chemiluminescence (SuperSignal; Pierce) as described previously (28). To detect the level of endogenous p65 and FLAG-tagged AO7 expression, rabbit polyclonal anti-p65 antibody (Santa Cruz) and mouse monoclonal anti-FLAG (Sigma) were used.

RESULTS

Identification of AO7 as a Novel p65-interacting Protein by Yeast Two-hybrid Screen—Although various p65-interacting proteins have been identified (6, 7, 10, 19), we have adopted Cytotrap™ two-hybrid screening method of the yeast to search for additional p65-interacting proteins. For this screening, the C-terminal region (residues 262–551) of NF-κB p65 subunit was fused to human Sos (hSos) and used as bait. The temperature-sensitive mutant *S. cerevisiae* strain cdc25H, containing a point mutation in the yeast homologue (cdc25) of the hSos gene, cannot grow at 37 °C but can grow at the permissive temperature (25 °C). This yeast strain was used to screen a human lung cDNA expression library fused to the v-Src myristoylation sequence, thus anchoring the fusion protein to the plasma membrane. When the bait and target proteins physically interact, the hSos protein is recruited to the membrane, and the cdc25H yeast strain is enabled to grow at 37 °C.

We screened ~3.3 million transformants from the human lung cDNA library. Nucleotide sequence determination and comparison with GenBank™ databases (National Center for Biotechnology Information) revealed human IκBα (seven clones) and only one clone encoding human homologue of mouse AO7 (the corresponding protein of AO7 in human and mouse has also been termed RNF25; GenBank™ accession number NM022453). We isolated a full-length human AO7 cDNA clone from the library derived from human neuroblastoma cell line SK-N-MC (25) for further study.

To confirm the interaction between p65 and AO7 *in vitro*, we performed the GST pull-down assay. GST fusion proteins with various portions of AO7 (Fig. 1*A*) were synthesized in *E. coli*, immobilized on glutathione-Sepharose beads and incubated

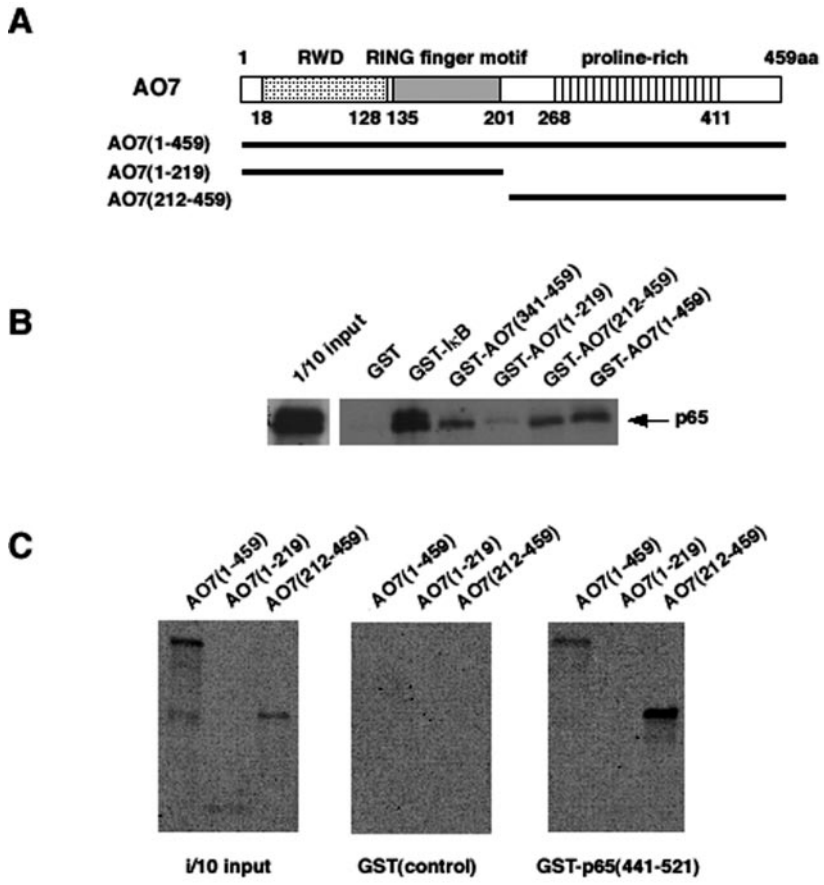


FIG. 1. **AO7 interacts with p65 *in vitro* and *in vivo*.** *A*, schematic illustration of AO7 and its mutants. AO7 contains RWD domain and the RING finger motif and the Pro-rich region as predicted by SMART (38, 39) and Motif Scan (40), respectively. *B* and *C*, Binding between p65 and AO7 *in vitro*. p65 and AO7 proteins were labeled with [^{35}S]methionine by *in vitro* transcription/translation. *B*, radiolabeled p65 was incubated with GST, GST-AO7-(1–459), GST-AO7-(1–219), GST-AO7-(212–459), GST-AO7-(341–459), or GST-IκBα (positive control) immobilized on glutathione-Sepharose beads. The bound protein was eluted, resolved by 10% SDS-PAGE, and visualized by autoradiography. *C*, radiolabeled AO7s were incubated with GST and GST-p65-(441–551) immobilized on glutathione-Sepharose beads. After incubation and further washing, the complexes were resolved by 10% SDS-PAGE and subjected to autoradiography.

with the [^{35}S]methionine-labeled p65 (full-length). Fig. 1*B* shows that p65 bound to GST-AO7-(1–459), GST-AO7-(212–459), and GST-AO7-(341–459), although the binding affinities of AO7 and some mutants with p65 appeared to be lower than that of IκBα. However, p65 did not significantly bind to GST-AO7-(1–219) containing the RING finger domain. No p65 binding was detected with GST alone. To further confirm this interaction, we also performed the reverse GST pull-down assay using GST-p65-(441–521) and the [^{35}S]methionine-labeled AO7-(1–459), AO7-(1–219), and AO7-(212–459). In Fig. 1*C*, GST-p65-(441–521) bound to AO7-(1–459) and AO7-(212–459) but not to AO7-(1–219). These results suggested that the minimal region of AO7 responsible for the interaction with the C-terminal region of p65 resides within the C-terminal amino acid sequence 341–459, overlapping with the Pro-rich region.

AO7 Interacts with p65 in Vivo—To examine whether AO7 interacts with p65 *in vivo*, 293 cells were transfected with pcDNA-AO7-(1–459), expressing the FLAG-tagged full-length AO7 and harvested for the co-immunoprecipitation assay. As demonstrated in Fig. 2, AO7-(1–459) was co-immunoprecipitated with p65, and this interaction was not significantly decreased even at the higher salt concentration (*lane 4*), suggesting the strong affinity between p65 and AO7. When stimulated with TNF, which causes the nuclear translocation of NF-κB and phosphorylation of p65 (29–31) a greater interaction between AO7 and p65 was observed (compare *lanes 2* and *6*), suggesting that AO7 interacts with p65 in the nucleus. Interestingly, this TNF-induced augmentation of the interaction between p65 and AO7 was significantly reduced at higher salt concentration (compare *lanes 6* and *8*), implying that the increased affinity between p65 and AO7 may be due to the increased electrostatic interaction such as by phosphorylation of p65.

AO7 Augments the NF-κB-dependent Gene Expression Induced by NIK—Since mouse AO7 was initially identified as an

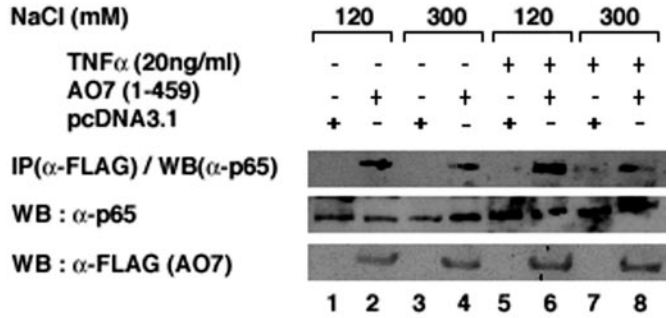


FIG. 2. **AO7 binds to p65 *in vivo*.** 293 cells were transfected with pAO7-(1–459) expressing the FLAG-tagged AO7. After 48 h of transfection, cells were stimulated with 20 ng/ml of TNF for 30 min in order to activate NF-κB. The AO7 was immunoprecipitated (*IP*) with anti-FLAG, the protein complex containing AO7 was resolved on 8% SDS-PAGE, and these proteins were probed with anti-p65 antibody (*upper panel*). Either low salt (120 mM NaCl) or high salt (300 mM NaCl) buffers were used for the immunoprecipitation to assess the binding affinity. The *middle* and *lower panels* show that equal amounts of p65 and AO7 were expressed in each transfection. *WB*, Western blot.

interacting protein with ubiquitin-conjugating enzyme (E2), UbcH5B, and involved in E2-dependent ubiquitination through its RING finger domain, we first asked if AO7 is involved in the NF-κB-induced transcription. We performed a transient luciferase assay using pGL3–4κB-luc reporter plasmid, in which luciferase reporter gene is under the control of NF-κB. Fig. 3 shows that cotransfection with pcDNA-AO7-(1–459) activated NF-κB-dependent gene expression upon NIK overexpression in a dose-dependent manner for the amount of AO7-expressing plasmid. Western blot analysis of the transfected cell lysate revealed no increase in the protein level of endogenous p65 (Fig. 3*A*). The similar effect of AO7 was observed when NF-κB was stimulated with IL-1β or TNF (Fig. 3*B*). To further inves-

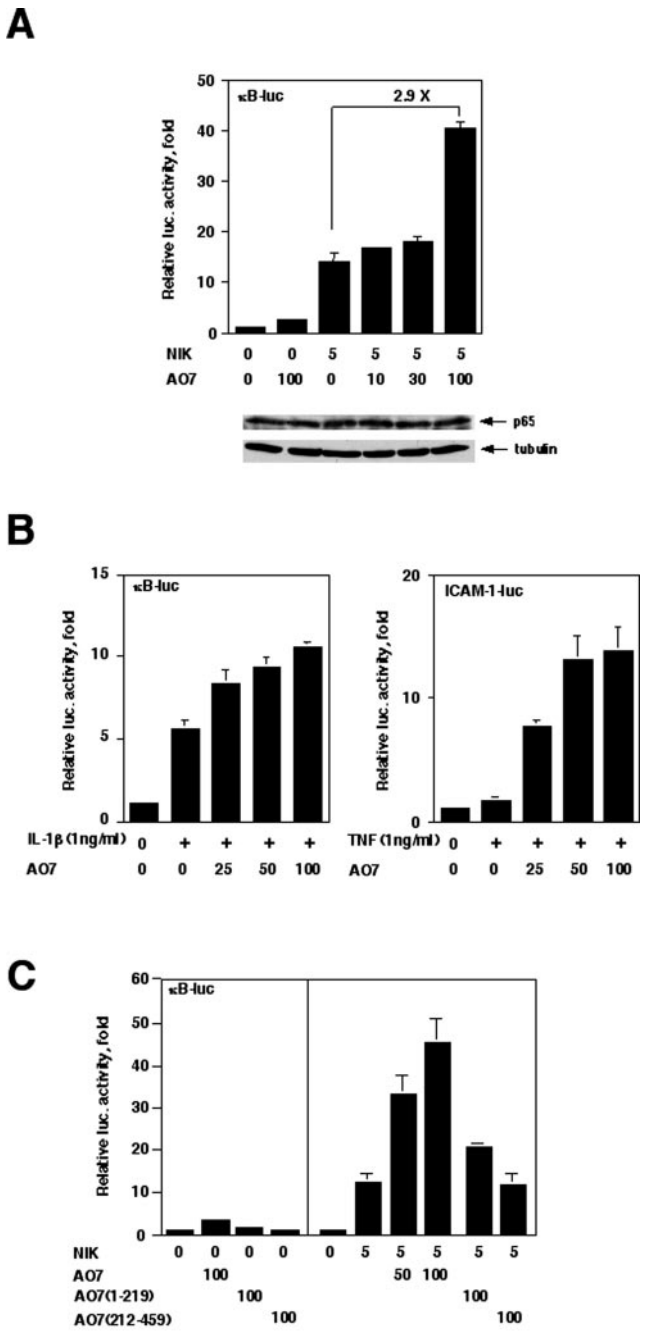


FIG. 3. **AO7 augments the NF-κB-dependent gene expression induced by NIK.** *A*, augmentation of the NIK-mediated NF-κB-dependent gene expression by AO7-expressing plasmid in a dose-dependent manner. 293 cells were transfected with 30 ng of 4κB-luc reporter plasmid together with the indicated amounts (in ng) of AO7-(1–459) and NIK expression plasmids. The *lower panel* shows that the level of p65, as detected by Western blotting (with β-tubulin as an internal control), was not changed by overexpression of NIK and/or AO7. *B*, effects of AO7 on the NF-κB-dependent gene expression stimulated by IL-1β or TNF. 293 cells were transfected with 30 ng of 4κB-luc reporter plasmid or ICAM-1-luc together with the indicated amounts (in ng) of AO7 expression plasmid. After 24 h of transfection, cells were stimulated with 1 ng/ml IL-1β or 1 ng/ml TNF. After additional incubation for 24 h, the cells were harvested, and the luciferase activity was determined. *C*, effects of AO7 mutants. 293 cells were transfected with 30 ng of 4κB-luc reporter plasmid together with the indicated amounts (in ng) of AO7-(1–459), AO7-(1–219), or AO7-(212–459) and NIK expression plasmids. CMV controls were included whenever appropriate such that all transfections had equal amounts of expression plasmid. Total plasmid DNA was kept at 500 ng with pUC19 plasmid. Cells were harvested 48 h after transfection, and luciferase activity was measured. The luciferase activity was measured with an equal amount (1 μg of total protein) of each cell lysate. Values are the mean ± S.D. of three independent transfections. Similar results were achieved repeatedly.

tigate the AO7 action, we created AO7 mutant constructs, and their effects on the NIK-induced NF-κB activation were examined. As shown in Fig. 3*C*, neither the C-terminal nor N-terminal truncated AO7 mutant enhanced NF-κB activation mediated by NIK or exhibited dominant negative effect. These findings indicate that both the C-terminal and the N-terminal regions of AO7 are required for its effect on the NF-κB activity.

Effect of AO7 Is Mediated by the p65 Subunit of NF-κB—To investigate whether AO7 enhances the action of NF-κB through acting on NIK-mediated signaling pathway or the transcriptional activity of p65, we examined the effect of AO7 on NF-κB-dependent gene expression when p65 is overexpressed. As shown in Fig. 4*A*, AO7 augmented the NF-κB-dependent gene expression when p65-expressing plasmid was cotransfected, indicating that the effect of AO7 does not depend on the signaling cascade involving NIK and IKK complex. Using a control reporter plasmid, 4κBm-luc, in which all four κB sites were mutated, no effect of AO7 was observed even upon overexpression of p65. These results indicate that AO7 stimulates the transcriptional activity of p65 but not through the basal transcriptional machinery.

AO7 Is Expressed Abundantly in the Nucleus—In order to examine the subcellular localization of AO7, the full-length AO7 in fusion with GFP was expressed in 293 cells. As shown in Fig. 4*B*, AO7 was detected predominantly in the nucleus, although the cytoplasmic localization was also evident. This intracellular distribution of AO7 was not altered by TNF stimulation. In addition, AO7 was colocalized with p65 when the nuclear translocation of NF-κB was induced by the treatment with TNF. These findings suggest that AO7 may interact with p65 in the nucleus.

AO7 Activates NF-κB through the Transactivation Domain of p65 (RelA)—To further investigate the effect of AO7 on NF-κB, various p65 mutants fused to Gal4BD (Fig. 5*A*) were cotransfected into 293 cells with the reporter plasmid, pFRluc, in which luciferase gene expression is under the control of Gal4, with or without the AO7 expression plasmid pcDNA-AO7-(1–459). As shown in Fig. 5*B*, AO7 enhanced the Gal4-p65-dependent gene expression in a dose-dependent manner, and truncation of either the N-terminal (containing RING finger motif and RWD domain) or C-terminal (containing the Pro-rich region) half of AO7 abolished its action. These findings indicate that both the N- and C-terminal regions of AO7 are indispensable for its action. Moreover, these AO7 mutants did not block the transcriptional activity of p65, which is consistent with the results in Fig. 3*B*. There was no significant effect of AO7 on the VP16-mediated transactivation (data not shown).

We then explored the region of p65 responsible for the AO7-mediated transcriptional enhancement using various Gal4-p65 mutants. There was no significant effect of AO7 on Gal4-p65N, containing p65-(1–286) and lacking the TA domain (Fig. 5*C*). In contrast, AO7 stimulated the transcriptional activity of Gal4-p65C, containing nuclear localization signal, TA1, and TA2, in a dose-dependent manner (Fig. 5*D*). Further truncation and deletion of the C-terminal region of p65 revealed that both TA1 and TA2 are required for the action of AO7 (Fig. 5, *E–G*). Notably, since the extent of augmentation by AO7 for pGal4-p65C2 (8.3-fold) was greater than that for pGal4-p65C (12-fold) (Fig. 5, compare *D* and *F*), the central region, containing nuclear localization signal and thus serving as the target of IκB, is fully dispensable for the action of AO7, indicating that AO7 is unlikely to be involved in the IκB degradation. Moreover, since p65-(441–521) is shown to be sufficient for the interaction with AO7 (Fig. 1*C*), at least *in vitro*, the action of AO7 may require additional protein(s) interacting with TA1.

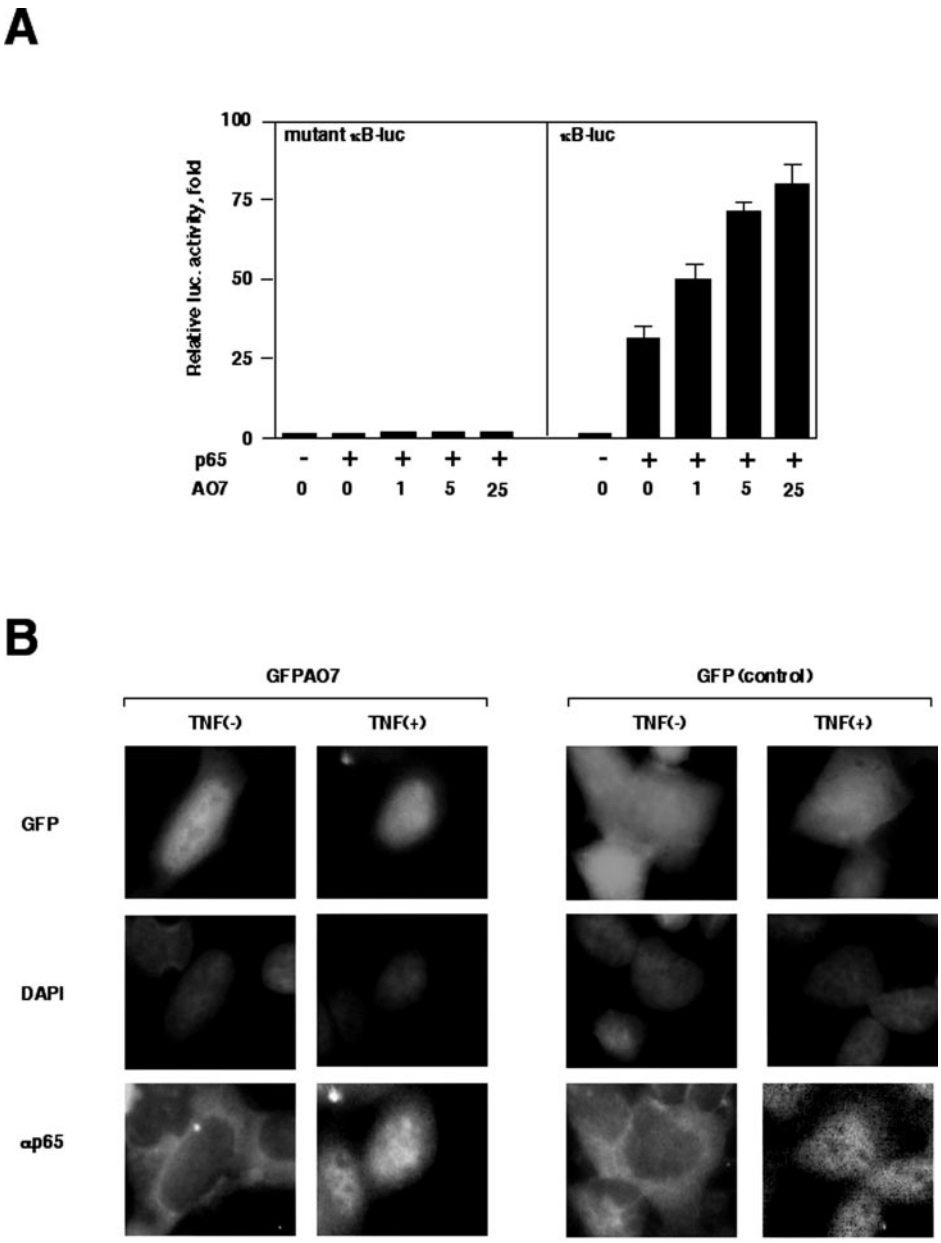
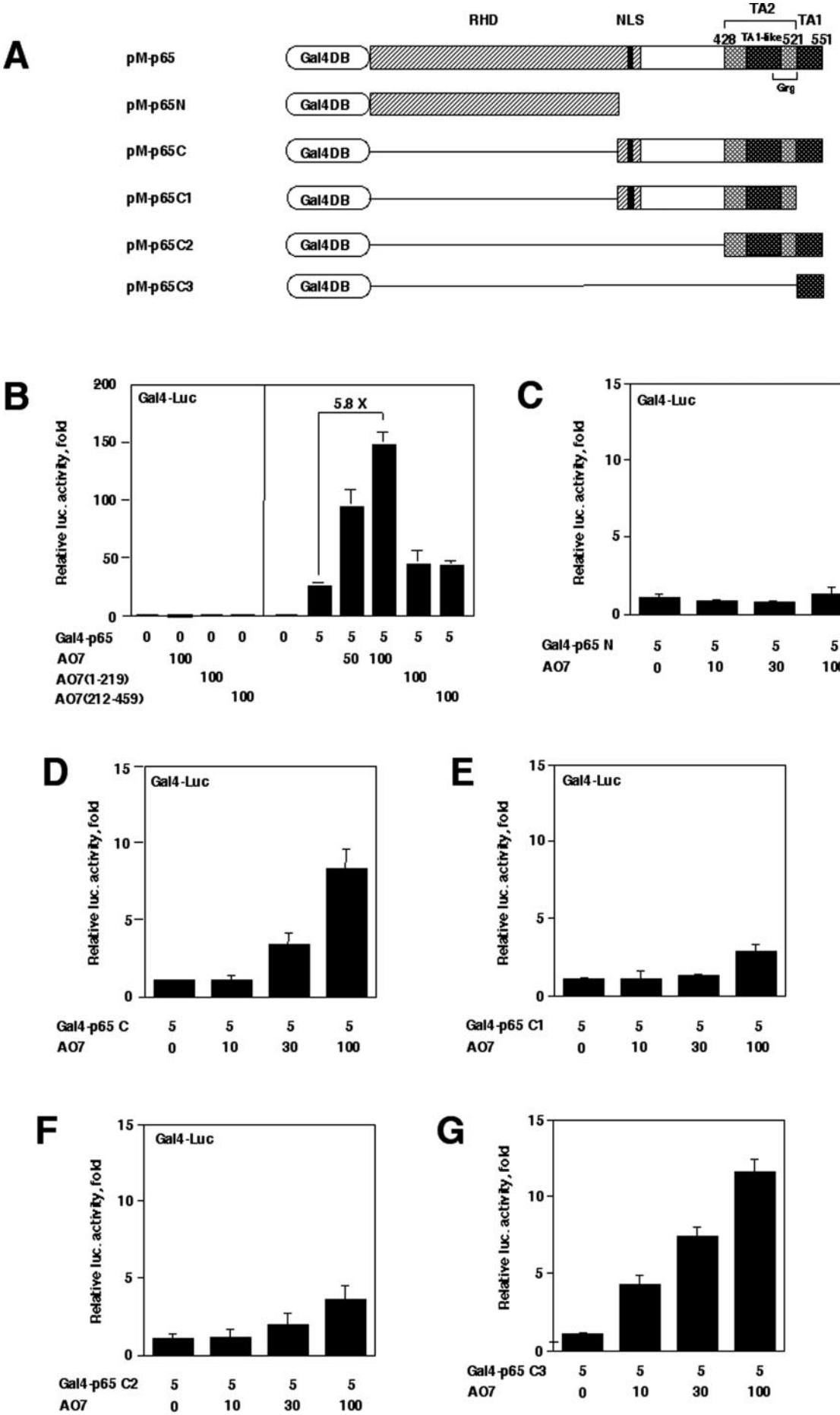


FIG. 4. **Augmentation of p65-mediated transactivation by AO7 and its cellular localization.** *A*, action of AO7 on the p65-mediated transactivation. 293 cells were transfected with 30 ng of 4κB w-luc (containing wild type NF-κB binding sites) or 4κB m-luc (containing mutated NF-κB binding sites) together with the indicated amounts (in ng) of pCMV-p65 and AO7 expression plasmids. Luciferase activity was measured as in Fig. 3. *B*, subcellular localization of AO7. pGFP-AO7 expressing GFP in fusion with AO7-(1–459) was transfected into 293 cells in the presence or the absence of TNF (20 ng/ml). The nucleus was counterstained by 4′,6-diamidino-2-phenylindole (*DAPI*). The localization of p65 was demonstrated by immunostaining with anti-p65 antibody under fluorescence microscopy.

Effects of Mutation in the RING Finger Domain of AO7—AO7 contains a typical RING finger domain of the RING-H2 category, forming an interleaved zinc-binding site with six Cys residues and two His residues in the middle (C3H2C3) (Fig. 6*A*). These Cys and His residues of AO7 were shown to be crucial for its function such as ubiquitination and E2 binding (20). We thus introduced Cys substitutions and yielded two mutants: AO7(C159S), in which Cys at the irrelevant amino acid position 159 for RING was substituted by Ser, and AO7(C161S), in which Cys at 161, a crucial amino acid for the formation of RING and indispensable for ubiquitination and E2 binding, was substituted by Ser (20).

In Fig. 6*B*, the effects of these AO7 mutants were examined. Whereas the Cys mutation at 159, AO7(C159S), did not significantly affect the action of AO7, the Cys mutation at 161, substituting a crucial Cys for RING-H2 formation, exhibited no such action. At the higher amounts of AO7(C161S), the gene expression mediated by Gal4-p65 was inhibited in a dose-dependent manner, suggesting the dominant negative characteristics for this mutant. These findings indicated that the RING-H2 domain of AO7 is important for the action of AO7 and suggested that ubiquitination might be necessary for the augmentation of p65 transcriptional activity by AO7.

Selective Action of AO7 on p65—To further analyze the action of AO7, we examined whether AO7 could activate transcription when tethered to DNA. As shown in Fig. 7*A*, Gal4-AO7, containing the full-length AO7 fused to Gal4BD, did not activate transcription from a minimal promoter containing Gal4 binding sites, suggesting that AO7 does not act as a general coactivator of transcription. When pGal4-AO7 was co-transfected with pSport-p65, expressing full-length p65, the extent of gene induction was greatly augmented (up to 10-fold)



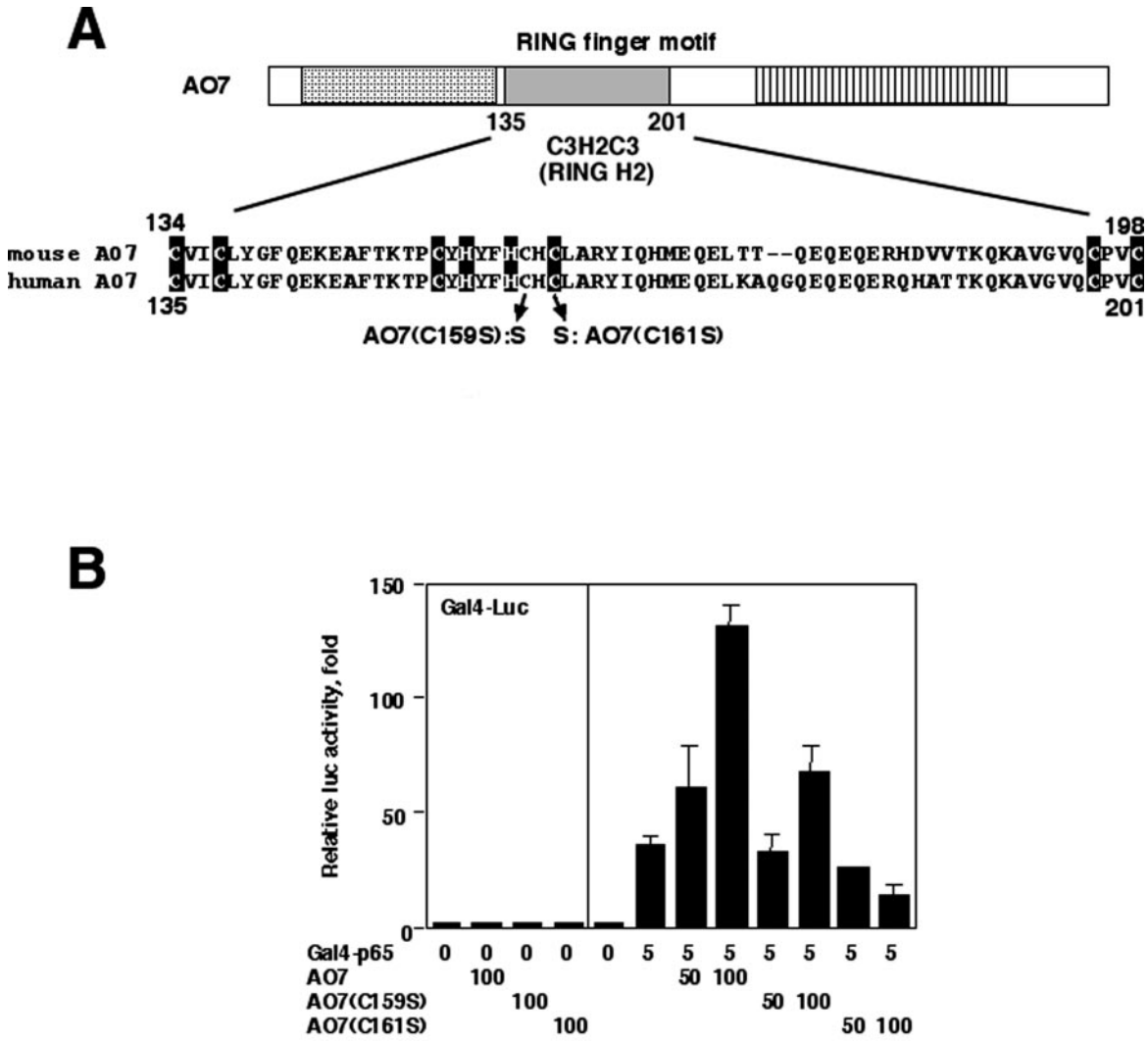


FIG. 6. **RING is important for the action of AO7 in supporting NF-κB transactivation.** *A*, amino acid sequence alignment of the RING finger domain of human and murine AO7. GenBank™ accession numbers of AAH15612 (*human*) and AAD48057 (*mouse*) are indicated. The potential zinc coordination residues are *boxed* and *numbered*. Note that the amino acid residue numbers are slightly different between humans and mice. The positions of mutations in AO7 mutants, AO7(C159S) and AO7(C161S), are indicated. *B*, effects of AO7 mutants on the transcriptional activity of Gal4-p65 protein. Experiments were similarly performed as in Fig. 5. Values are the mean ± S.D. of three independent transfections. Similar results were achieved repeatedly.

in a dose-dependent manner for the amount of pSport-p65 (Fig. 7*B*). When it was cotransfected with pVP16-p65, expressing a p65-VP16 fusion protein, a similar extent of gene induction (6-fold) was observed. However, when pVP16 was cotransfected with pGal4-AO7, no such effect was observed, presumably because of the lack of AO7 binding to VP16. Together with the results in Fig. 5, these observations indicate that AO7 interacts with p65 in cells and selectively supports the transactivation of p65.

DISCUSSION

Various factors modify the NF-κB transcriptional activity through interaction with p65 TAD. When NF-κB stimulates the transcription of target genes, transcriptional coactivators, such as p300/CREB-binding protein (8, 9) and FUS/TLS (10), and basal factors, including TBP (11) and TFIIB (7, 12), interact with p65 TAD. It is likely that p65 TAD acts as an essential platform for the protein interaction. To further identify other protein(s) interacting with p65 TAD and elucidate the precise mechanism of gene activation, we adopted the CytoTrap™ yeast two-hybrid system because p65 TAD was fully functional in the conventional yeast two-hybrid assay utilizing the Gal4-transcription system (10, 19). Using the C-terminal region of p65 containing p65 TAD and nuclear localization signal as bait, we identified AO7 as an interacting partner as well as IκBα. The interaction between p65 and AO7 was confirmed both *in vitro* and *in vivo*. Further mapping of the p65 interaction do-

FIG. 5. **p65 TAD is responsible for the AO7 action.** *A*, schematic illustration of Gal4-p65 and its mutants. *Gal4BD*, Gal4-DNA binding domain; *RHD*, Rel homology domain; *NLS*, nuclear localization signal; *TA1*, transactivation domain 1; *TA2*, transactivation domain 2; *TA1-like*, TA1-like domain; *Grg*, the interacting region with Groucho-related genes, or "Groucho-interacting region" (10, 19). Amino acid positions of TADs are indicated. *B–G*, effects of AO7 and mutants on the transcriptional activities of various Gal4-p65 proteins. 293 cells were transfected with 30 ng of pFR-luc, a luciferase reporter plasmid controlled by Gal4, together with various amounts (ng) of pcDNA-AO7-(1–459), pcDNA-AO7-(1–219), pcDNA- AO7-(212–459), and the pGal4-p65 constructs. Total plasmid DNA was kept at 500 ng with pUC19 plasmid. Cells were harvested 48 h after transfection, and the luciferase activity was measured with an equal amount (1 μg of total protein) of each cell lysate. Values are the mean ± S.D. of three independent transfections. Similar results were achieved repeatedly.

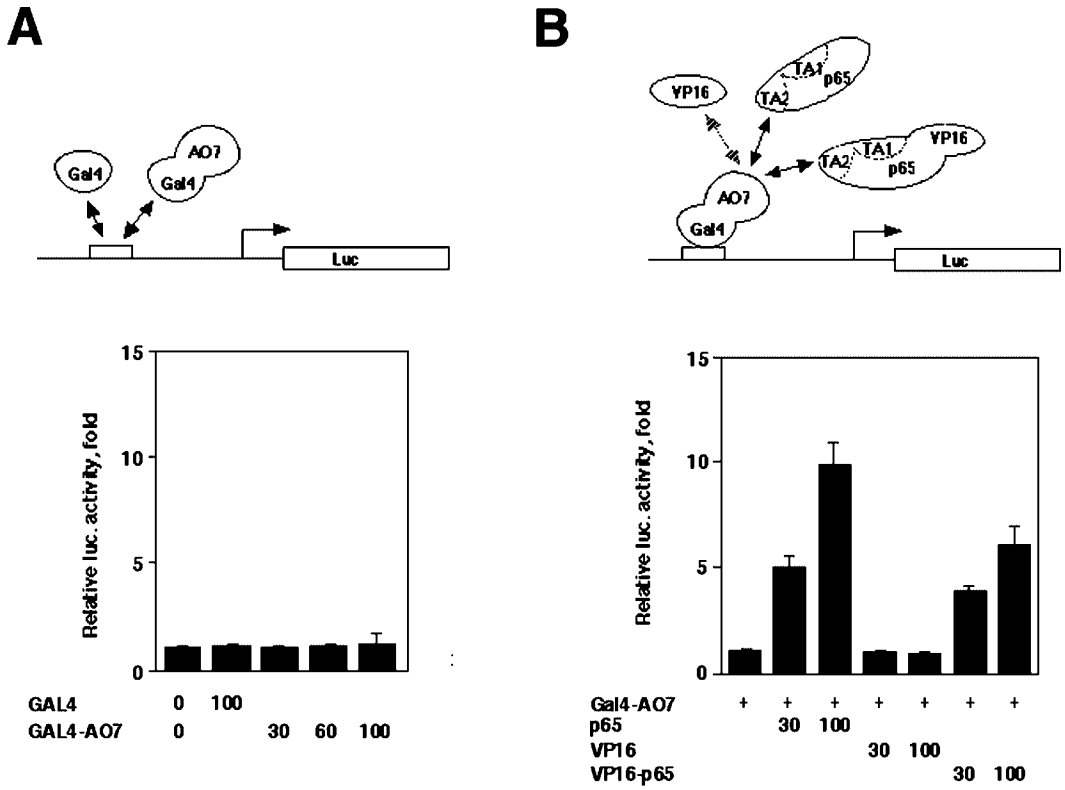


FIG. 7. **Action of AO7 when tethered to DNA.** *A*, AO7 does not act as a general coactivator. 293 cells were cotransfected with pFR-luc reporter plasmid (30 ng), under the control of Gal4, and pGal4-AO7 or pGal4 (expressing the Gal4 DNA-binding domain). *B*, effects of p65, VP16, and p65-VP16 on the transcriptional activity of Gal4-AO7. Cells were cotransfected with pFR-luc and pGal4 or pGal4-AO7 (400 ng) together with the indicated amounts (in ng) of pSport-p65, pVP16, or pVP16-p65. The luciferase activity was measured as in Fig. 3. Values are the mean ± S.D. of three independent transfections. Similar results were achieved repeatedly.

main revealed that a portion of TAD (from aa 441 to 521 localized in TA2) is responsible for the interaction with AO7 *in vitro*. AO7 was localized predominantly in the nucleus and interacted with p65 *in vivo* upon stimulation with TNF. Interestingly, when AO7 was overexpressed, the transcriptional activity of p65 was greatly augmented, which was independent from the NF-κB activation cascade including TNF signaling and NIK. Using a heterologous transactivation system with the Gal4 DNA-binding domain fused with various portions of p65, we found that both TA1 and TA2 were required for the effect of AO7. Therefore, in addition to the AO7 binding to p65 TA2, other factors interacting with TA1, such as p300/CREB-binding protein coactivators (8, 9), may be involved in the AO7-mediated transcriptional activation.

AO7 was initially isolated as E2-interacting protein by conventional yeast two-hybrid screen and shown to bind UbcH5b through its RING finger motif (20). Although AO7 is known to accept the ubiquitin modification by E2 (UbcH5b), its biological function *in vivo* has not been elucidated. It was thus proposed that AO7 might act as an E3-like factor. However, its target proteins have not been found. In this study, we found that the RING finger motif of AO7 was not involved in the binding to p65 but required for the p65-mediated transactivation, suggesting a possibility that the AO7 might mediate the ubiquitination of p65 and enhance its transcriptional activity. However, we could not detect direct ubiquitination of p65 by AO7 or any change in the stability of p65 (data not shown). Therefore, the mechanism by which AO7 participates in the NF-κB-mediated transactivation is currently unknown.

Accumulating evidence that ubiquitin-modification plays a role in transcriptional regulation has been demonstrated (13–17). Ubiquitination appears to control transcription not only by mechanisms involving ubiquitin-dependent destruction of transcription factors or their inhibitors by proteasome but also by an intriguing mechanism independent of the proteasome (for a review, see Ref. 18). For example, most of the NF-κB activation pathways involve ubiquitination of its inhibitor IκBs followed by their degradation by proteasome and the generation of p50 and p52, DNA-binding subunits of NF-κB, from their precursors is mediated by ubiquitination-dependent proteolysis (3, 4). It is possible that AO7 may facilitate protein processing of these proteins. Another possibility by which AO7 activates NF-κB is ubiquitination and degradation of corepressors such as Sin3A, N-CoR, or, more selectively, Groucho family proteins (19). In fact, Siah-2, a RING finger containing E3-like protein, is known to promote the ubiquitination of N-CoR and derepresses the gene expression (32).

There are many proteins that possess E3 ligase activity and inhibit the action of transcription factors. For example, the p53 inhibitor Mdm2, a RING finger-containing protein, inhibits the transcriptional activity of p53 by promoting ubiquitination and degradation of p53 (33). Another E3 protein, RING finger LIM domain-binding protein, inhibits the transcriptional activity of LIM homeodomain transcription factor family by interacting with the LIM cofactor and promoting its ubiquitination (34). Similarly, WWP1, a HECT family E3 ligase, binds to the lung Krüppel-like factor through its inhibitory domain and inhibits its transcriptional activity, although ubiquitination has not been demonstrated (35).

Interestingly, there are a number of reports suggesting a positive correlation between the ubiquitin-modification and the transcriptional activation. For example, although direct ubiquitination has not been demonstrated, Molinari *et al.* (36) demonstrated that half-lives of heterologous transactivators such as Gal4-VP16, Gal4-p65, Gal4-p53, and Gal4-CTF inversely correlated with the potency of their TADs. In addition, it was

demonstrated that the transactivation of VP16 TAD requires SCF^{Met30}, a yeast E3, and fusion of a single ubiquitin moiety was sufficient to complement the deficiency of the SCF^{Met30} gene (14). These findings indicate that TAD-dependent ubiquitination may not only serve as a signal for its destruction but also be required for its function as an activator. Other examples include the involvement of E3 ligases, Rsp5/hPRF, E6-AP, and SNURF/RNF4, in the transactivation of various nuclear receptors (15–17). In addition, since a $TAF_{II}250$ mutant lacking the ubiquitination catalytic activity failed to support the transactivation of Dorsal, a *Drosophila* homologue of RelA (37), the ubiquitination of histone H1 by $TAF_{II}250$ is considered to be essential for the action of Dorsal.

Taken together, our findings raise a possibility that AO7 may support the transcriptional activity of NF-κB by several mechanisms. One such mechanism may be that the activated NF-κB interacts in the nucleus with various factors including general transcription factors, coactivators, and AO7. Formation of this complex may allow AO7 to direct ubiquitin modification of some of these factors, thus dissociating the transcriptional activator complex to initiate a subsequent step of transcription such as elongation. AO7 may also act as a scaffold protein for the interaction of transcription factors involved in the initiation complex. Further investigations are required to elucidate the mechanism by which AO7 supports the action of NF-κB and its biochemical actions.

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RING Finger Protein AO7 Supports NF-κB-mediated Transcription by Interacting with the Transactivation Domain of the p65 Subunit

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