Inhibition of Nuclear Factor-κB-mediated Transcription by Association with the Amino-terminal Enhancer of Split, a Groucho-related Protein Lacking WD40 Repeats

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The amino-terminal enhancer of split (AES) encodes a 197-amino acid protein that is homologous to the NH2-terminal domain of the Drosophila Groucho protein but lacks COOH-terminal WD40 repeats. Although the Drosophila Groucho protein and its mammalian homologs, transducin-like enhancer of split proteins, are known to act as non-DNA binding corepressors, the role of the AES protein remains unclear. Using the yeast two-hybrid system, we have identified the protein-protein interaction between AES and the p65 (RelA) subunit of the transcription factor nuclear factor κB (NF-κB), which activates various target genes involved in inflammation, apoptosis, and embryonic development. The interaction between AES and p65 was confirmed by in vitro glutathione S-transferase pull-down assay and by in vivo co-immunoprecipitation study. In transient transfection assays, AES repressed p65-driven gene expression. AES also inhibited NF-κB-dependent gene expression induced by tumor necrosis factor α, interleukin-1β, and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1, which is an upstream kinase for NF-κB activation. These data indicate that AES acts as a corepressor for NF-κB and suggest that AES may play a pivotal role in the regulation of NF-κB target genes.

Nuclear factor κB (NF-κB)§¶ is an inducible cellular transcription factor that regulates a wide variety of cellular and viral genes including several cytokines, cell adhesion molecules, and human immunodeficiency virus (HIV) (1–4). The members of the NF-κB family in mammalian cells include the proto-oncogene c-Rel, Rel A (p65), Rel B, NFκB1 (p50/105), and NFκB2 (p52/p100). These proteins share a conserved 300-amino acids region known as the Rel homology domain, which is responsible for DNA binding, dimerization, and nuclear translocation of NF-κB (1–4). In most cells, Rel family members form hetero- and homodimers with distinct specificities in various combinations. p65, Rel B, and c-Rel are transcriptionally active members of the NF-κB family, whereas p50 and p52 primarily serve as mere DNA binding subunits (1–4). The transactivation domains of p65, Rel B, and c-Rel have been mapped in their unique COOH-terminal regions. p65 was shown to contain at least two independent transactivation domains within its COOH-terminal 120 amino acids (5–8). One p65 activation domain, TA1, is confined to the COOH-terminal 30 amino acids of p65. The second domain TA2 is contained within the NH2-terminally adjacent 90 amino acids.

A common feature of the regulation of NF-κB family is their sequestration in the cytoplasm as inactive complexes with a class of inhibitory molecules known as IκBs (1–4). Treatment of cells with a variety of inducers such as phorbol esters, interleukin-1, and tumor necrosis factor (TNF) results in phosphorylation, ubiquitination, and degradation of the IκB proteins (4, 9, 10). The degradation of IκB proteins exposes the nuclear localization sequence in the remaining NF-κB dimers, leading to nuclear translocation and subsequent binding of NF-κB to the DNA regulatory element of target genes (1–4).

In addition to the nuclear translocation and DNA binding of NF-κB, its transcriptional activity is also regulated by coactivators and corepressors in the nucleus. It has been recently demonstrated that the coactivators, CREB-binding protein and p300, physically interacts with p65 and increase its transcriptional activity (11, 12). Thus, using the yeast two-hybrid screening system, we searched for protein molecules interacting with the p65 subunit of NF-κB. We have found that p65 physically interacts with the amino-terminal enhancer of split (AES), a 197-amino acid protein homologous to the NH2-terminal region of the Drosophila Groucho protein but lacking COOH-terminal WD40 repeats (13, 14).

The Groucho family includes three types of proteins (15, 16). The larger proteins such as Groucho and its mammalian homologs, transducin-like enhancer of split (TLE) 1 through 3, share five domain structures. These proteins exhibit a common feature including an amino-terminal glutamine-rich region (Q domain), a glycine/proline-rich region (GP domain), a Ccn domain containing a casein kinase II site and nuclear localization sequence, a serine/proline-rich region (SP domain), and COOH-terminal WD40 repeats (13, 15–17). Three of these domains the Q, Ccn, WD40 domains are mostly highly conserved. A shorter
protein, the human TLE4, contains all the domains except for the amino-terminal Q domain. Shortest proteins in the Groucho family, which contain only the Q domain and the PCR domain, are designated as AES or the Groucho-related gene (Grg) (13, 14). Significant homology is observed in the Q domain between AES and other Groucho proteins except for TLE4. Drosophila Groucho protein and its mammalian homologs, TLEs, have been shown to serve as non-DNA binding corepressors for several transcription factors including the Hairy-related proteins, Runt domain proteins, Engrailed, Dorsal, and lymphoid enhancer factor-1/T-cell factor (18–23). The WD40 repeats in Groucho and TLEs appear to mediate protein-protein interaction with relevant DNA-binding proteins, such as Engrailed and Hairy (19, 20).

The function of AES, a Groucho-related protein lacking WD40 repeats, remains controversial. Mallo et al. (24) have demonstrated that in the in vitro transcription activity of the HeLa nuclear extract was diminished when AES was depleted. Roose et al. (23) have shown that XGrg-5, a Xenopus AES homolog lacking WD40 repeats, augmented transcriptional activation of T-cell factor-dependent reporter genes, whereas the XGrg-4, a Xenopus Groucho homolog containing WD40 repeats, inhibited TCF-dependent gene expression. From these observations it was suggested that AES may act as an inhibitor of Groucho family corepressors by dominant negative mechanisms. On the other hand, Ren et al. (25) have shown that AES mediates positive regulatory domain I-binding factor 1/Blimp-1-dependent repression of β-interferon gene and that AES itself can function as a potent repressor when tethered to DNA through the Gal4 DNA-binding domain.

Here we demonstrate that AES physically interacts with the p65 subunit of NF-κB. The interaction between AES and p65 was confirmed by in vitro glutathione S-transferase pull down assay and by in vivo co-immunoprecipitation assay. In transient transfection assays, AES repressed NF-κB-dependent gene expression. These results indicate that AES, even lacking the WD40 repeats can interact with and inhibit NF-κB-dependent gene expression.

**EXPERIMENTAL PROCEDURES**

**Plasmids—Mammalian expression vectors, pCMV-p65, pRSV-CREB, pCMV-ΔMEKK1, and pL6-luc were generous gifts from Drs. Yoshiaki Ito (Kyoto University), Masatashi Hagiwarra (Tokyo Medical and Dental University), Gary L. Johnson (University of Colorado), and Shizuo Akira (Hyogo Medical College), respectively. The cDNA of human p65 (amino acids 1–551) was amplified by PCR using pCMV-p65 as a template with oligonucleotides containing BamHI sites (forward, 5′-CCCCCGGATCCCCGGCCATGGACGAACTGTTC-3′ and reverse, 5′-CCACATCTCCTGAGCTAGTAGTACTCATAGACGTG-3′) containing BamHI and XhoI sites, respectively. The resulting PCR product was digested with BamHI and XhoI and ligated in frame into pCDNA3.1/HisA vector at the BamHI-XhoI site to form pCMV-AES. To construct the mammalian expression vector for TLE1, the cDNA fragment of TLE1 corresponding to amino acids 1–770 was amplified by PCR from cDNA encoding TLE1 (a generous gift from Dr. Stefano et al.). oligonucleotides (forward, 5′-CACGGATCCGGTCTTCCGAGACGCGCTGAC-3′ and reverse, 5′-CCACATCTCCTGAGCTAGTAGTACTCATAGACGTG-3′) containing BamHI and XhoI sites, respectively. The resulting PCR product was digested with BamHI and XhoI and ligated in frame into pCDNA3.1/HisA vector at the BamHI-XhoI site to form pCMV-TLE1. To generate the FLAG-tagged mammalian expression vectors, pFLAG-AES and pFLAG-TLE1, the cDNA of AES and TLE1 cDNA inserts were excised from pCMV-AES and pCMV-TLE1 with BamHI and XhoI and ligated in frame into pCMV-Tag1 expression vector (Stratagene) at the BglII-XhoI site, respectively.

Construction of a luciferase reporter plasmid, 4x κB-SV40-luc (4x κBw-luc), containing four tandem copies of the HIV κB sequence upstream of minimal SV40 promoter has been described previously (26). Other luciferase reporter plasmids including 5x κB-TATA-luc (p NFκB-luc), 4x CRE-TATA-luc (pCRE-luc), and 5x Gal4-TATA-luc (pFR-luc) were purchased from Stratagene. These plasmids contain five tandem copies of the NF-κB binding site, four tandem copies of cAMP response element (CRE) and five tandem copies of the Gal4 binding site upstream of the TATA box, respectively.

**Two-Hybrid Screen—Yeast Two-Hybrid Screen for Protein-Protein Interaction Assay—** The yeast two-hybrid screening was performed as described previously (27). The various portions of the p65 COOH-terminal regions corresponding to amino acids 286–551, 286–521, 286–470, and 286–470 were fused in-frame to Gal4 DNA-binding domain (1–147) against the pGBT9 vector (CLONTECH). They were tested for activation of the Gal4-dependent lacZ expression (β-galactosidase activity). Among them, pGBT-p65-(286–442/521) was chosen as a bait for library screening, because it had undetectable background, whereas other clones had significant background levels in β-galactosidase assay. Yeast strain Y190 was transformed with pGBT-p65-(286–442/521) and the human placenta cDNA expression library fused to the Gal4 transcription activation domain in the pACT2 vector (CLONTECH). Approximately one million transformants were screened for the ability to grow on the plates with medium lacking tryptophan/leucine/histidine and containing 25 μM 3-aminotriazole. Plasmids were rescued from clones that were positive for β-galactosidase activity and identified by nucleotide sequencing, cDNA sequences and their amino acid sequences were compared with GenBank™ and SwissProt data bases for identification of the interacting proteins.

**Cell Culture and Transfection—**Jurkat T lymphocytes were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin, 100 μg/ml streptomycin. 293 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotics. Cells were transfected using SuperFect transfection reagent (Qiagen, Hilden, Germany) according to manufacturer’s recommendations. At 48 h posttransfection, the cells were harvested, the extracts were prepared for luciferase assay. Luciferase activity was measured by the Luciferase Assay System (Promega, Madison, WI) as described previously (27). Transfection efficiency was monitored by Renilla luciferase activity using the pRL-TK plasmid (Promega) as an internal control. The data are presented as the fold increase in luciferase activities (mean ± S.E.) relative to control of three independent transfections.

**Protein-Protein Interaction Assay—** Bacterial expression of GST fusion proteins utilized pGEX expression vectors. To generate pGEX-AES, which expresses GST-AES, the AES cDNA insert was excised from pCMV-AES with BamHI and XhoI and ligated in frame into pGEX-2X-2 vector (Amersham Pharmacia Biotech) at the BamHI-XhoI site. GST fusion proteins were expressed in Escherichia coli strain DH5α and purified as described (27). In vitro protein-protein interaction assays were carried out as described previously (27). AES, Luciferase proteins were labeled with [35S]methionine by the in vitro transcription/translation procedure using the TNT wheat germ extract coupled system (Promega) according to the manufacturer’s protocol. Approximately 20 μg of GST fusion proteins immobilized on 20 μl of glutathione-agarose beads was mixed with two transformed human embryonic kidney 293 cells treated with two-fold modified HEMKN buffer (40 mM HEPES at pH 7.5, 50 mM KCl, 5 mM MgCl2, 0.2 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The beads were left in 0.6 ml of HEMKN after the final wash. The beads were incubated with the radiolabeled proteins overnight at 4 °C with gentle mixing. The beads were then washed five times with 1 ml of HEMKN buffer. Bound radiolabeled proteins were eluted with 30 μl of Laemmli sample buffer, boiled for 3 min, and
RESULTS

*p65 Interacts with Groucho-related Protein, AES*—Because the nuclear translocation and DNA binding of NF-κB were not sufficient for full activation of NF-κB (30, 31), it was suggested that modification of the transactivation domains of p65 by phosphorylation and/or protein-protein interactions with other molecules might play a critical role in controlling its transactivation activity. Thus, we searched for protein molecules interacting with the unique COOH-terminal region adjacent to the transactivation domain of NF-κB p65 using the yeast two-hybrid screening system. As shown in Fig. 1A, various portions of p65, i.e. amino acids 286–551, 286–521, 286–470, and 286–442/477–521 were fused to Gal4 DNA-binding domain in the pGBT9 vector (CLONTECH). Among these clones, pGBT-p65-(286–442/477–521) was chosen as a bait, because it had no detectable background in β-galactosidase assay, whereas other clones had significant background levels (Table I). Yeast strain Y190 was used to screen human placenta cDNA expression libraries fused to the Gal4 transcriptional activation domain in the pACT2 vector (CLONTECH). Approximately one million transformants of cDNA library were screened, and about 90 transformants were streaked on selective medium. Various portions of p65 correspond to pGBT-p65 constructs, which encode the fusion protein between Gal4 DNA-binding domain and indicated portions of p65. AES indicates the pACT2 clone obtained from screening, which encodes the fusion protein between Gal4 transcriptional activation domain and AES (amino acids 59–197). Yeast strain Y190 was transformed with these plasmids, and yeast two-hybrid interaction assays between p65 and AES were carried out to confirm the interactions between p65 and AES and to determine the region of p65 necessary for binding to AES. Yeast strain Y190 cells cotransformed with expression vectors encoding various proteins fused to Gal4 DNA-binding domain (Gal4-DDB) and Gal4 transcriptional activation domain (Gal4-AD). pACT2-AES is a rescued clone, which encodes AES (amino acids 59–197), fused to Gal4 AD. Leu- Trp+ transformants were streaked on selective medium lacking leucine/tryptophan and allowed to grow for 2 days at 30 °C. At least three colonies of each transformed yeast were tested for β-galactosidase activity using X-gal colony filter assay (Clontech). “+” indicates positive for β-galactosidase activity (blue colony) after 25 h; “−” indicates no β-galactosidase activity (white colony) after 24 h; N.D. indicates samples not determined.

![Image](https://example.com/image.png)

**FIG. 1. Yeast two-hybrid interaction assay between p65 and AES.** A, domains of p65 and AES constructs. Gal4-AD, Gal4 transcrip- tional activation domain; RHD, rel homology domain; NLS, nuclear localization signal; TA1, transactivation domain 1; TA2, transactivation domain 2; Q, glutamine-rich domain; GP, glycine- and proline-rich domain. B, growth of transformants coexpressing p65 and AES on selective medium. Various portions of p65 correspond to pGBT-p65 constructs, which encode the fusion protein between Gal4 DNA-binding domain and indicated portions of p65. AES indicates the pACT2 clone obtained from screening, which encodes the fusion protein between Gal4 transcriptional activation domain and AES (amino acids 59–197). Yeast strain Y190 was transformed with these plasmids, and individual Leu− Trp+ transformants were streaked on synthetic medium lacking leucine, tryptophan, and histidine and containing 25 mM 3-aminotriazole.

**TABLE I**

| Gal4-DDB hybrid | pACT2 | pACT2-AES | pACT2-IeBo |
|-----------------|-------|-----------|-------------|
| pGBT9           | −     | −         | −           |
| pGBT-p65-(1–286)| +     | N.D.      | N.D.        |
| pGBT-p65-(286–551)| +     | N.D.      | N.D.        |
| pGBT-p65-(286–521)| +     | N.D.      | N.D.        |
| pGBT-p65-(286–470)| +     | N.D.      | N.D.        |
| pGBT-p65-(286–442)| +     | +         | +           |
| pGBT-p65-(286–442/477–521)| + | + | + |
| pGBT-p65-(473–522)| − | − | − |

The human AES protein encodes a nuclear protein of 197 amino acids that is homologous to NH2-terminal regions of Groucho family corepressors but lacking COOH-terminal WD40 repeats. (13, 14, 32, 33). The isolated AES lacked a NH2-terminal region (amino acids 1–58), which contains the leucine zipper-like motif (13, 34). Thus, the leucine zipper-like motif in AES appears not to be required for the interaction with p65.

To confirm the interactions between p65 and AES and to determine the region of p65 necessary for binding to AES, various regions of p65 fused to the Gal4 DNA-binding domain in the pGBT9 vector were reintroduced into yeast cells along with pACT2-AES, which is a rescued clone encoding AES (amino acids 59–197) fused to the Gal4 transcriptional activation domain. Interactions were tested by growth on plates with medium lacking histidine, leucine, and tryptophan and containing 25 mM 3-aminotriazole (Fig. 1B) as well as by β-galactosidase activity (Table I). pGBT-p65-(1–286), pGBT-p65-(286–442), pGBT-p65-(286–442/477–521), and pGBT-p65-(473–522) were fused to pGBT9. Yeast strain Y190 was transformed with these plasmids, and individual Leu− Trp+ transformants were streaked on synthetic medium lacking leucine/tryptophan and allowed to grow for 2 days at 30 °C. At least three colonies of each transformed yeast were tested for β-galactosidase activity using X-gal colony filter assay (Clontech). “+” indicates positive for β-galactosidase activity (blue colony) after 25 h; “−” indicates no β-galactosidase activity (white colony) after 24 h; N.D. indicates samples not determined.

For co-immunoprecipitation assays, 293 cells were transfected with pCMV-p65 and pFLAG-AES or pFLAG-TLE1 expression vectors. Whole cell extract was harvested with whole cell extract buffer (20 mM HEPES at pH 7.9, 300 mM NaCl, 5 mM MgCl2, 0.2 mM EDTA, 10% glycerol, 0.3% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). The whole cell extract was incubated with 1 μg of anti-FLAG mouse monoclonal antibody (Kodak) or control mouse IgG (Santa Cruz) overnight at 4 °C in immunoprecipitation (IP) buffer (20 mM HEPES at pH 7.9, 100 mM NaCl, 5 mM MgCl2, 0.2 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). 10 μl of protein G-agarose, 0.5 mM phenylmethylsulfonyl fluoride beads were washed further for 1 h. The beads were washed five times with 1 ml of IP buffer. Antibody-bound complexes were eluted by boiling in 2 × Laemmli sample buffer. Supernatants were resolved by SDS-PAGE and transferred on nitrocellulose membrane (Hybond-C, Amersham Pharmacia Biotech). The membrane was incubated with anti-p65 antibody (Santa Cruz), and immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) as described previously (28, 29).

For protein-protein interaction assay using the p65 protein expressed in mammalian cells, the whole cell extract from p65-transfected 293 cells was incubated with GST or GST-AES immobilized on glutathione-Sepharose beads for 3 h at 4 °C in IP buffer. The beads were washed three times with IP buffer and two more times with IP buffer containing 300 mM NaCl. Bound p65 proteins were resolved by SDS-PAGE and immunoblotted using anti-p65 antibody as described above.

Nucleotide sequence determination and comparison with GenBank™ and SwissProt data bases revealed that six clones encoded the IκB family, i.e. IκBα/MAD3 (59–197). Yeast strain Y190 was transformed with these plasmids, and yeast two-hybrid interaction assays between p65 and AES were carried out to confirm the interactions between p65 and AES and to determine the region of p65 necessary for binding to AES. Yeast strain Y190 was transformed with these plasmids, and individual Leu− Trp+ transformants were streaked on synthetic medium lacking leucine, tryptophan, and histidine and containing 25 mM 3-aminotriazole.
Groucho-related Proteins Inhibit NF-κB Activity

Fig. 2. AES interacts with p65 in vitro and in vivo. A, p65 binds to AES and IκBα in vitro. p65 and luciferase (a negative control protein) were labeled with [35S]methionine by in vitro transcription/translation. Radiolabeled p65 and luciferase were incubated with GST, GST-AES, or GST-IκBα immobilized on glutathione-Sepharose beads. After incubation and further washing, the complexes were resolved by SDS-PAGE and subjected to autoradiography. B, p65 and AES bind to GST-AES in vitro. Radiolabeled p65 and AES were incubated with GST or GST-AES immobilized on glutathione-Sepharose beads. After incubation and further washing, the complexes were resolved by SDS-PAGE and subjected to autoradiography. C, p65 expressed in mammalian cells binds to AES in vitro. 293 cells were transfected with pCMV-p65 expression vector, and whole cell extract was harvested 48 h after transfection. The whole cell extract was incubated with GST or GST-AES immobilized on glutathione-Sepharose beads. After incubation and further washing, the complexes were resolved by SDS-PAGE and immunoblotted with anti-p65 antibody. D, AES interacts with p65 in vivo. 293 cells were transfected with pFLAG-AES and pCMV-p65 expression vectors. Whole cell extract was harvested 48 h after transfection, and the FLAG-tagged AES was immunoprecipitated with anti-FLAG mouse monoclonal IgG or with control mouse IgG. The immunoprecipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-p65 antibody.

Fig. 3. AES inhibits p65-mediated transcription but not basal transcription. A, AES represses NF-κB-dependent luciferase gene expression induced by p65 in a dose-dependent manner. The data are presented as the fold increases in luciferase activities relative to control transfection. B, AES represses p65-mediated transcription but not basal transcription. Percent inhibition of basal transcription and p65-mediated transcription by AES were indicated. Jurkat cells were transfected with 50 ng of 4xκB-SV40-luc reporter plasmid and indicated amounts of pCMV-p65 and pCMV-AES expression plasmids. CMV control plasmids were included such that all transfections had equivalent amounts or pCMV-p65 and pCMV-AES expression plasmids. CMV control plasmids were included such that all transfections had equivalent amounts of expression plasmid. Total DNA was kept at 1 μg with plUC19 plasmid. Cells were harvested 48 h after transfection, and luciferase activity was measured. Values are the mean ± S.E. of three independent transfections. Similar results were achieved repeatedly.

To further investigate the interaction between AES and p65, we performed an in vitro protein-protein interaction assay using GST fusion proteins. AES, p65, and luciferase (a negative control) proteins were radiolabeled with [35S]methionine by in vitro transcription/translation. These products were incubated with GST fusion proteins immobilized on glutathione-Sepharose beads. The radiolabeled p65 bound to GST-AES and GST-IκBα but not to GST (Fig. 2A). The radiolabeled AES also bound to GST-AES (Fig. 2B), indicating that AES can make homodimers. No binding was observed between radiolabeled luciferase and GST-AES or GST-IκBα (Fig. 2A). To test whether AES interacts with the p65 protein expressed in mammalian cells, GST-AES or GST was incubated with the cell extract from p65-transfected 293 cells. The p65 protein expressed in mammalian cells was found to interact with GST-AES but not with GST (Fig. 2C). To examine whether AES interacts with p65 in cultured cells, we performed the co-immunoprecipitation experiment. 293 cells were transfected with p65 and FLAG-tagged AES expression plasmids, and whole cell extract was prepared. Immunoprecipitation with anti-FLAG antibody followed by Western blot analysis with anti-p65 antibody revealed that AES interacted with p65 in vivo (Fig. 2D).

Taken together, these results demonstrate that AES specifically interacts with the p65 subunit of NF-κB in vivo as well as in vitro.

AES Inhibits NF-κB-dependent Gene Expression—We then investigated the effect of AES on NF-κB-dependent gene expression, as we found that AES interacts with the vicinity of the COOH-terminal transactivation domain of p65. The luciferase reporter plasmid (4xκB-SV40-luc) containing four tandem copies of the κB sequence upstream of minimal SV40 promoter was co-transfected with an expression vectors for AES and/or p65 into Jurkat cells. As shown in Fig. 3A, AES inhibited the p65-induced luciferase gene expression in a dose-dependent manner. The inhibitory effect of AES on κB-dependent gene expression was considered not due to the effect on SV40 promoter, because overexpression of AES did not inhibit basal 4xκB-SV40-luc expression in the absence of p65 (Fig. 3B).

To further examine whether the inhibitory effect of AES is specific for NF-κB, we compared the effects of AES on gene expression under the control of NF-κB (p65), CREB, or Gal4/VP16. In contrast to p65-mediated transactivation, AES did not
inhibit CREB-mediated or Gal4/VP16-mediated transcription (Fig. 4, B and C). These data suggest that the inhibitory effect of AES on transcription is relatively specific for NF-κB-mediated transcription.

AES also inhibited signal-induced NF-κB-dependent gene expression. In Fig. 5A, Jurkat cells were cotransfected with 4x κB-SV40-luc reporter plasmid and pCMV-AES expression plasmid. 24 h later, cells were stimulated with a proinflammatory cytokine, TNFα, and incubated for another 24 h. Expression of AES inhibited the TNFα-induced NF-κB-dependent luciferase gene expression. We then determined the effect of AES on NF-κB-dependent gene expression induced by mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (MEKK1), which is one of the upstream kinases of the NF-κB activating pathway (35–37). Plasmids expressing AES and the activated form of MEKK1 (ΔMEKK1) were transfected into Jurkat cells along with 5x κB-TATA-luc reporter plasmid (Stratagene). AES inhibited κB-dependent luciferase gene expression induced by ΔMEKK1 in a dose-dependent manner (Fig. 5B). Again, no significant repression was observed on the basal promoter activity.

**TLE1 Associates with p65 and Inhibits p65 Transactivation**—Because there is an extensive homology between AES and other members of the Groucho family, it is likely that p65 also interacts with other Groucho family proteins. To test this hypothesis, we examined whether a larger form of Groucho protein, TLE1 (13, 17), interacts with NF-κB and modulates the transcriptional activity. FLAG-tagged TLE1 and p65 were transiently coexpressed in 293 cells, and whole cell extract was harvested. Immunoprecipitation with anti-FLAG antibody followed by Western blot with anti-p65 antibody revealed that...
NF-κB-driven gene expression (Fig. 6). TLE1 repressed the transient transfection experiment, TLE1 repressed the luciferase activity was measured. Values are the mean ± S.E. of three independent transfections. Similar results were achieved repeatedly.

The promoter of the human IL-6 gene contains an NF-κB binding site, and NF-κB is indispensable for IL-1β-induced IL-6 gene induction (3). The long terminal repeat (LTR) of HIV-1 also contains two tandem NF-κB binding sites that are critical for HIV replication from latently infected cells (3). Thus, to determine whether AES acts as a corepressor for NF-κB in such biological processes, we examined the effect of AES on the promoter activities of the IL-6 gene and HIV-1-LTR. As shown in Fig. 7A, AES inhibited IL-1β-induced IL-6 promoter activity in a dose-dependent manner. In addition, AES inhibited the TNF-α-induced promoter activity of HIV-1-LTR (Fig. 7B). These data indicate that AES actually acts as a corepressor for NF-κB in biological processes.

DISCUSSION

The Drosophila Groucho protein and its mammalian homologs, TLEs, have been shown to function as corepressors for specific subsets of transcription factors (15, 16). However, the function of AES, a shorter Groucho-related protein lacking the WD40 repeats, has not been clarified (13, 14, 33). Here we have demonstrated the identification and characterization of protein-protein interaction between AES and the NF-κB p65 subunit. Molecular interaction between p65 and AES was confirmed by in vitro GST pull down assay and in vivo co-immunoprecipitation study. Transient transfection assays revealed that AES represses NF-κB-mediated gene expression. Furthermore, p65 was co-immunoprecipitated with TLE1, a mammalian Groucho homolog containing both NH2-terminal domains and COOH-terminal WD40 repeats (13, 17). We found that TLE1 also repressed NF-κB-driven gene expression. These data indicate that AES and TLE1 can act as corepressors for NF-κB.

It was demonstrated that a Drosophila homolog of NF-κB, Dorsal, serves as both an activator and a repressor of transcription (38). It activates twist and snail but simultaneously represses decapentaplegic and zerknöllt to establish dorso-ventral axis during early Drosophila development. It has been elucidated that the Drosophila corepressor, Groucho, directly binds to Dorsal and converts it from a transcriptional activator to a repressor (21). However, the role of Groucho family proteins in regulation of mammalian NF-κB has not been elucidated. The present study has demonstrated for the first time that mammalian Groucho homologs interact with mma-
lian NF-κB and repress its transcriptional activity. We also demonstrated that AES, the shorter Groucho homolog lacking the WD40 repeats, is sufficient for repressing the NF-κB activity.

These findings suggest that the transcriptional activity of NF-κB may be regulated by a balance between the counteracting effects of Groucho corepressors and coactivators, because it was previously found that NF-κB binds to coactivator proteins p300/CREB-binding protein (11, 12). It is also speculated, although not yet proven, that the interactions of NF-κB with coactivators or corepressors may be modulated by signal-induced modification of these proteins.

Our findings together with those of others suggest a putative role of vertebrate NF-κB in transcriptional repression. A recent observation has shown that inhibition of NF-κB activity by localized expression of transdominant-negative IkBα in chick embryo resulted in the arrest of limb growth (39, 40). In this phenotype, inhibition of NF-κB lead to inhibition of twist expression but derepression of a vertebrate decapentaplegic homolog, the bone morphogenetic protein-4 (40). Taken together the vertebrate NF-κB may also act as a transcriptional repressor by recruiting Groucho family corepressors, such as AES and TLEs.

In the present study, it was noted that AES and TLE1 inhibited NF-κB-mediated gene expression but did not actively repress NF-κB-driven gene expression to less than basal level (the level of gene expression without NF-κB and AES/TLE1). Lack of active repression may be because we used NF-κB-dependent reporter constructs, which do not contain additional repression elements. In Drosophila, recruitment of Groucho is obligatory but is not sufficient for Dorsal-mediated repression. Dorsal-mediated repression requires additional repression elements in the proximity of the Dorsal binding sites and the binding of other DNA-binding repressor proteins to these elements (38, 41). In the recent study using the zerknüllt promoter, Valentine et al. (41) demonstrated that Dorsal-mediated repression requires the formation of a multiprotein DNA-bound complex that includes Groucho, Dorsal, and additional DNA-binding proteins, such as cut and dead ringer. Because NFT-1/Grainyhead is known to bind to the repression elements in decapentaplegic promoter (42), and it may play a role in decapentaplegic repression that is analogous to the role of cut and dead ringer in zerknüllt repression. Thus, if NF-κB acts as an active repressor in vertebrates, it is likely that there are additional repressor binding sites and the respective DNA-binding proteins.

Many members of the Groucho family can act as corepressors for a specific subset of DNA-binding transcriptional regulators. These include the Hairy-related basic helix-loop-helix proteins, Runt domain proteins, Engrailed, and Dorsal, and lymphoid enhancer factor-1/T-cell factor (18–23). Whereas the interactions between Groucho/TLE and Hairy-related proteins or Runt domain proteins rely on common WRPWMWRY motifs, the interaction of Groucho with Engrailed is mediated by the eh1 domain (18–20). In Drosophila, the interaction between Groucho and Dorsal is mediated by rel homology domain (21). Our results suggested that the interaction between human p65 and human AES may be mediated by the vicinity of the p65 transactivation domain. Thus, interactions between DNA-binding transcription factors and Groucho proteins appears to be mediated through multiple protein motifs.

At present, little is known about the mechanism by which Groucho family proteins function as euchromatic corepressors in higher eukaryotes. The structural resemblance of Groucho and TLE proteins to Tup1, a general transcriptional repressor in yeast, suggests that these proteins may function by similar mechanisms (15, 43, 44). Both Groucho family proteins and Tup1 lack DNA-binding domains and must therefore be recruited to DNA through interactions with DNA-binding transcriptional regulators. Tup1 was shown to mediate transcriptional repression through at least two mechanisms. Transcriptional repression by Tup1 may occur via changes in chromatin structure. Mutations in Tup1 result in derepression and disturbance in nucleosome stability and positioning on DNA (45). The repression domain of Tup1 was shown to bind directly to the histones H3 and H4 (46). In addition to contacting chromatin, Tup1 appears to function by interacting directly with components of the general transcriptional machinery (47, 48). It is possible that Groucho family proteins may mediate repression by similar mechanisms. It was reported that TLE proteins are associated with chromatin in live cells and that TLE proteins can specifically interact with the histone H3 (49). Thus, Groucho proteins may regulate transcription by promoting formation of a repressive chromatin configuration in the vicinity of their target sites. Alternatively, Groucho proteins may interact with components in the general transcription machinery to inhibit transcription, as in the case of Tup1.

We demonstrated that AES inhibits the transcriptional activity of IL-6 and HIV-1 genes, which are under the control of NF-κB (3, 50, 51). NF-κB is an important regulatory factor in many cellular processes including inflammation, immune responses, and viral replication such as HIV-1, apoptosis, and embryonic development. Thus mammalian Groucho proteins, AES and TLEs, may play a pivotal role in these biological processes by modulating transcriptional activity of NF-κB.

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REFERENCES

1. Baldwin, A. S., Jr. (1996) Annu. Rev. Immunol. 14, 649–683
2. Baeuerle, P. A., and Baichwal, V. R. (1997) Adv. Immunol. 65, 111–137
3. Okamoto, T., Sakurada, S., Yang, J. P., and Merin, J. P. (1997) Curr. Top. Cell. Regul. 35, 149–161
4. Ghosh, S., May, M. J., and Kopp, E. B. (1998) Annu. Rev. Immunol. 16, 225–260
5. Moore, P. A., Ruben, S. M., and Rosen, C. A. (1993) Mol. Cell. Biol. 13, 1660–1674
6. Schiltz, M. L., and Baeuerle, P. A. (1991) EMBO J. 10, 3805–3817
7. Schmitz, M. L., dos Santos Silva, M. A., and Baeuerle, P. A. (1995) J. Biol. Chem. 270, 15576–15584
8. Schmitz, M. L., Stetzer, G., Altman, H., Meisterernst, M., and Baeuerle, P. A. (1995) J. Biol. Chem. 270, 7219–7226
9. Mercuro, F., and Manning, A. M. (1999) Curr. Opin. Cell Biol. 11, 226–232
10. Zandi, E., and Karin, M. (1999) Mol. Cell. Biol. 19, 4547–4551
11. Gernsheim, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2927–2932
12. Perkins, N. D., Felzien, L. K., Betts, J. C., Leung, K., Beach, D. H., and Nabel, G. J. (1997) Science 275, 523–527
13. Miyasaka, H., Choudhury, B. K., Hou, E. W., and Li, S. S. (1993) Eur. J. Biochem. 216, 343–352
14. Mello, M., Franco del Amo, F., and Gridley, T. (1993) Mech. Dev. 42, 67–76
15. Fisher, A. L., and Caudy, M. (1998) Genes Dev. 12, 1931–1940
16. Parkhurst, S. M. (1998) Trends Genet. 14, 130–132
17. Stifani, S., Blaumuelle, C. M., Redhead, N. J., Hill, R. E., and Artavanis-Tsakonas, S. (1992) Nat. Genet. 2, 119–127
18. Paroush, Z., Finley, R. L., Jr., Kidd, T., Wainwright, S. M., Ingham, P. W., Brent, R., and Ish-Horowicz, D. (1994) Cell 79, 805–815
19. Jenner, G., Paroush, Z., and Ish-Horowicz, D. (1997) Genes Dev. 11, 3072–3082
20. Tolkunova, E. N., Fujioka, M., Kobayashi, M., Deka, D., and Jaynes, J. B. (1998) Mol. Cell. Biol. 18, 2804–2814
21. Dubinett, T., Valentine, S. A., Chen, G., Shi, T., Lengyel, J. A., Paroush, Z., and Covery, A. J. (1997) Genes Dev. 11, 2952–2957
22. Cavallo, R. A., Cox, R. T., Moline, M. M., Roose, J., Polevoy, G. A., Clevers, H., Peifer, M., and Beach, D. (1998) Nature 395, 604–608
23. Roze, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O., and Clevers, H. (1998) Nature 395, 608–612
24. Mello, M., Lieberman, P. M., and Gridley, T. (1995) Cell. Mol. Biol. Res. 41, 435–440
25. Ren, B., Chee, K. J., Kim, T. H., and Maniatis, T. (1999) Genes Dev. 13, 125–137
26. Sato, T., Asamitsu, K., Yang, J. P., Takahashi, N., Tetsuka, T., Yoneyama, T., Kanagawa, A., and Okamoto, T. (1998) AIDS Res. Hum. Retroviruses 14, 293–298
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27. Yang, J. P., Hori, M., Sanda, T., and Okamoto, T. (1999) *J. Biol. Chem.* **274**, 15662–15670
28. Tetsuka, T., Baier, L. D., and Morrison, A. R. (1996) *J. Biol. Chem.* **271**, 11689–11693
29. Tetsuka, T., Daphna-Iken, D., Miller, B. W., Guan, Z., Baier, L. D., and Morrison, A. R. (1996) *J. Clin. Invest.* **97**, 2051–2056
30. Yoza, R. K., Hu, J. Y. Q., and McCall, C. E. (1996) *J. Biol. Chem.* **271**, 18306–18309
31. Bergmann, M., Hart, L., Lindsay, M., Barnes, P. J., and Newton, R. (1998) *J. Biol. Chem.* **273**, 6607–6610
32. Hartley, D. A., Preiss, A., and Artavanis-Tsakonas, S. (1988) *Cell* **55**, 785–795
33. Schmidt, C. J., and Sladek, T. E. (1993) *J. Biol. Chem.* **268**, 25681–25686
34. Pinto, M., and Lobe, C. G. (1996) *J. Biol. Chem.* **271**, 33026–33031
35. Hirano, M., Osada, S., Aoki, T., Hirai, S., Hosaka, M., Inoue, J., and Ohno, S. (1996) *J. Biol. Chem.* **271**, 13234–13238
36. Meyer, C. F., Wang, X., Chang, C., Templeton, D., and Tan, T. H. (1996) *J. Biol. Chem.* **271**, 8971–8976
37. Nakano, H., Shindo, M., Sakon, S., Nishinaka, S., Mihara, M., Yagita, H., and Okumura, K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 35457–35452
38. Courrey, A. J., and Huang, J. D. (1995) *Biochim. Biophys. Acta* **1261**, 1–18
39. Kanegas, Y., Tavares, A. T., Izpisua-Belmonte, J. C., and Verma, I. M. (1998) *Nature* **392**, 611–614
40. Bushdid, P. B., Brantley, D. M., Yull, F. E., Blaue, G. L., Hoffman, L. H., Niswander, L., and Kerr, L. D. (1998) *Nature* **392**, 615–618
41. Valentine, S. A., Chen, G., Shandala, T., Fernandez, J., Mische, S., Saint, R., and Courrey, A. J. (1998) *Mol. Cell. Biol.* **18**, 6584–6594
42. Huang, J. D., Dubnicoff, T., Liaw, G. J., Bai, Y., Valentine, S. A., Hirokawa, J. M., Leung, J. A., and Courrey, A. J. (1995) *Genes Dev.* **9**, 3177–3189
43. Johnson, A. D. (1995) *Cell* **81**, 655–658
44. Keleher, C. A., Redd, M. J., Schultz, J., Carlson, M., and Johnson, A. D. (1992) *Cell* **81**, 101–110
45. Cooper, J. P., Roth, S. Y., and Simpson, R. T. (1994) *Genes Dev.* **8**, 1400–1410
46. Edmondson, D. G., Smith, M. M., and Roth, S. Y. (1996) *Genes Dev.* **10**, 1247–1259
47. Song, W., Treisch, I., Qian, N., Kuchin, S., and Carlson, M. (1996) *Mol. Cell. Biol.* **16**, 115–120
48. Wahi, M., and Johnson, A. D. (1995) *Genetics* **140**, 79–90
49. Palaparti, A., Baratz, A., and Stifani, S. (1997) *J. Biol. Chem.* **272**, 26694–26699
50. Yoshida, S., Katoh, T., Tetsuka, T., Uno, K., Matsu, N., and Okamoto, T. (1999) *J. Immunol.* **163**, 351–358
51. Okamoto, T., Matsuyama, T., Mori, S., Hamamoto, Y., Kobayashi, N., Yamamoto, N., Josephs, S. F., Weng-Staal, F., and Shimotohno, K. (1989) *AIDS Res. Hum. Retroviruses* **5**, 131–138