Genes: Structure and Regulation:

FBI-1 Enhances Transcription of the Nuclear Factor- κB (NF-κB)-responsive E-selectin Gene by Nuclear Localization of the p65 Subunit of NF-κB

Dong-Kee Lee, Jae-Eun Kang, Hye-Jin Park, Myung-Hwa Kim, Tae-Hee Yim, Jung-Min Kim, Min-Kyu Heo, Kyu-Yeun Kim, Ho Jeong Kwon and Man-Wook Hur

J. Biol. Chem. 2005, 280:27783-27791.
doi: 10.1074/jbc.M504909200 originally published online May 24, 2005

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 30, Issue of July 29, pp. 27783–27791, 2005

Printed in U.S.A.

FBI-1 Enhances Transcription of the Nuclear Factor-κB (NF-κB)-responsive E-selectin Gene by Nuclear Localization of the p65 Subunit of NF-κB*

Received for publication, May 4, 2005
Published, JBC Papers in Press, May 24, 2005, DOI 10.1074/jbc.M504909200

Dong-Kee Lee‡, Jae-Eun Kang‡, Hye-Jin Park‡, Myung-Hwa Kim‡, Tae-Hee Yim‡, Jung-Min Kim‡, Min-Kyu Heo‡, Kyu-Yeun Kim§, Ho Jeong Kwon¶, and Man-Wook Hur‡‖**

From the ‡Department of Biochemistry and Molecular Biology and the Institute of Genetic Sciences, BK21 Project for Medical Sciences, Yonsei University School of Medicine, 134 ShinChon-Dong, SeoDaeMoon-Ku, Seoul 120-752, Korea, the §Department of Biological Sciences, Ewha Womans University, DaeHyun-Dong, Seoul 120-750, Korea, and the ¶Department of Biological Engineering, Sejong University, KunJa-Dong, KwangJin-Ku, Seoul 143-747, Korea

The POZ domain is a highly conserved protein-protein interaction motif found in many regulatory proteins. Nuclear factor-κB (NF-κB) plays a key role in the expression of a variety of genes in response to infection, inflammation, and stressful conditions. We found that the POZ domain of FBI-1 (factor that binds to the inducer of short transcripts of human immunodeficiency virus-1) interacted with the Rel homology domain of the p65 subunit of NF-κB in both *in vivo* and *in vitro* protein-protein interaction assays. FBI-1 enhanced NF-κB-mediated transcription of E-selectin genes in HeLa cells upon phorbol 12-myristate 13-acetate stimulation and overcame gene repression by IκBα or IκBβ. In contrast, the POZ domain of FBI-1, which is a dominant-negative form of FBI-1, repressed NF-κB-mediated transcription, and the repression was cooperative with IκBα or IκBβ. In contrast, the POZ domain tagged with a nuclear localization sequence polypeptide of FBI-1 enhanced NF-κB-responsive gene transcription, suggesting that the molecular interaction between the POZ domain and the Rel homology domain of p65 and the nuclear localization by the nuclear localization sequence are important in the transcription enhancement mediated by FBI-1. Confocal microscopy showed that FBI-1 increased NF-κB movement into the nucleus and increased the stability of NF-κB in the nucleus, which enhanced NF-κB-mediated transcription of the E-selectin gene. FBI-1 also interacted with IκBα and IκBβ.

The BTB/POZ (broad complex, Tramtrack, and bric-a-brac/poxvirus and zinc finger) domain is an evolutionarily conserved protein-protein interaction domain that is found at the N terminus of various cellular and viral regulatory proteins. The proteins containing the BTB/POZ domain have several C-terminal structures important in their biological functions, such as the zinc finger, actin-binding repeats, and ion channel motifs (1–3). The POZ domains of PLZF (promyelocytic leukemia zinc finger) and Bcl-6 (B-cell lymphoma-6) have been shown to interact with SMRT (silencing mediator for retinoid and thyroid hormone receptors)/N-CoR (nuclear receptor co-repressor), mSin3A, and histone deacetylases (4, 5).

FBI-1 (factor that binds to the inducer of short transcripts of human immunodeficiency virus-1) was purified as a cellular factor that binds specifically to the wild-type IST (inducer of short transcripts) elements of human immunodeficiency virus-1 long terminal repeats and the proximal promoter of the *ADH5/FDH* gene, and its cDNA was cloned (6–9). FBI-1 is a ubiquitous transcription factor that contains a BTB/POZ domain at its N terminus and Krüppel-like zinc fingers at its C terminus. There have been several recent reports on the function of FBI-1. FBI-1 stimulates Tat (transactivator of transcription) activity on the human immunodeficiency virus-1 long terminal repeat (8) and represses human *ADH5/FDH* gene expression by interacting with Sp1 zinc fingers (9). The mouse counterpart of FBI-1, LRF (leukemia/lymphoma-related factor), is co-immunoprecipitated and co-localized with Bcl-6 (10). The rat homolog of FBI-1, OCZF (osteoclast-derived zinc finger), is a transcription repressor and is involved in osteoclastogenesis (11). SAGE (serial analysis of gene expression) analysis shows that the expression of FBI-1 is increased in cancer tissues (available at www.ncbi.nlm.nih.gov/UniGene/clust.cgi?ORG=Hs&CID=104640).

Nuclear factor-κB (NF-κB)[1] exists in homo- or heterodimeric form and is an inducible transcription factor that activates the transcription of a variety of genes with the 10-bp consensus sequence GGGRNNYYCC (where R is purine, Y is pyrimidine, and N is any base) in the promoter in response to infection, inflammation, and stress conditions (12–15). NF-κB exists in the cytoplasm of most cell types as the homo- or heterodimer of structurally related proteins called the Rel or Rel/NF-κB proteins. It appears that various Rel/NF-κB complexes have different NF-κB binding site specificities and that the extent of transcription induced by individual subunit combinations is, in part, dependent upon the nature of binding sites that they recognize. The Rel/NF-κB proteins contain a highly conserved N-terminal 300-amino acid region called the Rel homology domain (RHD). The RHD contains important domains for DNA

* This work was supported by a National Research Laboratory research grant (to M.-W. H.) from the Korea Institute of Science & Technology Evaluation and Planning, the Korea Ministry of Science and Technology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** Current address: Baylor College of Medicine, Dept. of Medicine, Division of Diabetes, Endocrinology, and Metabolism, One Baylor Plaza, Houston, TX 77030.

‖ To whom correspondence should be addressed. Tel.: 82-2-361-5188; Fax: 82-2-312-5041; E-mail: mwhur2@yumc.yonsei.ac.kr.

[1] The abbreviations used are: NF-κB, nuclear factor-κB; RHD, Rel homology domain; NLS, nuclear localization sequence; TNFα, tumor necrosis factor-α; GST, glutathione *S*-transferase; FITC, fluorescein isothiocyanate; PDTC, ammonium pyrrolidinedithiocarbamate; PMA, phorbol 12-myristate 13-acetate; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PBS, phosphate-buffered saline.

binding, dimerization, the nuclear localization sequence (NLS), and interaction with IκB (inhibitor of NF-κB) family members (12–15). In non-stimulated cells, NF-κB complexes are sequestered in the cytoplasm in an inactive form via interaction with a monomer of an inhibitory protein called IκB, which itself belongs to a structurally and functionally related family of proteins (14, 16, 17).

The IκB family of inhibitory proteins of NF-κB includes IκBα, IκBβ, IκBγ, IκBϵ, Bcl-3, and the precursor Rel proteins p100 and p105 (14, 18). These proteins not only specifically and reversibly inhibit DNA binding by NF-κB, but also actively dissociate DNA-bound NF-κB *in vitro* and export back to the cytoplasm (19). IκBα and IκBβ are important regulators of mammalian NF-κB (20). All IκB proteins contain three to seven ankyrin repeat sequences of 30–33 amino acids, and the repeats mediate the binding of IκB proteins to the RHD of NF-κB. The direct protein-protein interaction between ankyrin repeats of IκB proteins and the RHD mask the NLS and prevent the nuclear translocation of NF-κB (12, 17, 22, 23). The C-terminal PEST sequences and N termini of IκB proteins contain serine phosphorylation and ubiquitination sites, which are important for the signal-induced degradation of IκB proteins and the release of NF-κB from cytoplasm trapping (24, 25).

In non-stimulated cells, NF-κB exists in an inactive form, having been bound to a member of the IκB family in the cytoplasm (18, 23, 26). When cells are exposed to various stimuli such as cytokines (*e.g.* tumor necrosis factor-α (TNFα) and interleukin-1), bacterial or viral products (*e.g.* lipopolysaccharide), and pro-apoptotic or necrotic stimuli (*e.g.* UV light and γ-irradiation) (Refs. 14, 18, and 29 and references therein), IκB is phosphorylated by a specific IκB kinase complex (27, 28). The phosphorylation of IκB proteins by the IκB kinase complex triggers polyubiquitination by a specific ubiquitin ligase belonging to the SCF (Skp-1/Cul/F-box) family (29). As soon as the IκB proteins are polyubiquitinated, they are rapidly degraded by the 26 S proteasome. The released NF-κB enters the nucleus, binds to target promoters, and activates transcription (14, 18, 29).

Here, we report that FBI-1 interacts with the RHD of the p65 subunit of NF-κB. The interactions increase the localization and stability of the p65 subunit in the nucleus, resulting in the transcription enhancement of an NF-κB-responsive target gene such as E-selectin.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and Reagents—Mammalian expression vectors for the human p65 subunit, IκBα, IκBβ, and NF-κB reporter plasmids were kind gifts from Drs. T. H. Lee and K. C. Jung (Yonsei University). The overexpression plasmids for the p65 subunit of NF-κB, IκBα, and IκBβ are under the control of the cytomegalovirus promoter, and the pELAM-Luc plasmid was described previously (24). pcDNA3.0-FBI-1 and pcDNA3.0-POZ_{FBI-1} were reported elsewhere (9). pcDNA3.0-POZ_{NLS} was prepared by attaching the NLS sequence (amino acids 487–505, KDGC-NGVPSRRGRKPRVRG) of FBI-1 to the POZ domain (amino acids 1–133). For bacterial overexpression, His_6-RHD and GST-POZ cDNAs were cloned into pQE30 (Qiagen Inc.) and pGEX4T3 (Amersham Biosciences) expression vectors. All plasmid constructs were verified by sequencing.

Anti-His_6 antibody was purchased from Qiagen Inc. Anti-p65 subunit, anti-phosphorylated p65 subunit, anti-IκBα, and anti-IκBβ antibodies were from New England Biolabs Inc. (Beverly, MA), Upstate Biotechnology Inc. (Charlottesville, VA), and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit and rhodamine-conjugated anti-mouse secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Horseradish peroxidase-conjugated anti-rabbit antibody was from Bio-Rad, and horseradish peroxidase-conjugated anti-mouse antibody was from Santa Cruz Biotechnology, Inc. Anti-glyceraldehyde-3-phosphate dehydrogenase antibody was purchased from Chemicon International, Inc. (Temecula, CA). TNFα, MG132 (benzyloxycarbonyl-leucinyl-leucinyl-leucinal), ammonium pyrrolidinedithiocarbamate (PDTC), Me_2SO, GSH-agarose, *p*-phenylenediamine, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma. Rabbit polyclonal antibody against the POZ domain of FBI-1 was prepared by us (9).

Yeast Two-hybrid Screening of Polypeptides Interacting with the POZ Domain of FBI-1—To perform the yeast two-hybrid screening, we used the Matchmaker® LexA two-hybrid system and cDNA library (Clontech). The POZ domain of FBI-1 was cloned into a pLexA-DB vector and used as bait. The PCR-amplified POZ domain of FBI-1 was cloned into the pLexA vector. The pLexA-POZ_{FBI-1} plasmid was introduced into yeast strain EGY48(p8op-lacZ) by a standard protocol, and yeast containing pLexA-POZ_{FBI-1} was selected on synthetic dextrose/−Ura/−His plates. Transformed yeast containing pLexA-POZ_{FBI-1} was prepared as competent yeast by a standard protocol. An amplified human liver Matchmaker® yeast LexA cDNA library was transformed into competent pLexA-POZ_{FBI-1}-containing yeast and screened on synthetic dextrose/−Ura/−His/−Trp plates. At 5 days post-transformation, 153 well grown colonies were selected and stored. The cotransformants of the 153 colonies were screened on synthetic dextrose/Gal/Raf/−Ura/−His/−Trp/−Leu + X-gal to identify the protein-protein interactions. After nutrition and X-gal double selection, 37 colonies were isolated. The plasmids in the yeast were isolated by miniscale preparation and transformed into *Escherichia coli* DH5α. The amplified plasmids from *E. coli* were purified, restriction enzyme-digested for mapping, and sequenced.

Molecular Interaction between IκBα or IκBβ and the POZ Domain of FBI-1 in Yeast Two-hybrid Assays—The POZ domain of FBI-1 was cloned into the pLexA-DB vector to prepare pLexA-POZ_{FBI-1} and used as bait. The pLexA-POZ_{FBI-1} plasmid was introduced into yeast strain EGY48(p8op-lacZ) by a standard protocol, and yeast containing pLexA-POZ_{FBI-1} was selected on synthetic dextrose/−Ura/−His plates. Transformed yeast containing pLexA-POZ_{FBI-1} was prepared as competent yeast by a standard protocol. To prepare the fish protein pB42AD-IκBβ plasmid, the coding sequence of IκBβ was amplified by PCR and cloned into the pB42AD vector in-frame with the B42 activation domain. Yeast cotransformants of two plasmids (pLexA-POZ_{FBI-1} and pB42AD-IκBβ) were streaked onto synthetic dextrose/Gal/Raf/−Ura/−His/−Trp + X-gal, and the *in vivo* protein-protein interactions were identified by blue colony formation.

Bacterial Overexpression of Fusion Proteins and in Vitro Protein-Protein Interaction Assays—For bacterial overexpression of fusion proteins, the pTrcHisB-RHD (the RHD of the p65 subunit of NF-κB) and pGEX4T3-POZ_{FBI-1} plasmids were transformed into *E. coli* DH5α. Bacteria were grown in 500 ml of LB broth to A_{600} = 0.8 at 37 °C and induced overnight with 0.2 mM isopropyl β-D-thiogalactopyranoside at 18 °C. Bacteria were collected by centrifugation and lysed in cold *E. coli* binding buffer (50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1% Nonidet P-40, 10 mM imidazole, and one tablet/50 ml protease inhibitor mixture (Roche Applied Science)). Soluble fractions from the lysate were incubated with 2 ml of GSH-agarose (for GST-POZ) or 500 μl of nickel-nitrilotriacetic acid (for His_6-RHD; Qiagen Inc.) for 1 h at 4 °C. After washing, bound proteins were eluted with 2 ml of elution buffer (for GST-POZ, 10 mM GSH and 50 mM Tris-HCl (pH 8.0); and for His_6-RHD, 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, and 100 or 250 mM imidazole) and dialyzed with cold phosphate-buffered saline (PBS) for 1 h at 4 °C.

For the *in vitro* protein-protein interaction assay, 9 μg of purified GST-POZ and GST were bound to GSH-agarose for 1 h in 500 μl of TNE buffer (50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 2 mM EDTA, and one tablet/50 ml protease inhibitor mixture) at 4 °C. Agarose-bound GST or GST fusion protein was collected by centrifugation at 3000 × *g* for 1 min at 4 °C and washed four times with cold TNE buffer. Agarose-bound GST protein was incubated with His_6-RHD in TNE buffer for 1 h at 4 °C. After the agarose-protein complex was washed three times with cold TNE buffer, the bound proteins were resolved by 15% SDS-PAGE and subjected to Western blot analysis using anti-Xpress tag antibody.

Recombinant GST-IκBα, GST-IκBβ, and His_6-POZ proteins were prepared as described above using the pGEX4T3-IκBα, pGEX4T3-IκBβ, and pQE30-His-POZ expression plasmids, respectively. For the *in vitro* protein-protein interaction assay, 9 μg of purified GST-IκBα or GST-IκBβ and GST were bound to GSH-agarose for 1 h in 500 μl of TNE buffer at 4 °C. Agarose-bound GST or GST fusion protein was collected by centrifugation at 3000 × *g* for 1 min at 4 °C and washed four times with cold TNE buffer. Agarose-bound GST protein was incubated with His_6-POZ in TNE buffer for 1 h at 4 °C. After the agarose-protein complex was washed three times with cold TNE buffer, the bound proteins were resolved by 15% SDS-PAGE and subjected to Western blot analysis using anti-His tag antibody.

Cell Culture and Transient Transfection—HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. For the transient transfection study, HeLa cells were seeded onto 6-well plates and grown for 24 h before transfection with a

total of 1 μg of DNA (0.6 μg of pELAM-Luc, 0.2 μg of FBI-1 or POZ domain expression plasmid, and 0.2 μg of pCMV-β-gal) using Lipofectamine Plus reagent (Invitrogen). Cells were harvested 48 h after transfection, and TNFα (20 ng/ml) or PMA (250 ng/ml) was introduced 4 h before harvesting the cells. Cells were lysed in reporter lysis buffer, and luciferase activities were measured using the luciferase assay system (Promega). Luciferase activity was normalized to β-galactosidase activity.

To investigate whether the POZ domain of FBI-1 is sufficient to affect the cellular localization of the p65 subunit of NF-κB and to activate transcription of the E-selectin promoter, HeLa cells were transfected with a total of 1.2 μg of DNA (0.2 μg of pELAM-Luc, 0.4 μg of POZ or POZ_{NLS} domain expression plasmid, 0.2 μg of pCMV-β-gal, and the appropriate amount of pcDNA3.0 to make a total of 1.2 μg of DNA) using Lipofectamine Plus reagent as described above.

Immunostaining—For immunostaining, PMA-stimulated or non-stimulated HeLa cells were grown on coverslips (Sunshine Works, Seoul, Korea), washed with cold PBS, and fixed in 99:3 cold methanol/formaldehyde for 20 min at −20 °C. Cells were permeabilized in 0.2% Triton X-100 for 10 min at room temperature and then washed three times for 10 min each with PBS. Cells were incubated in 5% normal goat serum (Invitrogen) for 30 min at room temperature. After blocking, the cells were incubated in a wet chamber with rabbit anti-POZ domain primary antibody (diluted to a final concentration of 5 μg/ml) in an incubation solution of 1% bovine serum albumin and 0.02% sodium azide in PBS for 2 h at room temperature. Cells were then rinsed three times for 10 min each with the incubation solution and further incubated with rhodamine-conjugated anti-rabbit IgG secondary antibody (diluted to 5 μg/ml; Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. For double staining, after the cells were washed with PBS, they were incubated with 3.7% formaldehyde. The cells were then incubated with different antibodies (anti-p65 subunit primary antibody (diluted to a final concentration of 5 μg/ml) and FITC-conjugated anti-mouse IgG secondary antibody) and washed three times for 10 min at room temperature, with the final wash containing 1 mg/ml 4′,6-diamidino-2-phenylindole. Cells were mounted onto slide glasses with mounting medium (90% (v/v) glycerol, 1 mg/ml *p*-phenylenediamine, and 0.02% sodium azide) and examined with a Carl Zeiss LSM 510 confocal laser scanning microscope.

Immunoprecipitation and Western Blot Analysis—One microgram of anti-p65 subunit polyclonal antibody, anti-IκBα or anti-IκBβ antibody, or control anti-IgG antibody was incubated overnight at 4 °C with 1 mg of cell extract prepared using lysis buffer (20 mM Tris (pH 7.9), 1.0% Triton X-100, 1 mM Na_3VO_4, 137 mM NaCl, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM sodium orthovanadate, 1 mM EGTA, 10 mM NaF, 1 mM tetrasodium pyrophosphate, 5 mM EDTA, 10% glycerol, 1 mM glycerophosphate, 0.1 g/ml *p*-nitrophenyl phosphate, and 0.2 mM phenylmethylsulfonyl fluoride). Forty microliters of a 1:1 suspension of protein A-Sepharose beads were added to the cell lysate and incubated for 2 h at 4 °C with gentle rotation. The beads were pelleted and washed three times with cell lysis buffer. Bound proteins were dissociated by boiling the samples in 1× PAGE sample buffer (12 mM Tris-HCl, 5% glycerol, 0.4% SDS, 2.88 mM mercaptoethanol, and 0.02% bromphenol blue). The whole cell lysates were separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Biosciences). The membranes were blocked in TBST buffer (20 mM Tris (pH 7.6), 137 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk for 1 h and then incubated overnight at 4 °C in 5% nonfat dry milk containing anti-POZ domain antibody. The membrane was washed several times with TBST buffer and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody. After 1 h, the blot was washed several times with TBST buffer and developed with ECL reagents (Amersham Biosciences).

RESULTS

FBI-1 Interacts with the p65 Subunit of NF-κB—We screened a human liver Matchmaker® yeast LexA two-hybrid cDNA library using the LexA-POZ domain fusion protein as bait. We isolated the p65 subunit of NF-κB as one of the potential proteins interacting with the POZ domain. Accordingly, we investigated whether FBI-1 can interact with NF-κB by *in vivo* co-immunoprecipitation and *in vitro* GST fusion protein pull-down assay. We transiently transfected HeLa cells with either the pcDNA3.0-FBI-1 overexpression plasmid or the mock expression plasmid (pcDNA3.0). We immunoprecipitated cell extracts with anti-p65 subunit antibody, and precipitants were

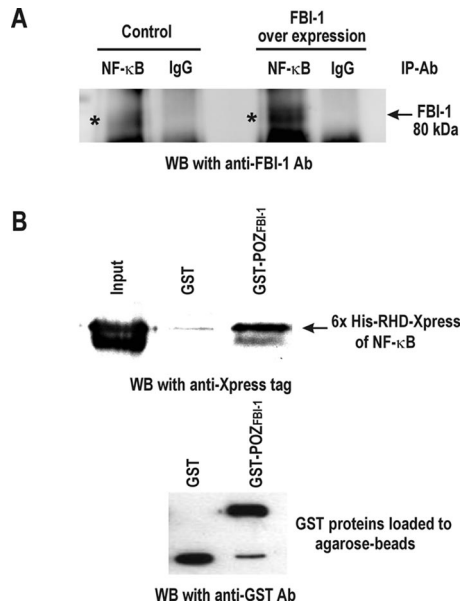

FIG. 1. **The POZ domain of FBI-1 interacts with the RHD of the p65 subunit of NF-κB.** *A*, co-immunoprecipitation of NF-κB and FBI-1. Cell lysates prepared from mock-transfected HeLa cells or HeLa cells transfected with the FBI-1 expression plasmid were immunoprecipitated (*IP*) with anti-p65 subunit antibody (*Ab*) and analyzed by Western blotting (*WB*) using FBI-1 antibody. FBI-1 interacted with the p65 subunit *in vivo*, and overexpression of FBI-1 resulted in a stronger interaction. The *asterisks* indicate the FBI-1 band at 80 kDa. *B*, *in vitro* GST fusion protein pull-down assay. GST or GST-POZ_{FBI-1} bound to agarose beads was incubated with His_6- and Xpress-tagged RHD, pulled down, and analyzed by Western blotting using anti-Xpress tag antibody. GST-POZ interacted with the RHD. However, GST did not bind the RHD. The same blot was also analyzed by Western blotting using anti-GST antibody to show equal loading of GST and GST-POZ in the binding reactions. This assay was repeated three times.

separated on a 12% SDS-polyacrylamide gel and analyzed by Western blotting using anti-FBI-1 antibody. p65 interacted with FBI-1, and overexpression of FBI-1 led to a stronger FBI-1 band at 75–80 kDa (Fig. 1*A*).

To investigate whether the protein-protein interaction between the POZ domain of FBI-1 and p65 is direct, we performed an *in vitro* GST fusion protein pull-down assay. Recombinant GST or GST-POZ was allowed to interact with His_6-RHD-Xpress, pulled down, and analyzed by Western blotting using anti-Xpress tag antibody. Our data show that the POZ domain of FBI-1 and the RHD of the p65 subunit interacted directly (Fig. 1*B*).

FBI-1 Enhances NF-κB-mediated Transcription of the E-selectin Promoter in HeLa Cells Stimulated with Either TNFα or PMA, and the POZ Domain of FBI-1 Represses Transcription—The molecular interaction between FBI-1 and the p65 subunit of NF-κB suggests that FBI-1 may play a role in NF-κB-mediated transcription activation. To investigate this possibility, we tested the effect of overexpression of FBI-1 and the POZ domain (the FBI-1 functional domain interacting with NF-κB) on NF-κB-mediated transcription using a luciferase reporter assay. After 44 h of cotransfection of HeLa cells with the NF-κB-responsive E-selectin promoter-luciferase reporter gene fusion plasmid (Fig. 2*A*) (30) and the FBI-1 or POZ domain expression plasmid, cells were stimulated with either TNFα or PMA for 4 h and harvested. In mock-transfected cells, reporter activity was increased by 6–10-fold due to the movement of NF-κB by the stimulants, as reported by others (40). Interestingly, full-length FBI-1 further enhanced transcription by 1.7–2-fold compared with the stimulated control (Fig. 2, *B* and *C*, *bars 2* and *3*). We overexpressed the POZ domain of FBI-1, which lacks the NLS and which is located only in the cytoplasm, and found that the domain repressed transcription by >70% compared with

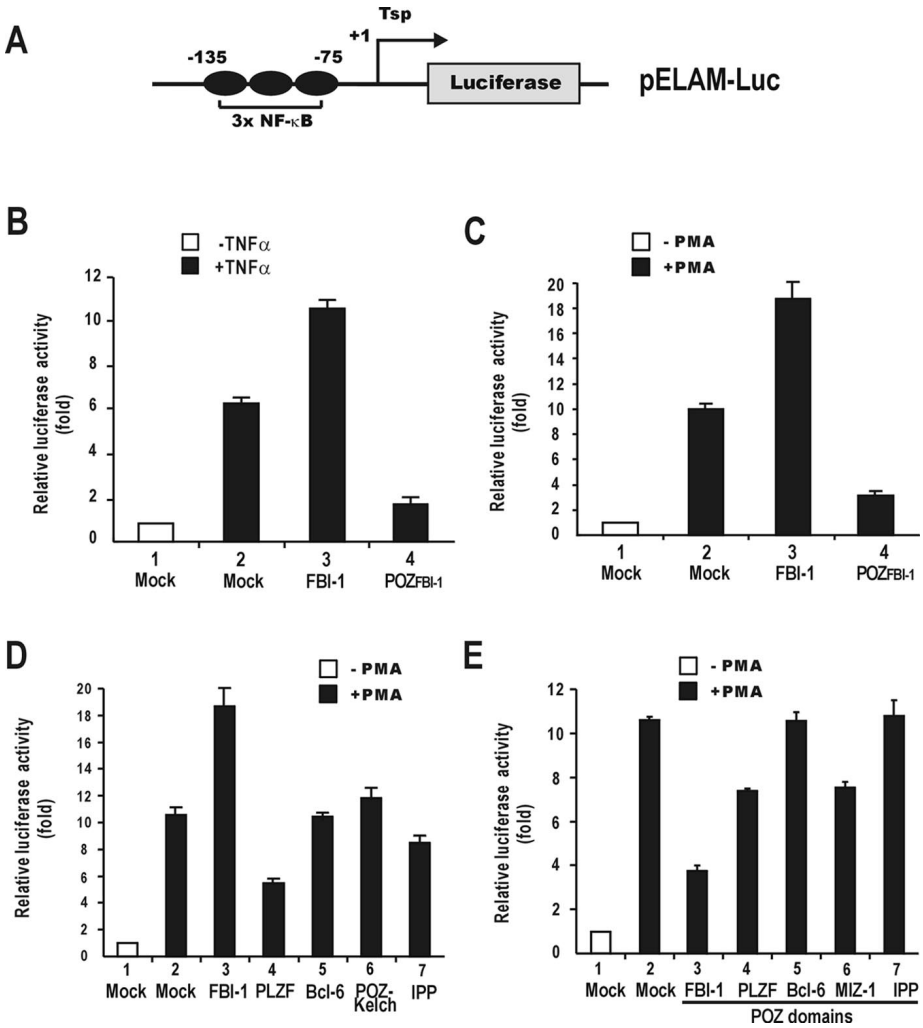

FIG. 2. **FBI-1 enhances and the POZ domain represses NF-κB-mediated E-selectin gene expression in HeLa cells stimulated with either TNFα or PMA.** *A*, shown is the structure of the NF-κB-responsive E-selectin gene promoter-luciferase gene fusion construct (pELAM-Luc). Three copies of the NF-κB-responsive elements located at bp −135 to −75 are indicated by *ovals*. *Tsp*, transcription start point (position +1). *B*, HeLa cells were transfected with the pELAM-1-Luc reporter plasmid and with an expression plasmid for the POZ domain (POZ_{FBI-1}) or full-length FBI-1. After 24 h, cells were treated with TNFα for 4 h. FBI-1 enhanced transcription, and the POZ domain repressed transcription. *C*, HeLa cells were treated as described for *B*, but the cells were treated with PMA. The results were the same as those shown in *B*. *D*, FBI-1 enhanced transcription, but other POZ domain proteins either repressed or had no effect on transcription of the pELAM E-selectin promoter. HeLa cells were cotransfected with the expression plasmids for the POZ domain proteins containing either zinc fingers (PLZF and Bcl-6) or Kelch repeats (Kelch protein and IPP) and the pELAM-1-Luc reporter plasmid and stimulated with PMA for 4 h. *E*, the POZ domains of FBI-1, PLZF, and MIZ-1 repressed transcription, but those of Bcl-6 and IPP did not upon stimulation with PMA for 4 h. *Error bars* represent S.D. Luciferase activity was normalized to β-galactosidase activity and is shown as the mean ± S.D. of three independent experiments.

the control mock-transfected cells treated with TNFα or PMA (Fig. 2, *B* and *C*, *bars 4*).

We then investigated whether the regulatory property of FBI-1 for NF-κB-mediated transcription is unique to FBI-1 or whether the property is generally applicable to other regulatory proteins with a POZ domain. We cotransfected HeLa cells with expression plasmids for various POZ domains or with full-length expression plasmids for PLZF, Bcl-6, MIZ-1 (Myc-interacting zinc finger protein-1), IPP (an actin-binding protein, murine intracisternal A-particle-promoted placenta-expressed protein; Swiss-Prot accession number P28575), and the NF-κB-responsive E-selectin promoter-reporter plasmid. Bcl-6, PLZF, and MIZ-1 have POZ domains at their N termini and zinc fingers at their C termini and are well characterized transcription factors (4, 5, 31). IPP has a POZ domain at its N terminus and Kelch repeats at its C terminus, which are critical in actin binding (32). Full-length PLZF and IPP repressed transcription by 50 and 23%, respectively (Fig. 2*D*, *bars 4* and *7*). However, other POZ domain proteins such as Bcl-6 and Kelch did not show any effect on transcription and did not affect transcription activation triggered by PMA. The POZ domains of PLZF and MIZ-1 repressed reporter activity by 30% (Fig. 2*E*, *bars 4* and *6*). However, the POZ domains of Bcl-6 and IPP did not show repression (Fig. 2*E*, *bars 5* and *7*). In general, the other POZ domains showed either weak or no repression, and the POZ domain of FBI-1 showed the most potent repression, suggesting that some POZ domains have variable degrees of regulatory properties for NF-κB-mediated transcription and that some POZ domains do not have transcription repressor activity.

These data suggest that the transcription enhancement by FBI-1 of the NF-κB-responsive E-selectin promoter is unique to FBI-1. Although some POZ domains showed repressive effects similar to the POZ domain of FBI-1, their activities in the full-length versions were different, and none of them enhanced transcription of the E-selectin promoter. PZLF repressed transcription both in the POZ domain form and in the full-length version. IPP did not affect transcription in the POZ domain form, but slightly repressed transcription in the full-length form. Bcl-6 did not show any effect in either form.

Full-length FBI-1 Antagonizes Transcription Repression by IκB Proteins, and the POZ Domain of FBI-1 Cooperates with IκBα or IκBβ in Transcription Repression—The POZ domain of FBI-1 interacted with the p65 subunit, and full-length FBI-1 enhanced transcription of the NF-κB-responsive E-selectin gene promoter. In contrast, the POZ domain repressed transcription of the same NF-κB-responsive E-selectin gene and, in this respect, acted as a dominant-negative mutant of full-length FBI-1. We were puzzled by this observation and tried to address how the molecular interaction among the POZ domain of FBI-1, NF-κB, and IκB affects the transcription process that involves NF-κB. As the molecular interaction between IκBα or IκBβ and NF-κB in the cytosol and the molecular events affecting the interaction are important in the nuclear localization of NF-κB and transcription activation, we investigated the relationship among the three factors (IκB proteins, the POZ domain, and NF-κB) by overexpressing each component and analyzing transcription of the NF-κB-responsive E-selectin gene. PMA treatment greatly activated transcription, and FBI-1 fur-

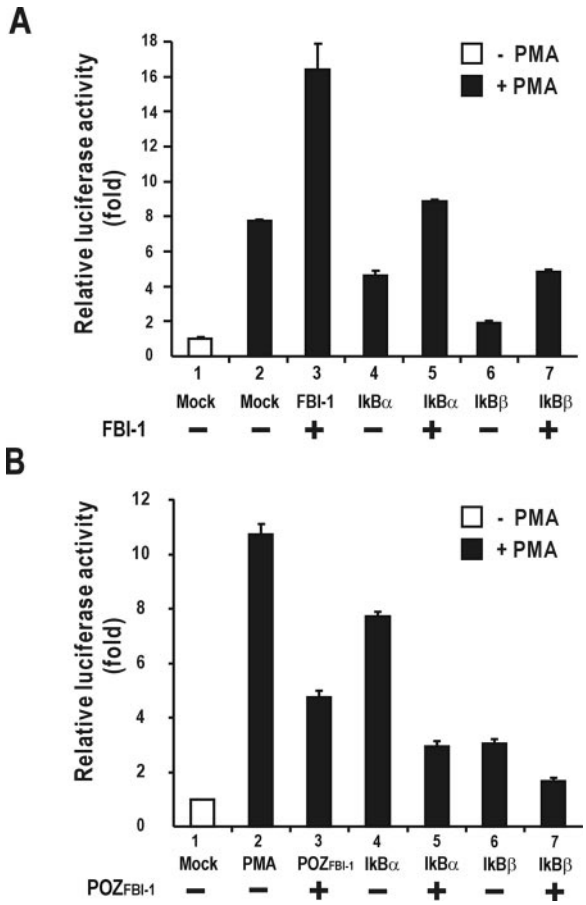

FIG. 3. **FBI-1 reverses transcription repression by IκBα or IκBβ.** The POZ domain of FBI-1 further enhanced transcription repression by IκBα and IκBβ. *A*, HeLa cells were transfected with the pELAM-1-Luc reporter plasmid and with an expression plasmid for IκBα or IκBβ in the presence or absence of FBI-1. After 24 h, cells were treated with PMA for 4 h before harvest and analyzed for luciferase reporter activity. *B*, cells were treated as described for *A*, but with a POZ domain (POZ_{FBI-1}) expression plasmid instead of FBI-1. Luciferase activity was normalized to β-galactosidase activity and is shown as the mean ± S.D. of three independent experiments.

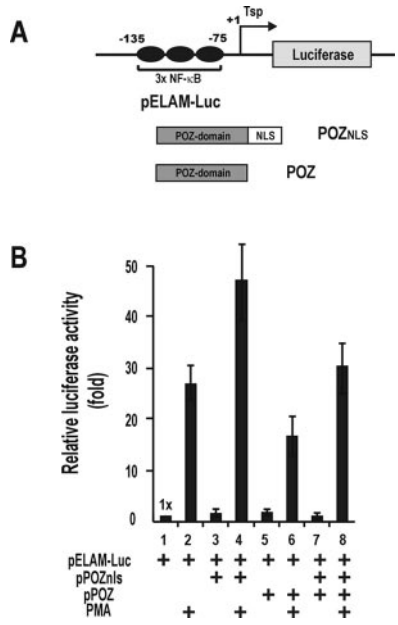

FIG. 4. **Transcription enhancement of the NF-κB-responsive E-selectin gene by the POZ_{NLS} domain.** *A*, shown is the structure of expression plasmids for the POZ domain, the POZ_{NLS} domain, and the pELAM-Luc reporter. *Tsp*, transcription start point (position +1). *B*, HeLa cells were transfected with pELAM-Luc and an expression plasmid for the POZ or POZ_{NLS} domain. After 24 h, cells were treated with PMA for 4 h. The POZ_{NLS} domain enhanced transcription, and the POZ domain repressed transcription enhancement by the POZ_{NLS} domain. Luciferase activity was normalized to β-galactosidase activity and is shown as the mean ± S.D. of three independent experiments.

ther increased transcription by 2-fold (Fig. 3*A*, *bars 2* and *3*). FBI-1 may increase nuclear import or stay free of NF-κB induced by PMA treatment. The transcription of mock-transfected cells (transfected only with the pELAM-Luc plasmid and treated with PMA) was repressed by the presence of IκBα or IκBβ, but this repression was reversed by cotransfection with the FBI-1 expression plasmid. FBI-1 significantly relieved transcription repression by IκBα and IκBβ by 26.7 and 18%, respectively, in the presence of PMA (Fig. 3*A*, *bars 4–7*), suggesting that, although ectopic IκB proteins inhibited transcription activation by PMA, FBI-1 probably affected the cellular localization of NF-κB freed by PMA treatment and enhanced transcription.

We next investigated the function of the POZ domain itself. The POZ domain, IκBα, and IκBβ were able to repress transcription by 56, 28, and 74%, respectively (Fig. 3*B*, *bars 3*, *4*, and *6*). Transcription repression by IκBα and IκBβ was further intensified by 74 and 85%, respectively, by the overexpressed POZ domain, suggesting a cooperative role in transcription repression (Fig. 3*A*, *bars 5* and *7*). The data suggest that the POZ domain most likely represses transcription via a mechanism similar to that of IκB proteins.

Full-length FBI-1 has an NLS, whereas the POZ domain does not, and this difference may explain the opposite behaviors in transcription of the NF-κB-responsive gene. Our results suggest that the molecular interaction between the POZ domain of FBI-1 and the p65 subunit of NF-κB affects NF-κB-mediated gene transcription. Full-length FBI-1 may increase the nuclear localization of NF-κB, whereas the POZ domain of FBI-1 may interact with and trap NF-κB in the cytoplasm, just like IκB proteins.

Therefore, we prepared a plasmid vector (pcDNA3.0-POZ_{NLS}) expressing the POZ domain fused to the NLS of FBI-1 (amino acids 487–505, KDGCNGVPSRRGRKPRVRG) and tested whether the POZ_{NLS} domain can, like full-length FBI-1, enhance transcription of the NF-κB-responsive E-selectin promoter (Fig. 4*A*) (9). Indeed, the POZ_{NLS} domain enhanced transcription by ~1.7-fold in the presence of PMA, just like FBI-1, whereas the POZ domain with no NLS repressed transcription by 40% (Fig. 4*B*). The presence of the POZ domain reduced transcription enhancement by the POZ_{NLS} domain to the POZ domain-only level (Fig. 4*B*, *bar 8*). This suggests that the POZ domain is the functional domain important both in protein-protein interactions with the p65 subunit and in transcription enhancement by FBI-1.

Transcription Activation of NF-κB by FBI-1 Is Inhibited by the NF-κB Inhibitors PDTC and MG132—We investigated whether FBI-1 transcription enhancement of NF-κB-responsive genes can be directly suppressed by inhibitors of NF-κB. MG132 and the antioxidant PDTC are inhibitors of NF-κB that work at the level of the proteasome and NF-κB, respectively. Cells transfected with the FBI-1 expression plasmid showed ~2–3-fold higher luciferase activity, even in the absence of PMA stimulation, compared with control cells (Fig. 5, *A* and *B*, *bars 1* and *5*). Treatment of the cells with PDTC or MG132 resulted in repression, probably by inhibiting low levels of free NF-κB (Fig. 5, *A* and *B*, *bars 2* and 6). PMA stimulation resulted in strong transcription activation (6-fold increase), and FBI-1 further enhanced transcription by 2–2.6-fold (Fig. 5, *A* and *B*, *bars 3* and *7*). Transcription activation by the combined actions of PMA and FBI-1 was completely reversed to the basal

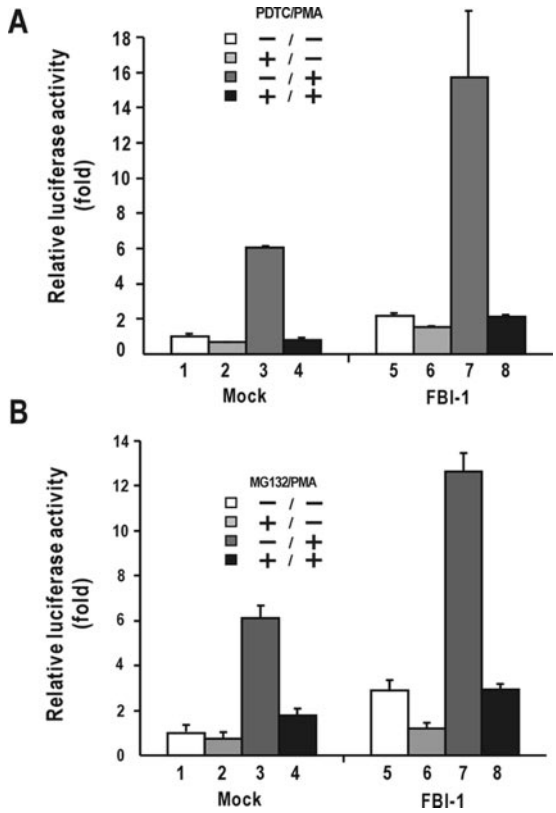

FIG. 5. **Transcription enhancement of the NF-κB-responsive E-selectin gene by FBI-1 can be efficiently blocked by the NF-κB inhibitors PDTC and MG132.** HeLa cells were cotransfected with pELAM-Luc and FBI-1 expression plasmids, pretreated for 1 h with PDTC (*A*) or for 6 h with MG132 (*B*), and stimulated with PMA for 4 h before harvest. *A*, PDTC treatment lowered transcription in both the absence and presence of FBI-1 and PMA. *B*, MG132 treatment also lowered transcription in the absence and presence of FBI-1 and PMA. Luciferase reporter activity was normalized to β-galactosidase activity and is shown as the mean ± S.D. of three independent experiments.

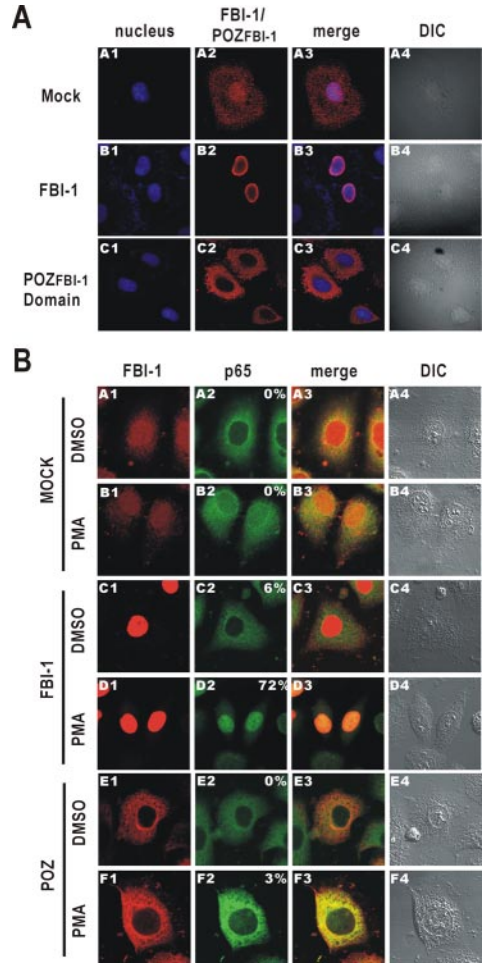

FIG. 6. **Cellular localization of FBI-1 and the POZ domain.** *A*, FBI-1 localized mainly in the nucleus (*panel A2*). The POZ domain localized entirely in the cytoplasm and trapped endogenous FBI-1 in the cytoplasm as well (*panel C2*). HeLa cells were transfected with the indicated plasmids for either FBI-1 or the POZ domain of FBI-1 (POZ_{FBI-1}). Cells were incubated with rabbit anti-POZ domain primary antibody and further incubated with rhodamine-conjugated anti-rabbit IgG secondary antibody. The cell nuclei were stained with 4′,6-diamidino-2-phenylindole. The merged images of cells stained with 4′,6-diamidino-2-phenylindole and rhodamine are shown. *DIC*, differential interference contrast optic images of the cells. *B*, HeLa cells were transfected with the indicated expression plasmids for FBI-1 or the p65 subunit of NF-κB. The cells were incubated with rabbit anti-POZ domain antibody and further incubated with rhodamine-conjugated anti-rabbit IgG secondary antibody. The cells were also incubated with mouse anti-p65 subunit primary antibody and further incubated with FITC-conjugated anti-mouse secondary antibody. In most HeLa cells (>72%) transfected with FBI-1, the p65 subunit localized mainly in the nucleus after PMA stimulation (*panel D2*). In contrast, in mock-transfected cells or cells transfected with the POZ domain expression plasmid, a low level of nuclear p65 (*panel B2*) or none (*panel F2*) was detected after PMA stimulation. The merged images of cells stained with FITC and rhodamine are shown. *DMSO*, dimethyl sulfoxide.

level by treatment with PDTC or MG132 (Fig. 5, *A* and *B*, *bars 4* and *8*). Our data suggest that FBI-1 enhanced the NF-κB-responsive E-selection gene via modulation of NF-κB activity, not by direct activation of the E-selection promoter. It appears that FBI-1 may enhance the transcription of NF-κB-responsive genes by modulating NF-κB, by increasing either the localization or stability of NF-κB in the nucleus.

FBI-1 Increases Translocation of the p65 Subunit of NF-κB into the Nucleus—Our transient transfection assays suggested that FBI-1 might enhance transcription by modulating the localization or stability of NF-κB in the nucleus because NF-κB inhibitors working at the level of NF-κB itself or of its proteasome repressed NF-κB-responsive gene transcription enhanced by FBI-1. We investigated this possibility by monitoring the movement of the p65 subunit of NF-κB, the POZ domain, and FBI-1 by immunohistochemical staining of each protein under a confocal microscope.

First, to determine the cellular localization of the POZ domain and endogenous and overexpressed FBI-1, HeLa cells overexpressing the POZ domain or FBI-1 were incubated with affinity-purified rabbit anti-POZ domain antibody, followed by detection with rhodamine-conjugated anti-rabbit secondary antibody. In control mock-transfected cells, FBI-1 localized mainly in the nucleus, and a low level of FBI-1 was detected in the cytoplasm (Fig. 6*A*, *panel A2*). Overexpressed FBI-1 was located in the nucleus (Fig. 6*A*, *panel B2*) (8). The POZ domain with no NLS localized mainly in the cytoplasm; the overexpressed POZ domain changed the localization of endogenous FBI-1, trapping FBI-1 in the cytoplasm. In this respect, the POZ domain acted as a dominant-negative form of FBI-1 (Fig. 6*A*, *panel C2*). Because the cellular localization of the POZ domain and FBI-1 was not changed by treatment with the NF-κB stimulant PMA (Fig. 6*B*, *panels B1*, *D1*, and *F1*), FBI-1 might play a role in the nuclear localization of the p65 subunit of NF-κB molecules freed by PMA treatment.

The nuclear translocation of NF-κB is a key regulatory step in the transcription activation of NF-κB-responsive genes. We suspected that FBI-1 might affect the localization of NF-κB and thereby enhance transcription. We investigated the cellular localization of p65 after transient overexpression of FBI-1 and

the POZ domain to study cellular movement of the proteins. The POZ domain altered the cellular localization of FBI-1 and acted as a dominant-negative mutant for FBI-1 in terms of cellular localization. The cells overexpressing the POZ domain or FBI-1 were incubated with rabbit anti-POZ domain primary antibody, followed by incubation with rhodamine-conjugated anti-rabbit IgG secondary antibody. Cells were also incubated with specific mouse primary antibodies against the NF-κB p65 subunit and then further incubated with FITC-conjugated anti-mouse IgG secondary antibody. In mock-transfected cells, p65 localized in the cytoplasm, and upon stimulation with PMA, p65 was detected in both the cytoplasm and nucleus (Fig. 6*B*, *panels A2* and *B2*). No cell showed intense nuclear localization of p65 in the cells overexpressing FBI-1; p65 localized mainly in the cytosol, as in the control, whereas its concentration might have been slightly increased in the nucleus. However, upon stimulation with PMA, most of the p65 subunit localized intensely in the nucleus, even after 4 h of PMA treatment, in the majority (72%) of cells overexpressing FBI-1 (Fig. 6*B*, *panel D2*). Although p65 existed both in the nucleus and cytoplasm of control PMA-treated cells, most of the p65 subunit was located in the nucleus in cells overexpressing FBI-1. We also examined the cellular localization of p65 in cells expressing the POZ domain. p65 was detected only in the cytosol, even in the presence of PMA (Fig. 6*B*, *panel F2*), suggesting that the POZ domain trapped free NF-κB in the cytosol.

Our data suggest that FBI-1 drastically increased the nuclear localization of the p65 subunit of NF-κB even after 4 h of PMA treatment and that the POZ domain prevented the nuclear localization of p65 (Fig. 6). This dramatic increase in the nuclear localization of p65 might explain the enhanced transcription of the NF-κB-responsive E-selectin gene by FBI-1 (Figs. 2–4).

FBI-1 Facilitates Nuclear Import of the p65 Subunit of NF-κB and Maintains High Levels of Nuclear p65 for a Prolonged Period—The increased nuclear localization of the p65 subunit by the combined action of FBI-1 and PMA may increase the transcription of NF-κB-responsive genes. The stability of p65 in the nucleus is also important. Other researchers have reported that nuclear NF-κB reaches a maximum level at 30 min after TNFα stimulation and gradually decreases to basal levels over a 1-h period (33). Under conditions of persistent stimulation, nuclear NF-κB levels oscillate and eventually decrease to about half of the maximum level (33). As our experiments were carried out under conditions of persistent stimulation with PMA, we investigated, by confocal microscopy, whether overexpressed FBI-1 changes the level of p65 in the nucleus over a 4-h period. In our control experiments, the NF-κB level in the nucleus reached its peak at 30 min, quickly decreased over 1–2 h, and decreased to the basal level by 4 h, and very little of the p65 subunit was detected in the nucleus (Fig. 7*A*, *panels A2–F2*). Interestingly, in HeLa cells transfected with FBI-1, the nuclear concentration of the p65 subunit was already very high even at 15 min, and this high level was maintained over a 4-h period in most of the cells overexpressing FBI-1. More than 92% of the cells overexpressing FBI-1 maintained high nuclear p65 levels over a period of 15 min to 1 h. In the majority of cells (>59%) overexpressing FBI-1, p65 still maintained a high level of nuclear localization without a significant reduction over a 1–4-h period (Fig. 7*B*). This observation is quite different from the changes in p65 subunit localization observed in the control cells, where most of the nuclear p65 subunit levels drastically decreased over a 1–4-h period (Fig. 7*A*). The fast nuclear import and lasting nuclear retention (Fig. 7*C*) of p65 subunits by FBI-1 may explain the more potent transcription activation by NF-κB in the presence of PMA and

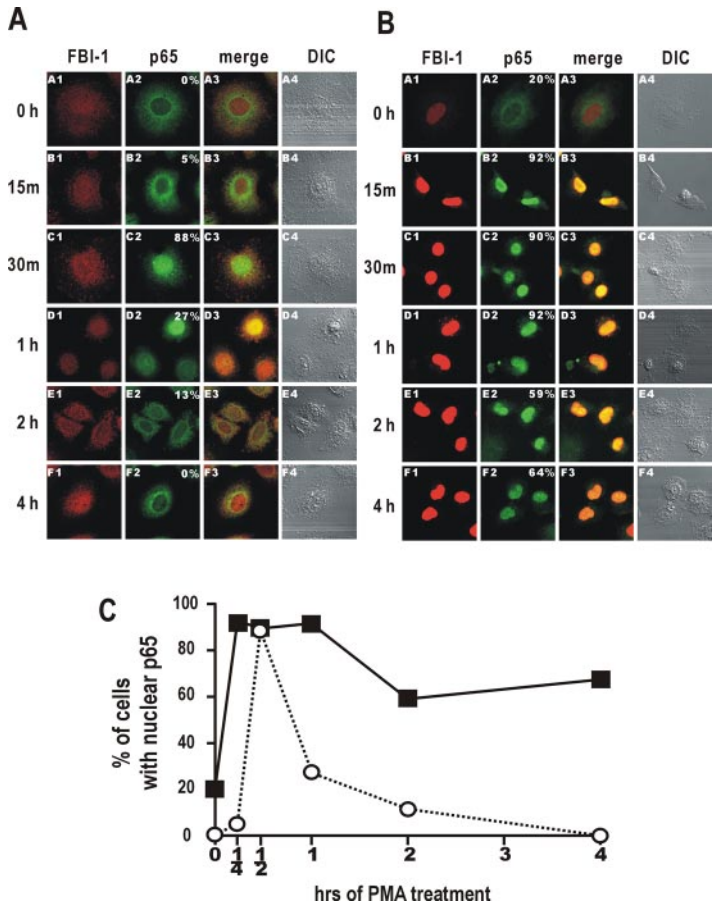

FIG. 7. **The p65 subunit of NF-κB is quickly moved into the nucleus and is stable in the presence of FBI-1 upon persistent PMA stimulation.** Shown are the results from a time course experiment of p65 subunit nuclear translocation. *A*, mock-transfected cells were stimulated with PMA for the indicated treatment times and fixed at various time points. The cells were stained with both rabbit antibody against the POZ domain and mouse antibody against the p65 subunit. At 15 min (*m*), p65 was detected in the nuclei of 5% of the cells. At 30 min, most of the cells (88%) showed clear nuclear p65. The p65 levels decreased rapidly over 1–2 h, and only 13–27% of the cells showed nuclear p65 during this time period. Virtually no cells showed nuclear p65 at 4 h. The merged images of cells stained with FITC and rhodamine staining are shown. *DIC*, differential interference contrast optic images of the cells. *B*, cells were transiently transfected with the FBI-1 expression plasmid and stimulated with PMA as described for *A*. Even at 15 min, in 92% of the cells overexpressing FBI-1, p65 subunits were detected in the nuclei, suggesting a fast and nearly complete movement of p65 into the nuclei. Over a period of 15 min to 1 h, >90% of the cells showed nuclear p65. At 4 h, the majority of the cells (>59%) still showed nuclear p65. *C*, the diagram shows the percentage of cells with nuclear p65 subunits at various time points of PMA treatment. ■, percentage of cells overexpressing FBI-1 and with nuclear p65 subunits of NF-κB; ○, percentage of cells with nuclear p65 subunits in mock-transfected cells. Cells were incubated with PMA for up to 4 h and stained with antibodies against FBI-1 and p65.

FBI-1 compared with the mock-transfected cells (Fig. 2, *B* and *C*, *bar 3*). This novel function of FBI-1 may be important in the NF-κB-mediated transcription activation of many genes in tissue or pathological states, where the expression of FBI-1 is increased.

FBI-1 Interacts with IκBα and IκBβ in Vivo and in Vitro—The RHD of NF-κB interacted with IκB and with the POZ domain of FBI-1. Accordingly, we investigated whether the POZ domain interacts with IκB by yeast LexA two-hybrid and GST fusion protein pull-down assays. We cloned a human IκBβ cDNA into the pB42AD vector and cotransformed pLexA-POZ_{FBI-1} and pB42AD-IκBβ into competent yeast strain EGY48(p8op-lacZ). Cotransformed yeast was grown on syn-

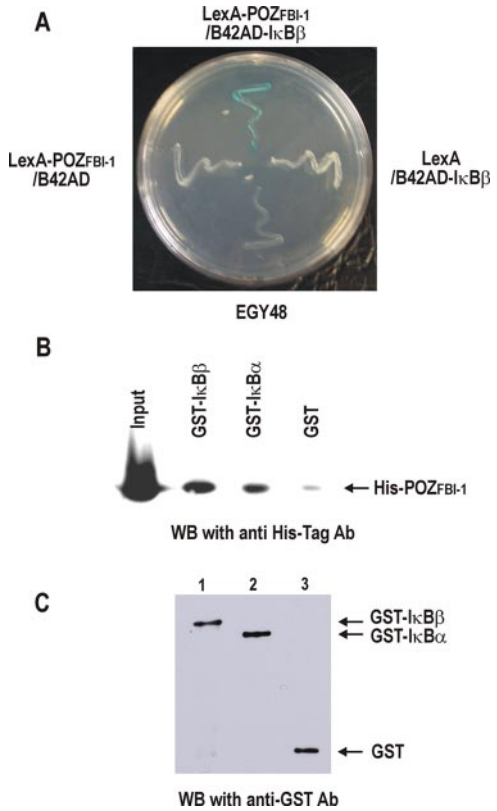

FIG. 8. **The POZ domain of FBI-1 interacts with IκBα or IκBβ.** *A*, yeast two-hybrid assay. The POZ domain cDNA was cloned into the pLexA vector, and IκBβ cDNA was cloned into the pB42AD vector. The cotransformants of pLexA-POZ_{FBI-1}/pB42AD and pLexA/pB42AD-IκBβ showed no protein-protein interaction (white colony formation), but the cotransformant of pLexA-POZ_{FBI-1} and pB42AD-IκBβ showed protein-protein interaction (blue colony formation). *B*, *in vitro* GST fusion protein pull-down assay. GST, GST-IκBα, and GST-IκBβ bound to agarose beads were incubated with His_6-POZ and pulled down. Western blot (*WB*) analysis of the precipitants with anti-His tag antibody (*Ab*) showed that GST-IκBα and GST-IκBβ pulled down His_6-POZ (His-POZ_{FBI-1}), suggesting a direct protein-protein interaction between IκBα or IκBβ and the POZ domain of FBI-1. In contrast, GST (negative control) did not pull down His_6-POZ. *C*, Western blot analysis of the same blot using anti-GST antibody showing that the same amounts of GST, GST-IκBα, and GST-IκBβ were used in the pull-down reactions.

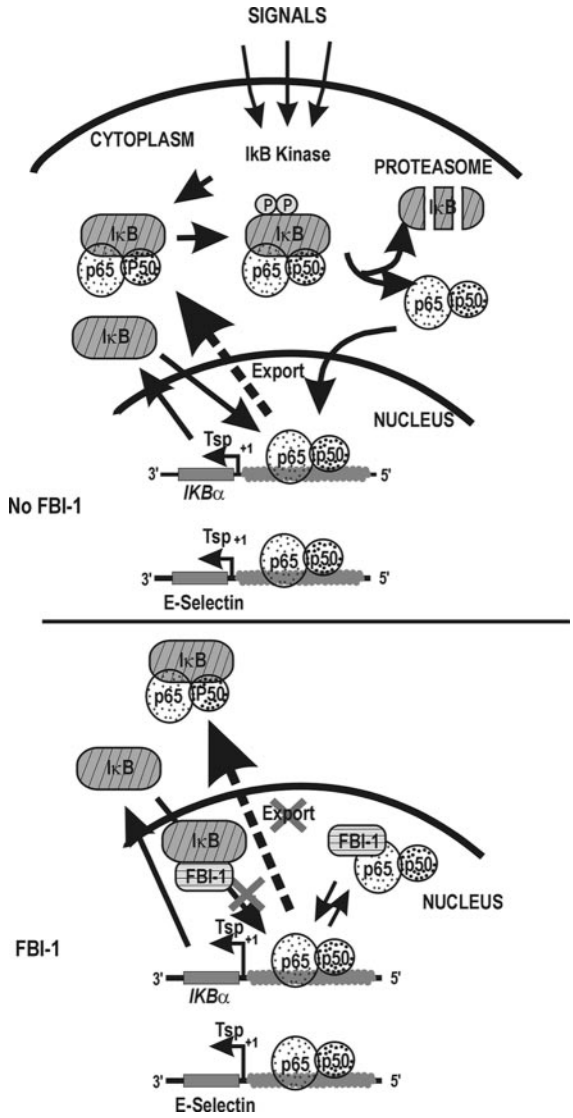

FIG. 9. **Hypothetical model of the transcription enhancement of the NF-κB-responsive gene by FBI-1.** NF-κB exists in the cytoplasm as an inactive form bound to IκB. Upon stimulation with various signals, IκB is phosphorylated by IκB kinase and is further degraded by proteasomes. Freed NF-κB, including the p65 subunit, moves quickly into the nucleus and activates the transcription of a variety of genes, including the IκBα gene. Newly synthesized IκB in the cytoplasm enters the nucleus, binds NF-κB, dissociates DNA-bound NF-κB, and exports NF-κB out of the nucleus in the absence of FBI-1. However, in the presence of FBI-1 in the nucleus, the POZ domain of FBI-1 may interact with the RHD of the p65 subunit, make p65 move into the nucleus quickly and stay longer in the nucleus either by blocking nuclear export or increasing stability, and enhance transcription by NF-κB. FBI-1 may also interact with IκB proteins; prevent IκB proteins from interacting with NF-κB; and retain NF-κB in the nucleus for prolonged periods, eventually enhancing transcription by NF-κB. *Tsp*, transcription start point (position +1).

thetic dextrose/Gal/Raf/−His/−Trp/−Ura + X-gal and showed strong β-galactosidase activity (blue colony formation), suggesting a protein-protein interaction between the POZ domain of FBI-1 and IκBβ. In contrast, the control cotransformants of pLexA-POZ_{FBI-1}/pB42AD and pLexA/pB42AD-IκBβ showed no protein-protein interaction (white colony formation) (Fig. 8*A*).

We also tested whether the protein-protein interaction between the POZ domain and IκBβ is direct by GST fusion protein pull-down assay. Recombinant GST or GST-IκBβ was incubated with His_6-POZ, pulled down, and analyzed by Western blotting using anti-His tag antibody. The POZ domain and IκBβ interacted directly. Another member of the IκB family, IκBα, also interacted directly with the POZ domain (Fig. 8*B*). The fact that the POZ domain directly interacted with both IκBα and IκBβ suggests that FBI-1 may regulate gene expression involving NF-κB probably by modulating the interaction between NF-κB and IκB proteins most likely in the nucleus, where FBI-1 is primarily located.

DISCUSSION

The POZ domain is a protein-protein interaction motif found in many regulatory proteins and is important in biological processes such as oncogenesis, signal transduction, apoptosis, and osteoclastogenesis (1–3). The regulatory proteins interacting with the POZ domain include histone deacetylases, N-CoR/SMRT, B-CoR (Bcl-6 co-repressor), and the Sp1 zinc finger (4, 5, 9). We have shown here, by co-immunoprecipitation and *in vitro* and *in vivo* protein-protein interaction assays, that the POZ domain of FBI-1 interacts with the RHD of the p65 subunit of NF-κB. The molecular interaction between the RHD and the POZ domain is important in increasing the nuclear localization of the p65 subunit and the resulting transcription activation of the NF-κB-responsive genes.

NF-κB plays important roles in many cellular processes such as development, cancer, apoptosis, immune responses, transformation, proliferation, invasion angiogenesis, and metastasis

of cancer (12–14, 34). Accordingly, controlling the activity of NF-κB is very important in cellular functions and is the target of intense research investigations. The most well known mechanism of controlling NF-κB is through control of the cellular localization of NF-κB by IκB proteins. IκB complexes with NF-κB, and NF-κB cannot then enter the nucleus because the NLS of NF-κB is masked by the interaction between IκB and NF-κB. Once IκB is degraded by ubiquitin-mediated processes, the repression is lifted, and NF-κB moves into the nucleus and activates transcription (12–15, 18). Newly synthesized IκBα accumulates in the cytoplasm but also in the nucleus, where it terminates NF-κB-dependent transcription. IκBα inhibits interactions between NF-κB and DNA and exports NF-κB back to the cytoplasm (19). There are many other examples of NF-κB activity regulation by reversible acetylation, deacetylation, and phosphorylation (21, 35–38).

FBI-1 markedly increased the localization and stability of NF-κB in the nucleus and thereby increased transcription of the NF-κB-responsive E-selectin gene. Cytoplasmic expression of the POZ domain lacking the NLS trapped NF-κB in the cytoplasm and inhibited transcription of the NF-κB-responsive gene. In contrast, the POZ_{NLS} domain (with the NLS sequence of FBI-1) acted just like FBI-1, enhancing transcription of the E-selectin promoter. FBI-1 may increase the expression of many cellular and viral genes that have NF-κB-responsive elements in their promoters by indirect mechanisms (Fig. 9). The nuclear retention of IκBα protects it from signal-induced degradation and inhibits NF-κB transcription activation (19, 22). Because the POZ domain of FBI-1 can also interact with IκBα or IκBβ, the interaction may inhibit IκB and prevent nuclear FBI-1 from being exported to the cytoplasm (Fig. 9). In this respect, FBI-1 may antagonize transcription repression by IκB in the nucleus by interaction with the RHD of the p65 subunit and IκB proteins via the N terminus of the POZ domain (Figs. 3 and 9). Our investigation may provide a novel mechanism of transcription activation that involves NF-κB, FBI-1, and possibly IκB.

We found that FBI-1 enhanced transcription most notably in the presence of PMA. This suggests that the primary function of FBI-1 was to trap the p65 subunit of NF-κB in the nucleus once the p65 subunit moved into the nucleus after the ubiquitin/proteasome-mediated degradation of IκB proteins. The ubiquitous transcription factor FBI-1 might also be important in maintaining the transcription of many genes with NF-κB-responsive elements by sustaining a low nuclear level of NF-κB in the absence of immunostimulant. Also, under conditions in which FBI-1 is highly expressed and immunostimulants exist, the potent transcription activation of NF-κB-responsive genes is possible due to the nuclear trapping of the p65 subunit by FBI-1 (Fig. 9).

Although the POZ domain used in our experiments does not exist in normal human cells, there are many proteins that contain only the POZ domain at their N termini. There are >300 POZ domain proteins, and although some of the proteins tested here did not show the same potent regulatory effect as FBI-1, some may possibly be involved in regulating NF-κB-mediated biological processes by controlling the cellular localization of the NF-κB family of proteins.

In particular, NF-κB activation mediates cellular transformation and proliferation, invasion angiogenesis, and metastasis of cancer (35). SAGE data from the NCBI Protein Database show that FBI-1 is highly expressed in carcinoma and adenocarcinoma cell lines. FBI-1 may play a role in cancer metastasis because FBI-1 potently increases the localization and stability of the p65 subunit of NF-κB in the nucleus and because FBI-1 increases the expression of NF-κB-responsive genes such as E-selectin, which was shown to be an important protein in tumorigenesis and metastasis.

REFERENCES

1. Albagli, O., Dhordain, P., Deweindt, C., Lecocq, G., and Leprince, D. (1995) *Cell Growth & Differ.* **6,** 1193–1198
2. Bardwell, V. J., and Treisman, R. (1994) *Genes Dev.* **8,** 1664–1677
3. Collins, T., Stone, J. R., and Williams, A. J. (2001) *Mol. Cell. Biol.* **21,** 3609–3615
4. David, G., Alland, L., Hong, S. H., Wong, C. W., DePinho, R. A., and Dejean, A. (1998) *Oncogene* **16,** 2549–2556
5. Huynh, K. D., and Bardwell, V. J. (1998) *Oncogene* **17,** 2473–2484
6. Pessler, F., Pendergrast, P. S., and Hernandez, N. (1997) *Mol. Cell. Biol.* **17,** 3786–3798
7. Morrison, D. J., Pendergrast, P. S., Stavropoulos, P., Colmenares, S. U., Kobayashi, R., and Hernandez, N. (1999) *Nucleic Acids Res.* **27,** 1251–1262
8. Pendergrast, P. S., Wang, C., Hernandez, N., and Huang, S. (2002) *Mol. Biol. Cell* **13,** 915–929
9. Lee, D.-K., Suh, D., Edenberg, H. J., and Hur, M.-W. (2002) *J. Biol. Chem.* **277,** 26761–26768
10. Davies, J. M., Hawe, N., Kabarowski, J., Huang, Q. H., Zhu, J., Brand, N. J., Leprince, D., Dhordain, P., Cook, M., Morriss-Kay, G, and Zelent, A. (1999) *Oncogene* **18,** 365–375
11. Kukita, A., Kukita, T., Ouchida, M., Maeda, H., Yatsuki, H., and Kohashi, O. (1999) *Blood* **94,** 1987–1997
12. May, M. J., and Ghosh, S. (1997) *Semin. Cancer Biol.* **8,** 63–73
13. Sen, R., and Baltimore, D. (1986) *Cell* **46,** 705–716
14. Ghosh, S., May, M., and Kopp, E. B. (1998) *Annu. Rev. Immunol.* **16,** 225–260
15. Ghosh, S., and Karin, M. (2002) *Cell* **109,** (suppl.) S81–S96
16. Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D., and Miyamoto, S. (1995) *Genes Dev.* **9,** 2723–2735
17. Phelps, C. B., Sengchanthalangsy, L. L., Huxford, T., and Ghosh, G. (2000) *J. Biol. Chem.* **275,** 29840–29846
18. Whiteside, S. T., and Israel, A. (1997) *Semin. Cancer Biol.* **8,** 75–82
19. Arenzana-Seisdedos, F., Thompson, J., Rodriguez, M. S., Bachelerie, F., Thomas, D., and Hay, R. T. (1995) *Mol. Cell. Biol.* **15,** 2689–2696
20. Tran, K., Merika, M., and Thanos, D. (1997) *Mol. Cell. Biol.* **17,** 5386–5399
21. Schmitz, M. L., Bacher, S., and Kracht, M. (2001) *Trends Biochem. Sci.* **26,** 186–190
22. Rodriguez, M. S., Thompson, J., Hay, R. T., and Dargemont, C. (1999) *J. Biol. Chem.* **274,** 9108–9115
23. Simeonidis, S., Stauber, D., Chen, G., Hendrickson, W. A., and Thanos, D. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 49–54
24. Brown, K., Franzoso, G., Baldi, L., Carlson, L., Mills, L., Lin, Y.-C., Gerstberger, S., and Siebenlist, U. (1997) *Mol. Cell. Biol.* **17,** 3021–3027
25. Kroll, M., Conconi, M., Desterro, M. J., Marin, A., Thomas, D., Friguet, B., Hay, R. T., Virelizier, J. L., Arenzana-Seisdedos, F., and Rodriguez, M. S. (1997) *Oncogene* **15,** 1841–1850
26. Turpin, P., Hay, R. T., and Dargemont, C. (1999) *J. Biol. Chem.* **274,** 6804–6812
27. Mercurio, F., and Manning, A. M. (1999) *Curr. Opin. Cell Biol.* **11,** 226–232
28. Sizemore, N., Lerner, N., Dombrowski, N., Sakurai, H., and Stark, G. R. (2002) *J. Biol. Chem.* **277,** 3863–3869
29. Karin, M., and Ben-Neriah, Y. (2000) *Annu. Rev. Immunol.* **18,** 621–663
30. Schindler, U., and Baichwal, V. R. (1994) *Mol. Cell. Biol.* **14,** 5820–5831
31. Peukert, K., Staller, P., Schneider, A., Carmichael, G., Hanel, F., and Eilers, M. (1997) *EMBO J.* **16,** 5672–5686
32. Kim, I. F., Mohammadi, E., and Huang, R. C. (1999) *Gene* (*Amst.*) **228,** 73–83
33. Hoffmann, A., Levchenko, A., Scott, M. L., and Baltimore, D. (2002) *Science* **298,** 1241–1245
34. Shishodia, S., and Aggarwal, B. B. (2004) *Cancer Treat. Res.* **119,** 139–173
35. Chen, L. F., Fischle, W., Verdin, E., and Greene, W. C. (2001) *Science* **293,** 1653–1657
36. Ashburner, B. P., Westerheide, S. D., and Baldwin, A. S., Jr. (2001) *Mol. Cell. Biol.* **21,** 65–77
37. Sakurai, H., Chiba, H., Miyoshi, H., Sugita, T., and Toriumi, W. (1999) *J. Biol. Chem.* **274,** 30353–30356
38. Karin, M. (1999) *Oncogene* **18,** 6867–6874