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*

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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 ADHS/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 ADHS/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)† 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 GGGRNNYYYCC (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

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†† 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; FTPC, fluorescein isothiocyanate; PTDTC, 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 (Skp1-Cullin-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 transcriptional 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 kindly 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-POZγ/FBI-1 were reported elsewhere (9). pCDNA3.0-POZNLS was selected on synthetic dextrose/His/Trp plates. Transformed yeast containing pLexA-POZγ/FBI-1 was prepared as competent yeast by a standard protocol, and yeast containing pLexA-POZγ/FBI-1 was selected on synthetic dextrose/Ura-/His-/Trp plates. Transformed yeast containing pLexA-POZγ/FBI-1 was prepared as competent yeast by a standard protocol. To prepare the fish protein pB42AD-IκBα, GST-IκBα coding sequence of IκBα was amplified by PCR annealed into the pB42AD vector in-frame with the B42 activation domain. Yeast transformants of plasmids (pLexA-POZγ/FBI-1 and pB42AD-IκBα) were streaked onto synthetic dextrose/Gal/Raft/-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 Esherichia coli DH5a. 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—**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 LexA-DB vector and used as bait. The PCR-amplified POZ domain of FBI-1 was cloned into the pLexA vector. The pLexA-POZγ/FBI-1 plasmid was introduced into yeast strain EGY48(p8op-lacZ) by a standard protocol, and yeast containing pLexA-POZγ/FBI-1 was selected on synthetic dextrose/-Ura/-His/-Trp. Transformed yeast containing pLexA-POZγ/FBI-1, yeast containing synthetic dextrose/-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 Esherichia coli DH5a. The amplified plasmids from E. coli were purified, restriction enzyme-digested for mapping, and sequenced.

**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 POZNLs 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, FAMA-stimulated or non-stimulated HeLa cells were grown on coverslips (Sunnahine Works, Seoul, Korea), washed with cold PBS, and fixed in 9:3 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, the incubation was repeated with incubation solution and further incubated with 3%, 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, 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 vitro 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 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. 1A).

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-POZB bound to agarose beads was incubated with His6- 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.

FBI-1 Enhances NF-κB-mediated Transcription of the E-selectin Promoter in HeLa Cells Stimulated with Either TNFa 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. 2A) (30) and the FBI-1 or POZ domain expression plasmid, cells were stimulated with either TNFa 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...
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), and 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. 2D, 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. 2E, bars 4 and 6). However, the POZ domains of Bcl-6 and IPP did not show repression (Fig. 2E, 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. PLZF 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 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-
ther increased transcription by 2-fold (Fig. 3A, 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 IxBα or IxBβ, but this repression was reversed by cotransfection with the FBI-1 expression plasmid. FBI-1 significantly relieved transcription repression by IxBα and IxBβ by 26.7 and 18%, respectively, in the presence of PMA (Fig. 3A, bars 4–7), suggesting that, although ectopic IxB 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, IxBα, and IxBβ were able to repress transcription by 56, 28, and 74%, respectively (Fig. 3B, bars 3, 4, and 6). Transcription repression by IxBα and IxBβ was further intensified by 74 and 85%, respectively, by the overexpressed POZ domain, suggesting a cooperative role in transcription repression (Fig. 3A, bars 5 and 7). The data suggest that the POZ domain most likely represses transcription via a mechanism similar to that of IxB 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 IxB proteins.

Therefore, we prepared a plasmid vector (pcDNA3.0-POZNLS) expressing the POZ domain fused to the NLS of FBI-1 (amino acids 487–505, KDGCGNGPSRRQPRVRG) and tested whether the POZNLS domain can, like full-length FBI-1, enhance transcription of the NF-κB-responsive E-selectin promoter (Fig. 4A) (9). Indeed, the POZNLS 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. 4B). The presence of the POZ domain reduced transcription enhancement by the POZNLS domain to the POZ domain-only level (Fig. 4B, 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...
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. 6A, panel A2). Overexpressed FBI-1 was located in the nucleus (Fig. 6A, 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. 6A, 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. 6B, 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

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**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 (POZFBI-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. 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.
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 antimouse 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. 6B, 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. 6B, 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. 6B, 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. 7A, 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. 7B). 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. 7A). The fast nuclear import and lasting nuclear retention (Fig. 7C) of p65 subunits by FBI-1 may explain the more potent transcription activation by NF-κB in the presence of PMA and 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 by yeast LexA two-hybrid and GST fusion protein pull-down assays. We cloned a human IκBα cDNA into the pB42AD vector and cotransformed pLexA-POZFBI-1 and pB42AD-IκBβ into competent yeast strain EGY48(p8op-lacZ). Cotransformed yeast was grown on syn-
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_{BFP}\_B and pLexA/IκBβ showed no protein-protein interaction (white colony formation), but the cotransformant of pLexA-POZ_{BFP} 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 His6-POZ and pulled down. Western blot (WB) analysis of the precipitates with anti-His tag antibody showed that GST-IκBα and GST-IκBβ pulled down His6-POZ (His6-POZ_{BFP}). 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.

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 interact-
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 IκBα in the nucleus by interaction with the RHD of the p65 subunit and IκBα 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.

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