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Requirements for Two Proximal NF-κB Binding Sites and IκB-ζ in IL-17A-induced Human β-Defensin 2 Expression by Conducting Airway Epithelium*

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Among a panel of 21 cytokines (IL-1α, -1β, -2–13, and -15–18; interferon-γ; granulocyte-macrophage colony-stimulating factor; and tumor necrosis factor α), we have recently observed that IL-17A is the most potent inducer for human β-defensin 2 (*hBD-2*) in conducting airway epithelial cells (Kao, C. Y., Chen, Y., Thai, P., Wachi, S., Huang, F., Kim, C., Harper, R. W., and Wu, R. (2004) *J. Immunol.* 173, 3482–3491). The molecular basis of this regulation is not known. In this study, we demonstrated a coordinated degradation of inhibitory κB (IκB)-α followed by a nuclear translocation of p50 and p65 NF-κB subunits and their binding to NF-κB sites of *hBD-2* promoter region. With site-directed mutagenesis, we demonstrated the requirement of two proximal NF-κB binding sites (pκB1, −205 to −186; pκB2, −596 to −572) but not the distal site (dκB, −2193 to −2182) in supporting IL-17A-induced *hBD-2* promoter activity. These results are consistent with the data of the chromatin immunoprecipitation assay, which showed enhanced p50 binding to these pκB sites but not the dκB site in cells after IL-17A treatment. We also found that the NF-κB binding cofactor, *IκB-ζ*, was up-regulated by IL-17A, and the knockdown of *IκB-ζ* significantly diminished the IL-17A-induced *hBD-2* expression. This is the first demonstration of the involvement of two proximal NF-κB sites and IκB-ζ in the regulation of *hBD-2* by IL-17A, two important genes responsible for host defense.

Interleukin-17 (IL-17/IL-17A)[2] was originally identified as cytotoxic T-cell lymphocyte-associated antigen 8 (CTLA-8) (2) and it is the prototype member of the other five IL-17 family members (IL-17B–F) that have been subsequently described (3, 4). Subsequent studies of human IL-17A demonstrated expression of this cytokine by activated memory T-cells predominantly of the prototypic $CD45^+$ RO^+ $CD4^+$ subtype (5). A recent mouse lung study demonstrated the potential role of Toll-like receptor 4 (TLR-4) and IL-23 in proximally mediating the stimulation of IL-17A production by $CD4^+$, $CD8^+$ T-cells and dendritic cells (6). IL-17A has been found to be associated with a variety of inflammatory conditions such as asthma and Gram-negative bacterial pneumonia (3, 7, 8) because IL-17A has a proinflammatory role in mediating pulmonary neutrophil migration in the context of local bacterial infections (9–11) and stimulates the production of proinflammatory cytokines, such as IL-1β, IL-6, IL-8, and tumor necrosis factor α (TNF-α) (12–14).

In our recent work we observed that IL-17A is the most potent inducer among a panel of 21 cytokines (IL-1α, -1β, -2–13, and -15–18; interferon-γ; granulocyte-macrophage colony-stimulating factor; and TNF-α) to stimulate airway mucin genes *MUC5AC* and *MUC5B* (15), *hBD-2* (1), and *CCL-20* (16) gene expressions in well differentiated primary human tracheobronchial epithelial (TBE) cells. Mucins are major components responsible for the elasticity of mucus, which is important for mucociliary clearance (17). Both hBD-2 and CCL-20 are vital in protecting the epithelium from infection, and they are the only peptides/chemokines known to interact with CCR6 (18, 19). CCR6 is known to have an important role in mediating dendritic cell localization and lymphocyte homeostasis in mucosal tissues (16). Therefore, IL-17A may either direct or amplify the airway inflammatory response from innate response processes to adaptive response mechanisms.

hBD-2 is the first human defensin produced by epithelial cells following contact with bacteria, viruses, or cytokines, such as IL-1β and TNF-α (20–26), providing a chemical shield against a broad spectrum of microorganism infections (27). The known function of hBD-2 in innate immunity is believed to be related to its antimicrobial activity and to its chemotactic effects on immature dendritic cells and memory T-cells (28). To date, the induction of hBD-2 by lipopolysaccharide and IL-1β has been reported to be modulated by both the mitogen-activated protein kinase (MAPK) and/or NF-κB pathways (29–31), although the nature of the regulation is not completely characterized. It has been indicated that NF-κB mediates IL-1β- or TNF-α-induced hBD-2 transcription in A549 cells via p65-p50 binding to a proximal NF-κB-responsive element, the pκB1 site. Furthermore using macrophage-like RAW264.7 cells, it has been shown that the p65-p50 heterodimer could bind to this site on stimulation of the cells with lipopolysaccharide (32). In contrast, the p65-p65 homodimer is reported to selectively bind to the pκB1 site in gastrointestinal cell lines exposed to *Helicobacter pylori* or flagella filament protein from *Salmonella enteritidis* (33, 34). Thus, transcriptional activa-

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[2] The abbreviations used are: IL, interleukin; TBE, tracheobronchial epithelial; hBD, human β-defensin; TNF-α, tumor necrosis factor α; IκB, inhibitory κB; PBS, phosphate-buffered saline; siRNA, small interfering RNA.

tion of *hBD-2* gene may be regulated by a combination of NF-κB subunits in a cell type- or stimulus-specific manner. For IL-17A-induced *hBD-2*, the molecular basis of the stimulation has not been resolved.

NF-κB has been shown to play a critical role in the mammalian immune system (35–37). The five members of the mammalian NF-κB family, p65 (RelA), RelB, c-Rel, p50, and p52, exist in unstimulated cells as homo- or heterodimers bound to inhibitory κB (IκB) family proteins. The classical NF-κB signaling pathway could be stimulated by IL-1β and activated via the activation of the IκB kinase complex, which then phosphorylates IκB proteins and then releases NF-κB to translocate into the nucleus. The p65-p50 heterodimer is usually the most abundant form of the NF-κB family and acts as a strong transactivator. Intriguingly a recently identified IκB family member, IκB-ζ, is up-regulated by IL-1 and lipopolysaccharide. IκB-ζ was also shown to be involved in inhibiting or activating some NF-κB-regulated genes.

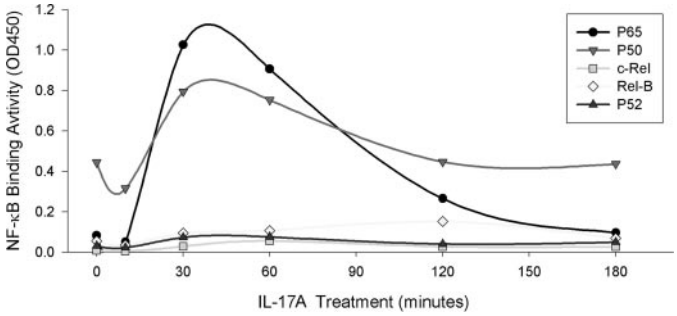


FIGURE 1. **Characterization of IL-17A effects on binding activity of different NF-κB subunits to consensus NF-κB probes in HBE1 cell cultures.** HBE1 cells were treated with IL-17A (20 ng/ml), and the nuclear fraction was collected at various times for enzyme-linked immunosorbent assay-based NF-κB binding analysis. The TransFactor NF-κB Family kit was used to detect DNA binding of NF-κB family members. Only p50 and p65 binding activity was stimulated by IL-17A. NF-κB p65 shows distinct stimulation kinetics different from that of p50.

Recently we have shown that inhibitors that affect NF-κB translocation and the DNA binding activity of its p65 NF-κB subunit could attenuate IL-17A-induced *hBD-2* expression in both primary TBE and an immortalized normal bronchial epithelial cell line, HBE1 (1). These findings support an NF-κB-mediated transcriptional mechanism for IL-17A-induced *hBD-2* expression. Despite this progress, very little information is available for both the *cis*- and *trans*-acting elements required in IL-17A-mediated *hBD-2* transcription. In this study, we undertook the task to elucidate the molecular basis of the transcriptional regulation of *hBD-2* by site-directed mutagenesis, chromatin immunoprecipitation assay, and DNA-protein interaction. Two proximal NF-κB binding sites at the 5′-flanking region of *hBD-2* were identified as important sites for IL-17A-induced DNA-protein interaction.

Given the intriguing and critical connections of hBD-2 and IL-17A in airway innate immunity, it is important to elucidate the molecular mechanism of the regulation of *hBD-2* expression by IL-17A. Although little information has yet to be uncovered about this mechanism, our study revealed that IL-17A activates NF-κB in airway epithelial cells. Further studies identified functional κB response elements in the promoter of the *hBD-2* gene. We then demonstrated that IL-17A stimulates *hBD-2* gene transcription via NF-κB, and this study may render the detailed mechanism useful for the treatment of airway innate immunity-related disease.

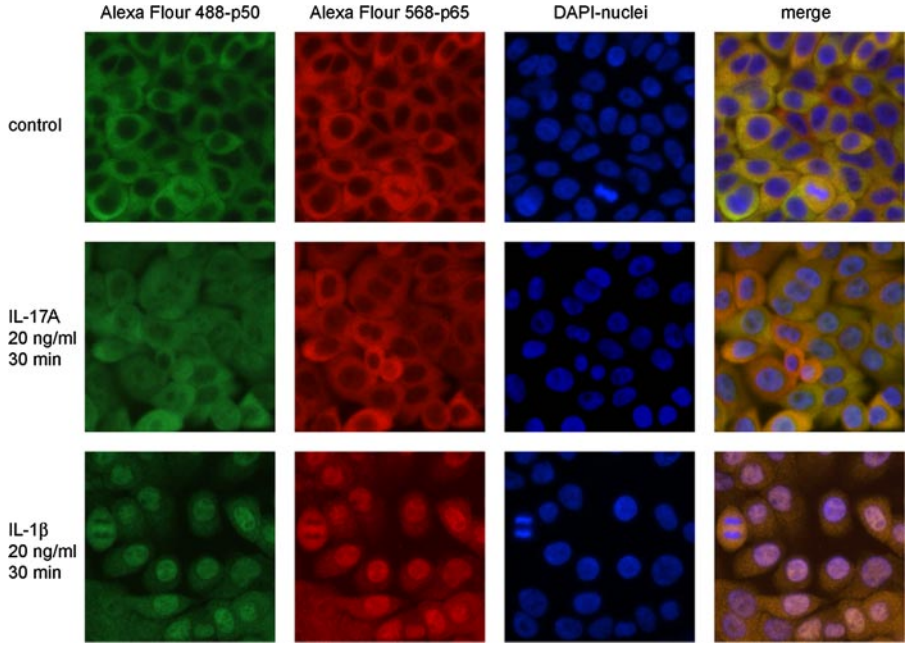


FIGURE 2. **Nuclear translocation of p50 and p65 subunits of NF-κB transcriptional factors in human HBE1 cells after IL-17A and IL-1β treatments.** HBE1 cells were plated on Lab-Tek II chamber slides and treated with IL-17A and IL-1β as described in the text. Thirty minutes after the treatment, these slides were fixed followed by staining with anti-p50 antibody, anti-p65 antibody, Alexa Flour 488-conjugated goat anti-mouse IgG antibody (*green* fluorescence), and Alexa Flour 568-conjugated goat anti-rabbit IgG antibody (*red* fluorescence) as described in the text. For counterstaining, nuclei were stained with 4′,6′-diamidino-2-phenylindole (*DAPI*) (*blue* fluorescence). These slides were examined by a Zeiss fluorescence microscope with a 40× lens under appropriate filters for anti-p50 (*green*, *first column*), anti-p65 (*red*, *second column*), 4′,6′-diamidino-2-phenylindole (*blue*, *third column*), and merge (*fourth column*). *Top panel*, control cultures without cytokine treatment; *middle panel*, IL-17A (20 ng/ml)-treated cultures; *bottom panel*, IL-1β (20 ng/ml)-treated cultures.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant human IL-1β and -17A were purchased from R&D Systems Inc. (Minneapolis, MN). NF-κB p65 mouse IgG, p65 rabbit IgG, and IκB-α mouse IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). NF-κB p50 mouse IgG was purchased from BioLegend (San Diego, CA). Nucleolin mouse IgG was purchased from Research Diagnostics Inc. (Concord, MA). β-Tubulin mouse IgG was purchased from Sigma-Aldrich. Actinomycin D was purchased from Calbiochem-Novabiochem.

Cell Culture and Cytokine Treatment—HBE1 cell line is an immortalized line of normal human bronchial epithelial cells (38). HBE1 cells were cultured on a plastic tissue culture surface in Ham's F-12/

Dulbecco's modified Eagle's medium (1:1) supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), epidermal growth factor (10 ng/ml), dexamethasone (0.1 μM), cholera toxin (10 ng/ml), bovine hypothalamus extract (15 μg/ml), and bovine serum albumin (0.5 mg/ml). Recombinant human cytokines were dissolved in phosphate-buffered saline (PBS) with 1% bovine serum albumin and added directly to media of the culture (20 ng/ml). The control treatments had the same amount of PBS, 1% bovine serum albumin added. The primary TBE cell culture condition was described in the previous studies (1). For the mRNA stability study, cells were pretreated with or without 20 ng/ml IL-17A for 24 h. Then 5 μg/ml actinomycin D was added to these cultures, which were harvested for RNA isolation at various hours as indicated.

Real Time Reverse Transcription-PCR Expression Analysis—The RNA extraction, cDNA generation, primer sequences of glyceraldehyde-3-phosphate dehydrogenase, β-actin, and *hBD-2* for real time PCR were described in our previous study (1, 16, 39). The following PCR primers were used for human *IκB-ζ*: forward, CATGGGAAATCCAATGAACAC; and reverse, GGCAACAGCAATATGAAGGAA.

NF-κB/Nuclear Extract Binding Enzyme-linked Immunosorbent Assay—BD™ TransFactor NF-κB Family Colorimetric kit from BD Biosciences Clontech was used to quantify the NF-κB-specific binding activity of nuclear extract. The quantification was carried out according to the manufacturer's protocol. Briefly to each well 20 μg of nuclear extract were added and incubated for 1 h at room temperature. Microtiter wells were then washed three times, and diluted primary antibodies against various NF-κB subunits were added (100 ml/well) and incubated further at room temperature for an hour. After extensive washing, diluted secondary antibody conjugated with horseradish peroxidase was added to each well and further incubated at room temperature for 30 min. After repeated washing, 100 μl of tetramethylbenzidine substrate solution were added to each well in the dark for the color development at room temperature. The reaction was quenched by 100 μl of 1 N HCl/well, and binding intensity was measured as absorbance at 450 nm using a microtiter plate reader.

Immunofluorescence Microscopy—HBE1 cells were plated to sterile Lab-Tek II chamber slides (Nalge Nunc International, Rochester, NY). At various times after IL-17A treatment, cells in slide chambers were fixed at 4 °C for overnight in PBS supplemented with 4% paraformaldehyde solution. The slide chambers were washed three times with PBS for 5 min each, permeabilized with 0.1% Triton X-100 in PBS for 30 min at 37 °C, and blocked with the blocking buffer containing 2% goat serum in PBS with Tween 20 for 30 min at 37 °C. The slide chambers were then stained with mouse anti-p50 monoclonal and rabbit anti-p65 polyclonal primary antibodies (1:200 dilution in blocking buffer) for 1 h at 37 °C, washed three times with PBS with Tween 20 for 5 min each, and incubated with fluorescently labeled Alexa Fluor 488-goat anti-mouse IgG and Alexa Fluor 568-goat anti-rabbit IgG secondary antibodies (1:500 dilution in blocking buffer) (Molecular Probes Inc., Eugene, OR) for 1 h at 37 °C. Nuclei were counterstained with VECTASHIELD® mounting medium with 4′,6′-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). The staining was visualized using a Zeiss AxioSkop fluorescence microscope (×40 objective).

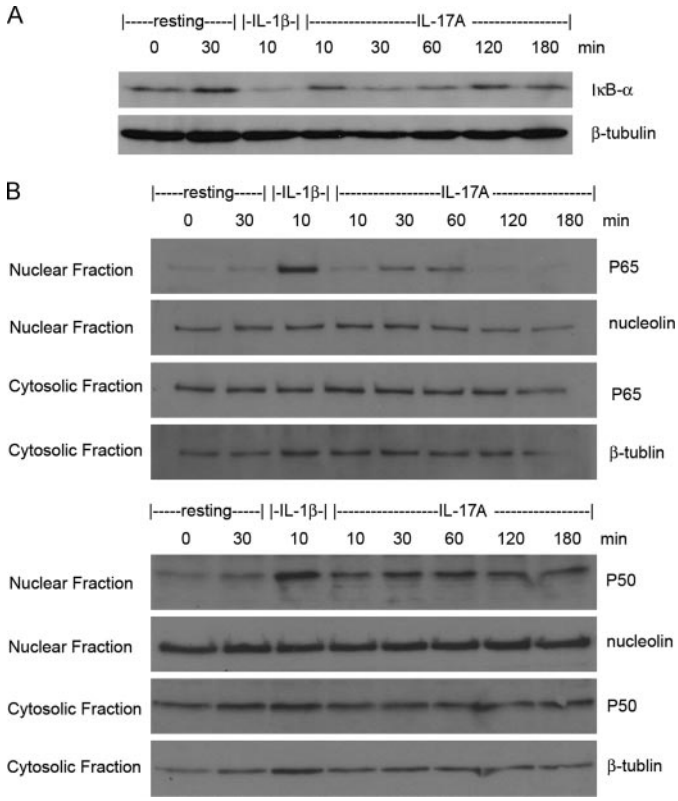


FIGURE 3. **IκB-α degradation and NF-κB p50 and p65 translocation primed by IL-17A in HBE1 cells.** HBE1 cells were treated with IL-17A (20 ng/ml) and harvested at the indicated times. Samples were resolved by PAGE, transferred to polyvinylidene difluoride membranes, and probed with the indicated antibodies. A representative of three independent experiments is shown. *A*, the total cell lysates were separated by SDS-PAGE and immunoblotted with an antibody against IκB-α. IL-17A was shown to prime for IκB-α protein degradation at 30–60 min, and then IκB-α returned to the basal level. *B*, nuclear and cytosolic fractions were prepared from the cells and subjected to immunoblot analyses using antibodies anti-p50, -p65, -nucleolin, and -β-tubulin. NF-κB p65 was stimulated by IL-17A to translocate into the nucleus at 30–60 min and then returned to the basal level. NF-κB p50 translocated into the nucleus in 10 min and was retained in the nucleus for at least 180 min.

Preparation of Nuclear Extracts—Nuclear lysates from cultured HBE1 cells were harvested according to the Panomics (Redwood City, CA) nuclear extraction protocol. In brief, HBE1 cells were lysed with a lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 10 mM EDTA, 1 mM dithiothreitol, 0.5% Igepal, and protease inhibitor mixture) on ice for 10 min and harvested using a sterile scraper. The cytosolic fraction was collected from the supernatant by centrifugation at 15,000 × *g* for 3 min at 4 °C. Pelleted nuclei were resuspended in extraction buffer (10 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and protease inhibitor mixture). After incubation at 4 °C for 2 h with gentle rocking, the nuclei were collected by centrifugation at 15,000 × *g* for 5 min at 4 °C. The resultant supernatants were collected and stored at −80 °C.

Western Blotting—HBE1 cells were treated with 20 ng/ml IL-17A and IL-1β, the positive control, at different time intervals. Cells were either harvested in radioimmune precipitation assay buffer followed by a brief sonication and centrifugation or with the Panomics Nuclear Extraction kit according to the

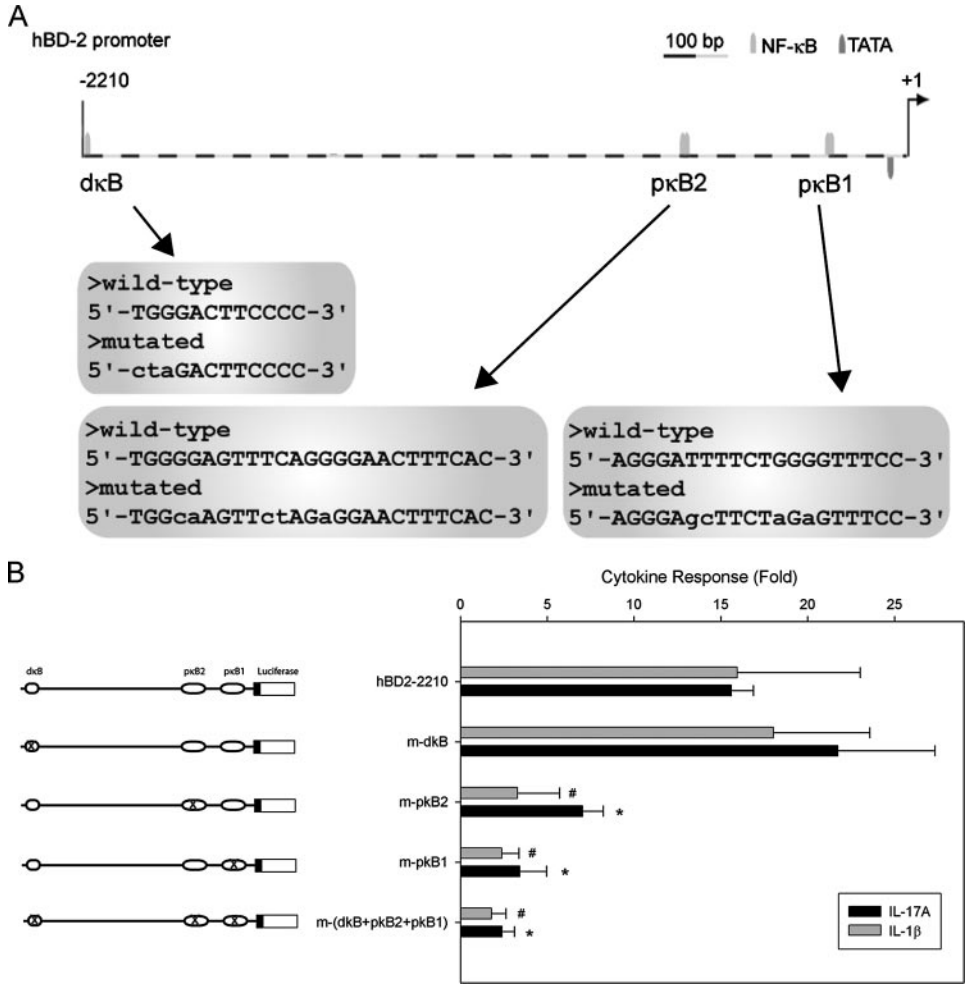


FIGURE 4. **Mutation analyses of effects of IL-17A on *hBD-2* promoter-reporter activity.** *A*, schematic representation of the *hBD-2* promoter depicting putative NF-κB sites and nucleotide sequences of those sites used for wild-type and mutated promoter constructs. The *arrow* indicates the potential transcription initiation site. The potential NF-κB binding sites and TATA boxes are *boxed*. *B*, HBE1 cells were transfected with the indicated luciferase reporter plasmids and pRL-TK. Two days after transfection, the cells were left unstimulated or stimulated with IL-17A or IL-1β (20 ng/ml) for 24 h, and the luciferase activities were measured. Luciferase activities were expressed as -fold activation normalized with cells without cytokine treatment. Only the mutant constructs with either pκB2 or pκB1 mutation abrogated the response to IL-17A and IL-1β. Data are expressed as mean ± S.E. from at least three different experiments. Group differences were calculated by *t* test, and *p* values <0.05 were considered significant (* and #).

manufacturer's protocol for nuclear and cytosolic protein extractions. The concentrations of the resulting total, nuclear, and cytosolic proteins were then determined by the Lowry method using bovine serum albumin as a standard. Twenty micrograms of protein extracts were subjected to 10% SDS-PAGE and blotted to polyvinylidene difluoride membrane for Western blot analysis.

Site-directed Mutagenesis of hBD-2 Promoter-Luciferase Reporter Plasmid and siRNA—The *hBD-2*-2210 promoter-luciferase reporter plasmid has been described in the previous study (1). The three individual NF-κB sequences in the *hBD-2* promoter construct, *hBD-2*-2210/Luc, dκB (−2193 to −2182), pκB2 (−596 to −572), and pκB1 (−205 to −186), were mutagenized by using the Transformer™ site-directed mutagenesis kit (BD Biosciences Clontech). The resulting mutated NF-κB constructs were termed dκB-mut/Luc, pκB2-mut/Luc, pκB1-mut/Luc, and dκB+pκB2+pκB1mut/Luc (mutations on all three NF-κB sites). The authenticity of these mutations was confirmed by DNA sequencing. siRNAs against *IκB-ζ* (identification number 33380) and random oligomer were purchased from Ambion (Austin, TX).

Transient Transfection and Luciferase Assay—HBE1 cells were seeded into 12-well plates at a density of 1×10^5 cells/well. One day after plating, cells were transfected with 0.5 μg of *hBD-2*-2210/Luc, dκB-mut/Luc, pκB2-mut/Luc, pκB1-mut/Luc, or dκB+pκB2+pκB1mut/Luc plasmid DNA and 50 ng of *Renilla* luciferase expression vector pRL-TK (Promega) using the FuGENE 6-based gene transfer protocol (Roche Diagnostics) according to the manufacturer's instructions. Eighteen hours after the transfection, cells were treated with 20 ng/ml IL-17A, and cell extracts were prepared for reporter gene assays 24 h after the IL-17A treatment. The reporter gene assays were carried out with the Dual-Glo™ Luciferase Assay System (Promega) according to the manufacturer's protocol. The relative *hBD-2* promoter activities were expressed as relative luciferase units after normalization to the internal control, *Renilla* luciferase activity. The results were averaged from triplicate wells of three separate experiments. For siRNA transfection, cells were plated at 40–60% density a day before transfection. An Oligofectamine-based transfection kit (Invitrogen) was used according to the manufacturer's instruction. Sixteen hours after transfection, the siRNA transfection mixture was replaced with fresh culture medium. Two days later, cultures were depleted of hormonal supplements 24 h before IL-17A treatment. At various times after the treatment, cells were harvested for gene expression analyses.

NoShift p50 and p65 Binding Assay—The NoShift transcriptional factor assay kit (Novagen, Inc., Madison, WI) was used to measure binding of NF-κB p50 and p65 proteins to three NF-κB binding sequences on *hBD-2* promoter as described in the manual. Briefly 10 μg each of sense and antisense 5′-biotinylated oligonucleotides (dκB-sense, 5′-CTTTGGGACTTCCCCAGCTA-3′; dκB-antisense, 5′-TAGCTGGGGAAGTCCCAAAG-3′; pκB2-sense, 5′-TGGGGAGTTTCAGGGGAACTTTCAC-3′, pκB2-antisense, 5′-GTGAAAGTTCCCCTGAAACTCCCCA-3′; pκB1-sense, 5′-AGGGATTTTCTGGGGTTTCC-3′, and pκB1-antisense, 5′-GGAAACCCCAGAAAATCCCT-3′) were dissolved to a final volume of 100 μl in a mixture of 0.5 M

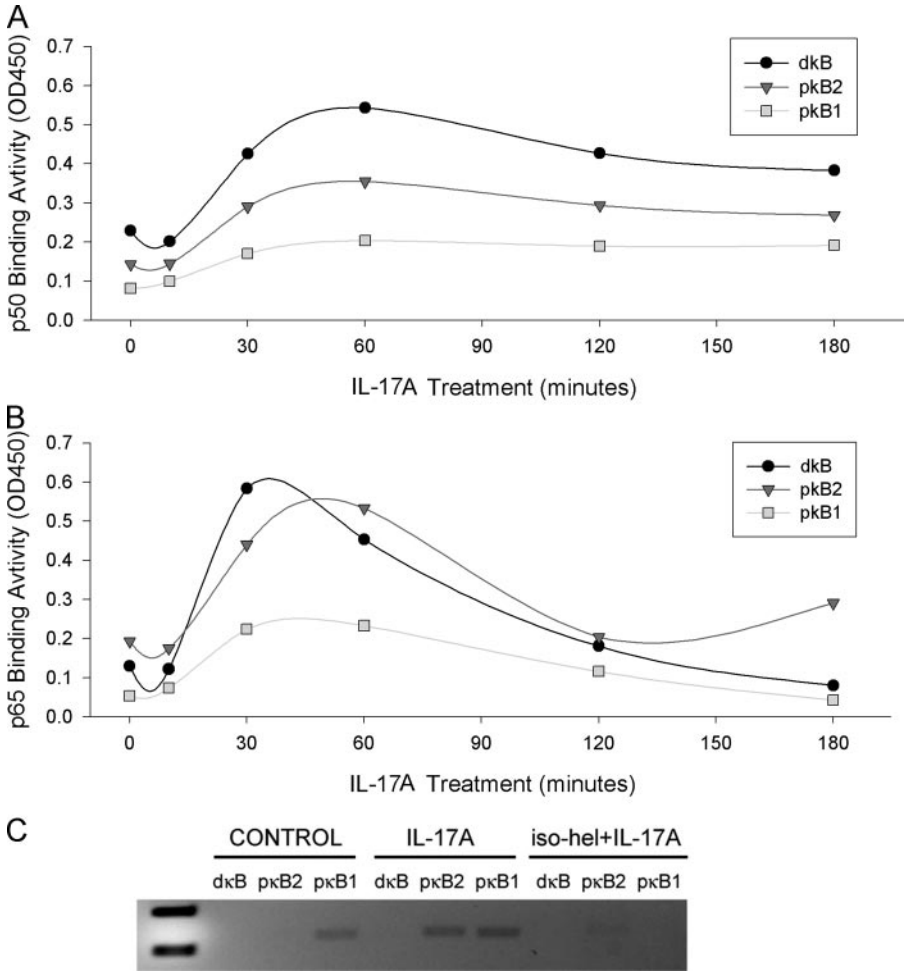


FIGURE 5. **Analysis of the *in vitro* and *in situ* occupancy of IL-17A-stimulated NF-κB p50 and p65 binding activity to the three NF-κB sites on *hBD-2* promoter.** HBE1 cells were treated with IL-17A (20 ng/ml), and the nuclear fraction was harvested at the indicated times. The Novagen NoShift Transcription Factor Assay kit was used to assay the p50 (*A*) and p65 (*B*) binding activity to all three NF-κB sites on the *hBD-2* promoter. All three *hBD-2* NF-κB probes showed stimulation by IL-17A at 30–60 min. *C*, formaldehyde-cross-linked chromatin samples from primary TBE cells were used for immunoprecipitation reaction with antibodies against p50. The cross-linking was reversed overnight at 65 °C, and immunoprecipitated DNA was purified for PCR by three different sets of primers as indicated. *iso-hel*, isohelenin.

SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 75 mM NaCl, and 7.5 mM sodium citrate (pH 7.0), heated to 100 °C (in boiling water bath) for 10 min, slowly cooled to room temperature, and then diluted to 10 pmol/μl. Nonbiotinylated oligonucleotides with the same sequence duplex that were used as competitor DNA were prepared in the same way except with a final concentration of 50 pmol/μl.

For measurement of binding affinity, the reaction mixtures mainly containing 1 pmol of biotinylated target DNA duplex, 20 μg of nuclear extract, and competitive nonbiotinylated DNA complexes were incubated on ice for 30 min. The reaction mixtures were then dispensed into freshly prepared streptavidin plates and incubated for 1 h at 37 °C. The binding of p50 and p65 was detected by incubation for 1 h at 37 °C with 100 μl of NF-κB p50 and p65 mouse IgG diluted 1:500 in NoShift antibody dilution buffer. After repeated washing of the plate, horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc.) was added (1:1,000 dilution in NoShift antibody dilution buffer). After 30 min of incubation at 37 °C, wells were washed thoroughly, and tetramethylbenzidine substrate was added. The reaction was quenched by 100 μl of 1 N HCl/well, and binding intensity was measured as absorbance at 450 nm.

Chromatin Immunoprecipitation Assay—Formaldehyde cross-linking and chromatin immunoprecipitation in TBE cells were performed according to standard protocols on Farnham laboratory web site. After reversing the cross-links, the DNA was purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA) and analyzed using PCR amplification. Five microliters of DNA were used for PCR to detect the presence of specific DNA segments with the following primer pairs: dκB: forward, 5′-CATCCCCCAGTCTCTTCA-TCT-3′; reverse, 5′-ATGAGACC-AGTGTCCAGGCTA-3′; pκB2: forward, 5′-GGTGTGAATGGAA-GGAACTCA-3′, reverse, 5′-TTC-AGCTCCTGGGGATGATAC-3′; and pκB1: forward, 5′-TGGCAGGT-TATAGGTCCTGAG-3′; reverse, 5′-ATAAAGGTCCTGGTCCCT-GGT-3′.

Statistical Analysis—Data are expressed as mean ± S.E. The number of repetitions for each experiment is given under "Results." Paired comparisons were carried out by *t* test. Differences were considered significant for *p* values less than or equal to 0.05.

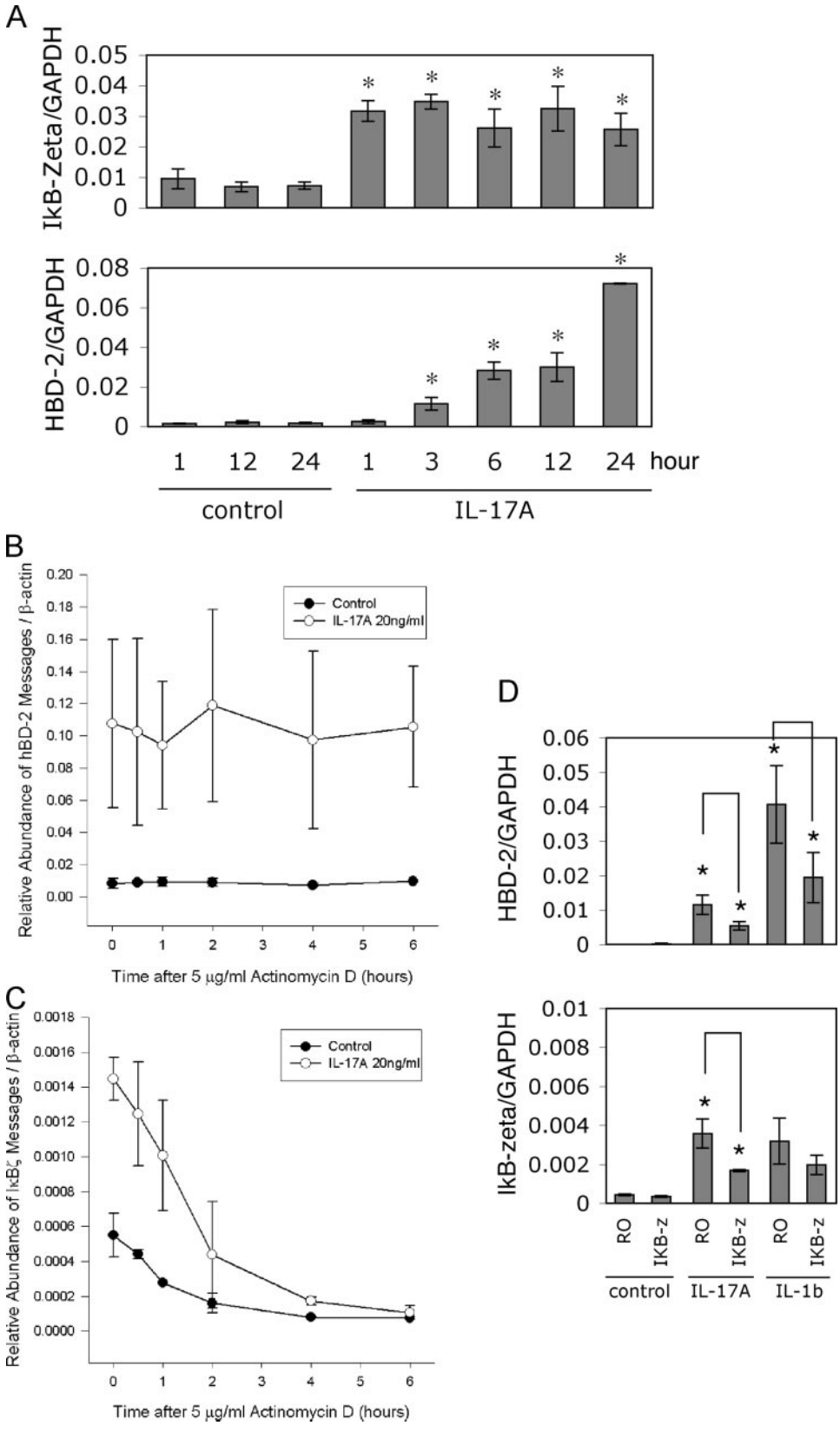
RESULTS

IL-17A Activates Nuclear Translocation of NF-κB in Airway Epithelial Cells—Our recent work has demonstrated that both helenalin, an inhibitor for the DNA binding activity of NF-κB p65 subunit (40), and sulfasalazine, an inhibitor shown to inhibit NF-κB activation via direct inhibition of IκB kinase (41), were very effective in abrogating IL-17A-induced *hBD-2* expression in primary TBE and HBE1 cells (15). However, the nature of the NF-κB-based transcriptional mechanism has not been resolved. Initially we sought to identify which NF-κB subunit(s) is involved. Using the BD TransFactor NF-κB Family Colorimetric kit, we observed a persistent basal level of p50-specific DNA binding activity in nuclear extracts prepared prior to IL-17A treatment (Fig. 1). For other NF-κB subunits, p65, p52, c-Rel, and Rel-B, the levels of their DNA binding activities were relatively low. However, after cytokine treatment, there was an early (30–60 min), transient increase of DNA binding activity for p50 and p65 subunits but not p52, c-Rel, and Rel-B in nuclear

extracts prepared from HBE1 cells treated with IL-17A. These increases are more than 2- and 20-fold for p50- and p65-specific DNA binding activity, respectively. Of note, IL-1β stimulated even stronger binding activities to p65 and p50 (data not shown) than did IL-17A, and a similar transitional increase phenomenon was observed in primary TBE cells after IL-17A treatment (data not included).

Because of the low levels of DNA binding activities for p52, c-Rel, and Rel-B, we focused on nuclear translocation of the other two NF-κB subunits, p50 and p65. Using an immunofluorescence microscope, we were able to observe the nuclear translocation of p50 and p65 in HBE1 cells 30 min after IL-17A treatment (Fig. 2). For p50, there was a low level presence in the nucleus prior to the cytokine treatment consistent with the above TransFactor enzyme-linked immunosorbent assay. As a control, IL-1β was more effective in promoting nuclear translocation of both p50 and p65 in treated cells (Fig. 2, *lower panels*). A similar nuclear translocation phenomenon was observed with primary TBE cells except that the cell morphologies were more heterogeneous compared with the cell line (data not included).

To further elucidate the molecular mechanism, Western blot analysis of both cytoplasmic and nuclear extracts from cultured cells after cytokine treatments was carried out. As shown in Fig. 3*A*, treatment with IL-17A caused a rapid degradation, within 10–30 min, of IκB-α, an inhibitor of NF-κB translocation (35) in HBE1 cells. However, this degradation was transient with a quick recovery at 120 and 180 min of treatments. A similar rapid decrease of IκB-α was observed in IL-1β-treated cultures. In contrast to the rapid degradation, nuclear translocation of p65 was increased transiently in cultures after IL-17A treatment (Fig. 3*B*). This increase was diminished at 120 and 180 min after the treatment. For cytoplasmic extracts, there was no apparent change in p65 protein level in cells treated with or without IL-17A. For p50, a similar transient increase of its presence in the nucleus was observed except that there was a higher than background presence of p50 in the nucleus prior to IL-17A treatment (Fig. 3*C*). The same Western



blot analysis as described in Fig. 3 was carried out in primary TBE cells, and consistent results were seen (data not included).

Determination of cis-κB-Elements in the hBD-2 Promoter Responsive to IL-17A Stimulation—To further determine whether the observed effect of IL-17A on *hBD-2* expression was due to an NF-κB-mediated transcriptional activation, a study of the effect of IL-17A on *hBD-2* promoter activity was carried out in HBE1 cells using a transient transfection approach with *hBD-2*-2210/Luc chimeric construct DNA. As shown in Fig. 4*A*, the *hBD-2* promoter contains three NF-κB binding motifs, a single distal site at −2193 to −2182 (dκB) and two proximal sites at −596 to −572 and −205 to −186 (pκB2 and pκB1, respectively). We have shown previously that there was a dose-dependent increase in the relative *hBD-2* promoter-based luciferase activity by IL-17A (1). To further elucidate the role of NF-κB sites in the transcriptional regulation of *hBD-2* gene, we generated four luciferase expression constructs containing mutated NF-κB promoters termed dκB-mut/Luc, pκB2-mut/Luc, pκB1-mut/Luc, and dκB+pκB2+pκB1mut/Luc. Luciferase reporter analysis showed that mutation of dκB (−2193 to −2182) of *hBD-2* promoters did not essentially reduce responsiveness to IL-17A, although the responsiveness of dκB/Luc was slightly increased compared with control (promoterless vector), suggesting the presence of basal promoter activity in this dκB/Luc construct. In contrast, mutations at the pκB1 site from −205 to −186 and at pκB2 (−596 to −572) significantly reduced the responsiveness to IL-17A. The triple mutation of the dκB, pκB1, and pκB2 sites almost completely abolished the up-regulation of promoter activity by IL-17A (Fig. 4*B*). We performed the same analysis with IL-1β in HBE1 cells, and similar results were observed. Taken together, our results clearly demonstrate that the NF-κB binding site of pκB1 is essential to the response of *hBD-2* promoter to IL-17A and IL-1β, and these results suggest that the NF-κB binding sites pκB1 (−205 to −186) and pκB2 (−596 to −572) mainly contribute to the IL-17A-induced *hBD-2* transcription in HBE1 cells.

Kinetics Studies of trans-Elements Bound to the NF-κB Sites in the hBD-2 Promoter—In Fig. 1, we have shown IL-17A stimulated the binding of p65 and p50 to generic NF-κB binding sites. To further elucidate the IL-17A-mediated p65 and p50 binding on the *hBD-2* promoter, an *in vitro* binding assay with an addition of anti-p65 antibody as well as anti-p50 antibody was performed. As shown in Fig. 5, no specific p65 binding was detected when all of the dκB, pκB2, and pκB1 oligonucleotide probes were incubated with nuclear extracts from unstimulated (resting) HBE1 cells. Similarly no p50 binding was observed using nuclear extracts from resting HBE1 cells. Time course studies revealed that the kinetics of IL-17A-induced p65 and p50 binding on all three NF-κB sites were different (Fig. 5). A significant level of stimulation of p65 binding by IL-17A (20 ng/ml) was seen. Peak stimulation occurred within 30 min to 1 h with a rapid decrease in binding at 2 and 3 h of treatment. In contrast, p50 binding upon IL-17A stimulation was different. The maximal stimulation was observed at 1 h, and the induction of binding was maintained for the duration of this time course. Remarkably for both p65 and p50 binding, the strongest stimulation was seen on dκB probe. The pκB1 probe somehow had the weakest binding activity. Furthermore although the p65 and p50 binding activity of the three probes showed a significant difference, importantly IL-17A stimulated the p65 and p50 binding activity on all three probes, and -fold inductions of IL-17A-induced binding activity on all three probes were about the same (3-fold at maximum). These observations suggest that NF-κB p65 and p50 can bind to the dκB, pκB2, and pκB1 sequences *in vitro* from HBE1 cell nuclear extract stimulated with IL-17A, and they all show the similar responses.

Identification of IL-17A-induced NF-κB Binding in Situ in hBD-2 Promoter—To gain an insight into the IL-17A-mediated activation on the hBD-2 promoter, the observation of increased endogenous *hBD-2* gene expression directly by p50 was further validated by the *in situ* occupancy of the NF-κB regulatory sites in the *hBD-2* gene promoter (Fig. 5*C*). Chromatin immunoprecipitation studies were performed in primary TBE cells. The results show that p50 subunit was not detected on the dκB element in cells prior to and after IL-17A treatment. For pκB2 site, there was no binding initially, but a significant binding at this site by p50 subunit could be demonstrated after IL-17A treatment. For pκB1 site, there was a basal binding, and this binding was greatly enhanced in cells after IL-17A treatment. Using isohelenin, an inhibitor that prevents IκB-α degradation and inhibition of IL-17A-induced *hBD-2* expression (39), the treatment abrogated the binding of p50 to both pκB1 and pκB2 sites. These results suggest the involvements of the DNA-protein interaction at both the pκB1 and pκB2 sites in IL-17A-induced *hBD-2* expression.

IL-17A-induced hBD-2 Expression Is Dependent on IκB-ζ—It was reported recently that IL-1β-specific up-regulation of *hBD-2* is dependent on a short lived NF-κB regulator, IκB-ζ, in A549 cells (42). IL-17A has also been shown to stabilize the mRNA of *IκB-ζ* in NIH3T3 cells (43). To elucidate whether IκB-ζ is involved in IL-17A-induced *hBD-2* expression, we performed a time course analysis on the IL-17A-induced *IκB-ζ*

FIGURE 6. **Knockdown of *IκB-ζ* expression causes a decrease of IL-17A-induced *hBD-2* expression.** *A*, a differential gene induction by IL-17A occurs in primary TBE cells. Primary TBE cells were cultured under biphasic condition and were stimulated with IL-17A (20 ng/ml) in a time course from 0 to 24 h. Expressions of *hBD-2* (*lower*) and *IκB-ζ* (*upper*) mRNA relative to a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), were determined by real time PCR in each time point. Student's *t* test was used for statistic analysis. * indicates $p < 0.05$ for each samples against the time 0 control. *B*, primary human TBE cells cultured under an air-liquid interface condition were treated with or without IL-17A (20 ng/ml). Twenty-four hours later, actinomycin D (5 μg/ml) was added to these cultures, and the cultures were harvested for RNA at 0.5, 1, 2, 4, and 6 h after the addition. Real time reverse transcription-PCR analysis was carried out for β-actin, *hBD-2*, and *IκB-ζ* messages in these RNA samples. The relative level of *hBD-2* message after normalization with the β-actin level in each RNA sample was plotted against the time of actinomycin D treatment. *C*, the relative level of *IκB-ζ* message after normalization with the β-actin level in each RNA sample was plotted against the time of actinomycin D treatment. Values are means ± S.E. of triplicates of one representative experiment. The experiment was repeated with two independent primary cultures derived from different donors. *D*, IκB-ζ is responsible for IL-17A- and IL-1β-induced *hBD-2* production. *IκB-ζ* siRNA or the random oligomers (*RO*; negative control) were transfected using Oligofectamine overnight in HBE1 cells. 48 h after transfection, cells were treated with IL-17A or IL-1β (10 ng/ml) overnight. Expression of *IκB-ζ* and *hBD-2* relative to glyceraldehyde-3-phosphate dehydrogenase was determined by real time PCR. Student's *t* test was used for statistic analysis. * indicates $p < 0.05$ as indicated by *brackets*.

expression. *IκB-ζ* expression was remarkably up-regulated after 1 h of IL-17A treatment and then peaked at least for 24 h, whereas *hBD-2* induction was delayed until 3 h (Fig. 6*A*). We then analyzed the relative mRNA stability of *hBD-2* and *IκB-ζ* transcripts compared with that of β-actin (Fig. 6, *B* and *C*). Our results indicate that *hBD-2* transcripts were as stable as β-actin mRNA, and IL-17A had no significant effect on the half-life of the *hBD-2* message. Interestingly the *IκB-ζ* messages showed the same quick turnover rate after the IL-17A treatment. We further transfected HBE1 cells with a specific siRNA against *IκB-ζ*, and the level of IL-17A-induced *hBD-2* expression was significantly diminished (Fig. 6*D*). A similar difference was seen for IL-1β/*IκB-ζ* siRNA-treated cells. These results suggested that IκB-ζ might positively mediate the IL-17A-induced *hBD-2* expression.

DISCUSSION

In the present study, we evaluated the roles of IL-17A in the activation of NF-κB and the induced *hBD-2* transcription in human airway epithelial cells. The known function of hBD-2 in innate immunity is believed to be related to its antimicrobial activity and to its chemotactic effects on immature dendritic cells and memory T-cells (28). Because hBD-2 is primarily expressed by epithelial cells, both primary TBE and HBE1 cells were used in this study to elucidate the molecular mechanism of its induction by IL-17A. Our data showed that IL-17A-mediated signaling, as compared with that of IL-1β, is not a strong activator for NF-κB translocation. However, such a weak translocation is sufficient to exert a huge induction of *hBD-2* transcription. We have provided the evidence to identify the "putative" κB sites at the *hBD-2* promoter region. We have also provided physical evidence of the interactions of these sites with NF-κB transcriptional factors, especially the p50; the attenuation of such a binding by the inhibitor isohelenin, which blocks the degradation of IκB-α to prevent nuclear translocation; and the crucial role of IκB-ζ in IL-17A-induced *hBD-2* expression.

In this report, we used a new approach to determine the NF-κB binding activity *in vitro*. The data suggested that IL-17A stimulated the binding of p65 and p50 to the consensus NF-κB sequences, whereas p52, Rel-B, and c-Rel had neither basal binding activity nor inducibility by IL-17A. The experiments with *hBD-2*-specific NF-κB probes showed a similar result. All three probes, dκB, pκB2, and pκB1, showed inducibility of binding to p65 and p50 separately by IL-17A treatment. dκB probes showed the strongest binding activity to p65 and p50, whereas pκB2 probes were weaker, and pκB1 probes showed the weakest binding activity by IL-17A stimulation. Besides the difference of binding activities, p65 and p50 showed a different kinetics. The p65 binding decreased dramatically after 1 h, but p50 binding activity remained until 3 h. The temporal change of binding activities is consistent with the IκB-α degradation and p65/p50 nuclear translocation data of Western blot analysis. Remarkably NF-κB oscillations have been described and predicted recently (44–46), although their biological role is not clear. The current hypothesis is that NF-κB oscillations might set a threshold to respond to the external stimulation and then lead to cellular response (46). Because our data could represent the first spike of the NF-κB oscillations of the predicted models (44–46), it is plausible that IL-17A could lead to NF-κB oscillations. Because the NF-κB oscillations are suggested to help the response to a small stimulation, it may also explain why IL-17A activates NF-κB less than IL-1β activates NF-κB based on our *in vitro* binding assays, immunofluorescence imaging, and immunoblots but still induces significant *hBD-2* expression. Further work with a longer time course on the binding assay, IκB-α degradation, and p65/p50 nuclear translocation would clarify whether IL-17A activates NF-κB oscillations and then drives differential regulation of downstream genes.

The site-directed mutagenesis study with the promoter-reporter gene expression assay demonstrated the requirement of the pκB1 site for the inducible promoter activity. Mutation at this site abrogated the promoter activity induced by IL-17A. For the pκB2 site, the mutation at this site reduced but did not abrogate all the inducible promoter activity. The simplest interpretation is that the pκB2 site is solely involved in further enhancing IL-17A-induced transcription. Our promoter analysis data also showed that dκB, which had the strongest binding with NF-κB, was not required for the effects of IL-17A. Thus, we speculate that the *in vitro* binding data could not completely explain the real situation *in vivo*. To address this possibility, we performed chromatin immunoprecipitation assays with an anti-p50 antibody in IL-17A-stimulated cells. The chromatin immunoprecipitation results were consistent with the promoter assay results. In the absence of IL-17A, there was a persistent interaction between the pκB1 site and the residual p50, whereas very little or no interaction can be seen at both pκB2 and dκB sites. However, after IL-17A treatment, such a protein-DNA interaction can be seen at the pκB2 site, not the dκB site. The binding to the pκB1 site is also enhanced by the IL-17A treatment. This is the first demonstration of the need of these two κB sites to maximize IL-17A-induced *hBD-2* expression.

We speculate that these pκB2 and pκB1 sites can function together as a module to regulate *hBD-2* gene activation. It is conceivable that the discrepancy between the data from the *in vitro* binding assays and promoter assays/chromatin immunoprecipitation assays is that IL-17A may use chromatin remodeling activities. Consequently the finding that dκB binding in the nuclear extracts is activated by IL-17A that does not use the formation of a chromatin structure is in accordance with this hypothesis.

The need for dual κB sites is different from the recent publication in which a gastric cancer cell line showed a single pκB1 site for *H. pylori*-induced *hBD-2* expression (34). The dual κB site requirement is also different from a study using the A549 model, a lung cancer cell line, in which the *hBD-2* expression stimulated by IL-1β and TNF-α is suggested to be through the single pκB1 site (31). Surprisingly our data showed that both pκB2 and pκB1 sites are required for the IL-1β-induced *hBD-2* promoter activity. The discrepancy in the NF-κB binding site requirement of *hBD-2* promoter activity may be related to differences in the cell systems used in these studies. It is interesting to note that A549 is a human alveolar epithelial cell line, whereas HBE1 is a bronchial epithelial cell line. Intriguingly IL-17A failed to induce *hBD-2* expression in A549 cells (data not shown), and our current results further emphasize the cell

type specificity of NF-κB signaling pathways. Nevertheless the minor difference of the mutated κB binding sites might contribute to the discrepancy (47). Furthermore our previous report that IL-17A is much more potent than IL-1β and TNF-α in stimulating *hBD-2* expression (1) was based on a study carried out in well differentiated primary TBE cells, a cell system that is highly relevant to *in vivo* conditions, but not in the cell lines used in other reports.

We also speculate that IL-17A may mediate a second pathway leading to NF-κB activation that is different from the signaling mediated by IκB degradation. Consistent with this notion, we recently demonstrated that IL-17A also activates phosphatidylinositol 3-kinase signaling through a Janus tyrosine kinase (JAK) 1/2-dependent pathway (39). Although this pathway is not involved in NF-κB activation, its signaling is required to exert NF-κB-based transcriptional activity.

Several previous reports have shown that IκB-ζ might be an important regulator for specific NF-κB-regulated genes (42, 43, 48). For example, IκB-ζ could inhibit transactivation of p65 or enhance certain gene expression (42, 49, 50). It has also been reported previously that *IκB-ζ* mRNA stabilization determines the stimulus specificity (43). In our study, it seems that a part of the IL-17A-stimulated *hBD-2* expression is mediated by IκB-ζ. However, results from the actinomycin D experiments did not support the possibility of *IκB-ζ* mRNA stabilization by IL-17A. Again this provides support that the NF-κB response is cell type-specific and that the details of the regulatory roles of IκB-ζ stimulated by IL-17A need to be further investigated.

In conclusion, definitive evidence of NF-κB activation of the *hBD-2* gene promoter has been provided by site-directed mutagenesis of the NF-κB binding site, *in vitro* binding assay, specific recruitment of NF-κB to the *hBD-2* promoter *in situ*, and the dependence on the NF-κB regulator IκB-ζ for gene expression induction. Taking these data together, we propose a pκB2- and pκB1-dependent NF-κB signaling pathway for the transcriptional induction of *hBD-2* gene by IL-17A in airway epithelial cells. To our knowledge, our results add to the growing body of evidence of the important role NF-κB plays in regulating *hBD-2* gene expression and host defense. We believe that the ability of IL-17A to induce *hBD-2* gene expression could play an important role in the airway host defense against bacterial pathogens. Because IL-17A is notable for its ability to stimulate IL-6/IL-8 secretion and to regulate neutrophil migration, it would be intriguing to speculate whether it may play a role in inflammatory airway diseases characterized by neutrophil infiltration such as chronic obstructive pulmonary disease and cystic fibrosis (51). Because NF-κB is one of the most potent transcription factors associated with microbial infections of the airways (51), we suggest that such a signaling mechanism may play a role in regulating and coordinating the adaptive and innate immune responses in the airways.

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