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Distal NF-kB binding motif functions as an enhancer for nontypeable *H. influenzae*-induced DEFB4 regulation in epithelial cells

Jeong-Im Woo [a], Sung-Hee Kil [a], Huiqi Pan [b], Yoo Jin Lee [a], David J. Lim [a], Sung K. Moon [a,*]

[a] Department of Head & Neck Surgery, David Geffen School of Medicine, University of California, Los Angeles, CA, USA
[b] House Research Institute, Los Angeles, CA, USA

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ABSTRACT

Among the antimicrobial molecules produced by epithelial cells, DEFB4 is inducible in response to pro-inflammatory signals such as cytokines and bacterial molecules. Nontypeable *Haemophilus influenzae* (NTHi) is an important human pathogen that exacerbates chronic obstructive pulmonary disease in adult and causes otitis media and sinusitis in children. Previously, we have demonstrated that DEFB4 effectively kills NTHi and is induced by NTHi via TLR2 signaling. The 5′-flanking region of DEFB4 contains several NF-κB binding motifs, but their NTHi-specific activity remains unclear. In this study, we aimed to elucidate molecular mechanism involved in DEFB4 regulation, focusing on the role of the distal NF-κB binding motif of DEFB4 responding to NTHi. Here, we show that the human middle ear epithelial cells up-regulate DEFB4 expression in response to NTHi via NF-κB activation mediated by IκKα/β–IκBα signaling. Deletion of the distal NF-κB binding motif led to a significant reduction in NTHi-induced DEFB4 up-regulation. A heterologous construct containing the distal NF-κB binding motif was found to increase the promoter activity in response to NTHi, indicating a NTHi-responding enhancer activity of the distal NF-κB binding motif. Furthermore, electrophoretic mobility shift assays and chromatin immunoprecipitation assays showed that the p65 domain of NF-κB binds to the distal NF-κB binding motif in response to NTHi. Taken together, our results suggest that NTHi-induced binding of p65 NF-κB to the distal NF-κB binding motif of DEFB4 enhances NTHi-induced DEFB4 regulation in epithelial cells.

1. Introduction

DEFB4, previously known as human β-defensin 2, is a small cationic antimicrobial peptide produced by a variety of epithelial cells [1–3]. DEFB4 predominantly kills Gram-negative bacteria by increasing permeability of the bacterial membrane through pore formation [4]. Unlike DEFB1, DEFB4 is inducible in response to inflammatory stimuli such as pro-inflammatory cytokines [3,5,6] and bacterial molecules [7–9]. A variety of signaling pathways is known to form a network for transcriptional regulation of DEFB4, including SRC-dependent ERK signaling [3], IL-17-dependent JAK signaling [6], and NF-κB signaling mediated by toll/IL-1 receptor [10] and protease-activated receptor 2 [11].

The gene regulatory region of the DEFB4 locus within 3000 base pairs upstream from the exon 1 contains several NF-κB binding motifs involved in DEFB4 regulation, including one distal and two proximal motifs [5,8,12]. Interestingly, a specific NF-κB binding motif contributing to transcriptional regulation of DEFB4 varies with the type of inflammatory stimuli. For example, *Fusobacterium nucleatum*-induced DEFB4 regulation is independent from NF-κB signaling [13] while *Helicobacter pylori* depends on NF-κB for DEFB4 regulation [14]. Furthermore, there exists controversy regarding the enhancer activity of the distal NF-κB binding motif of DEFB4. The distal NF-κB binding motif (−2193/−2184) of DEFB4 is known to significantly contribute to *Pseudomonas aerusinosa*-induced DEFB4 regulation, but not to IL-1β- or LPS-induced DEFB4 regulation [15,16], which led us to focus on the enhancer of DEFB4 responding to NTHi.

NTHi is a small Gram-negative bacterium and an opportunistic pathogen existing as a commensal organism in the human nasopharynx [17]. Unlike *Haemophilus influenzae* type b, NTHi is nontypeable since it lacks a polysaccharide capsule used for typing and rarely causes life-threatening infections [18]. Nevertheless, NTHi is a clinically important pathogen that exacerbates chronic obstructive pulmonary disease in adults, and causes otitis media and sinusitis in children [19,20]. In contrast to lipopolysaccharide (LPS) utilized in other studies [5,8,16], NTHi contains an unique atypical endotoxin, lipooligosaccharide (LOS), structurally different from LPS of the common Gram-negative bacteria. NTHi is predominantly recognized by TLR2 signaling [21] while LPS triggers TLR4 signaling [22]. In our prior study, we demonstrated that DEFB4

* Corresponding author. Address: Department of Head & Neck Surgery, David Geffen School of Medicine, University of California, Los Angeles, 2100W. Third St., Los Angeles, CA 90057, USA.
E-mail address: skmoon@mednet.ucla.edu (S.K. Moon).

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has a potent antimicrobial effect on NTHi [23,24]. We also showed that the middle ear epithelial cells up-regulate DEFB4 in response to NTHi via TLR2/MyD88 signaling and p38 MAPK signaling [7,24]. NF-κB is known to be involved in transcriptional regulation of DEFB4 [5,8,15,16]; however, it remains unclear if the distal NF-κB binding motif of DEFB4 functions as an enhancer for NTHi-induced DEFB4 regulation.

Here, we present that NF-κB activation mediates NTHi-induced DEFB4 regulation through IκKα/β–IκBα signaling. Furthermore, we demonstrate that the NTHi-activated nuclear components interact with the distal NF-κB binding motif to augment DEFB4 up-regulation in response to NTHi. This study will enable us to better understand molecular mechanism involved in transcriptional regulation of DEFB4 in epithelial cells.

2. Experimental procedures

2.1. Plasmid construction, transfection and luciferase assay

The 5′-flanking region (−2625 to +1) of the DEFB4 gene was subcloned to the pGL3-basic (Promega, Madison, WI) as previously described [3], which was named as pDEFB4/−2625/luc. Nested deletions of pDEFB4/−2625/luc (1.7, 1.1 and 0.7 kb) were obtained using the Erase-a-Base® System (Promega) in which exonuclease III was used to specifically digest DNA from a blunt restriction site of BstZ17I (New England Biolabs, Ipswich, MA) and the opposite end was protected from digestion by a 4-base 3′ overhang restriction site of KpnI (New England Biolabs). 200 bp-sized segments of the DEFB4 5′-flanking region (−2503 to −2301, −2324 to −2121, −2131 to −1919, −1938 to −1732 and −1751 to −1642) were subcloned upstream of pTAL-luc, a vector containing the luciferase gene with a TATA-like promoter region from the Herpes simplex virus thymidine kinase (TK) promoter (Clontech, Mountain View, CA). pNFκB-luc was purchased from Clontech, containing multiple copies of the NF-κB consensus sequence fused to pTAL-luc. A transdominant mutant IκKα/β–IκBα [(S32/36A)] was previously described [21,25], and pcDNA 3.1(+) was purchased from Invitrogen. For luciferase assay, cells were seeded into six-well plates at a density of 1.5×10^5 cell/well and transfected at ~60% confluence. pRL-TK vector (Promega) was co-transfected to normalize for transfection efficiency. Luciferase activity was measured using a luminometer (Pharmingen, La Jolla, CA) after adding harvested cell lysate to the luciferase substrate (Promega). Results were expressed as fold-induction of luciferase activity, taking the value of the non-treated group as 1.

2.2. Electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP) assays

Cells were treated with NTHi lysate for 1.5 and 3 h after overnight starvation. Nuclear protein was extracted using the NE-PER® Nuclear extraction reagent (Pierce Biotechnologies) and the protein concentration was determined using a BCA™ protein assay kit (Pierce Biotechnologies) [25]. 5′-Biotin labeled double-stranded oligonucleotide probes were purchased from Integrated DNA Technologies, Inc. (Coralville, IA) as follows: wild type NF-κB 1 (5′-gttactttgggacttccccagctatg-3′) and mutated NF-κB 1 (5′-gttacttt ctagcttccccagctatg-3′). In vitro binding of NF-κB to the DEFB4 promoter was determined using the LightShift® chemiluminescent EMSA kit (Pierce Biotechnologies) according to the manufacturer's instruction. Briefly, nuclear proteins (4 μg) were incubated with a biotin-labeled target DNA probe (20 fmol) in 20 μl of binding buffer for 20 min at RT. Samples were applied to 6% polyacrylamide gels under native conditions in high ionic strength buffer and electrophoresis was performed. As a positive and a negative control, a biotinylated Ebstein–Barr nuclear antigen (EBNA) control DNA (5′-TAGGCATATGCTA-3′) was applied with or without the EBNA extract. To detect biotin-labeled DNA, a 1:300 dilution of Streptavidin-HRP conjugate was applied to the membranes for 15 min. After washing, the chemiluminescent substrate was added and the signal was detected with exposure to X-ray films. To determine if NTHi induces NF-κB p65 to bind with an enhancer in vivo, ChIP assays were performed using Chip-IT™ Express (Active Motif, Carlsbad, CA). Briefly, cells were exposed to NTHi lysate for 2 h and fixed with 1% formaldehyde for 10 min. After lysing cells, we collected the nuclei pellet and chromatins were sheared with the provided enzyme cocktail. 10 μl of the sheared DNA samples were named as "Input DNA" for use as controls in PCR analysis. 1 μg of anti-NF-κB p65 antibody and 25 μl of protein G magnetic beads were added to 10 μg of sheared DNA samples and incubated for 4 h at 4 °C. The samples were placed on the magnetic stand to pellet beads and the supernatant was discarded carefully. After washing, the pelleted beads were resuspended and named as "Chip DNA." After purifying Chip DNA and Input DNA, PCR analysis was performed with the specific primer pairs as follows: NF-κB1 (−2369/−2108: 5′-agtacagcagcagtgatagtgcca-3′ and 5′-ttggtgctgat gtctgagcctt-3′), the positive control (NF-κB2, −687/-423: 5′-ttctcag aggaaggaagtgggcat-3′ and 5′-acagtctcaggccaaattgagagc-3′) and the negative control (−1775/−1400: 5′-tcagcacacaaggaaacaaagccc-3′ and 5′-agcatggtggcttacacctgtcat-3′).

3. Results

3.1. NF-κB activation is required for NTHi-induced DEFB4 regulation in human epithelial cells

NF-κB is critically involved in transcriptional regulation of DEFB4 induced by proinflammatory cytokines and LPS [5,8,16]. NTHi is known to up-regulate DEFB4 expression via TLR2/MyD88 signaling [23] and activate NF-κB via TLR2/TAK1 signaling [21]. Hence, we sought to determine if NF-κB activation is required for NTHi-induced DEFB4 up-regulation in human epithelial cells. The HMEEC cells were exposed to the NTHi lysate, and immunolabeling was performed using an anti-p65 NF-κB antibody. p65 NF-κB was found to translocate into the nucleus upon exposure to the NTHi lysate (Fig. 1A). Moreover, NTHi-induced NF-κB translocation was blocked by CAPE (an inhibitor of NF-κB activation) and Wedelolactone (a selective and irreversible inhibitor of IκKα and β kinase activity). To investigate NTHi-induced NF-κB activation, luciferase assays were performed using a pNFkB-luc construct containing multiple copies of the NF-κB consensus sequence fused to pTAL-luc. As shown in Fig. 1B, NTHi increases NF-κB activity in human epithelial cell lines such as HMEEC (a human middle ear cell line), A549 (a human lung adenocarcinoma epithelial cell line) and HeLa (a human cervical carcinoma cell line). Next, we sought to determine if NF-κB activation is required for NTHi-induced DEFB4 up-regulation. Cells were pre-treated with chemical inhibitors of NF-κB activation, CAPE and JSH-23, before exposure to the NTHi lysate. Real-time quantitative PCR analysis showed that NTHi-induced DEFB4 up-regulation is inhibited more than 70% by either CAPE or JSH-23 (Fig. 1C).

3.2. IκKα/β-mediated IκBα phosphorylation is involved in NTHi-induced DEFB4 regulation

Since IκKα/β–IκBα signaling has been shown to mediate NTHi-induced NF-κB activation [21] we sought to determine if epithelial cells suppress NTHi-induced DEFB4 up-regulation upon exposure to chemical inhibitors of IκKα/β and IκBα. Real-time quantitative PCR analysis showed that NTHi-induced DEFB4