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Opposing roles of PAK2 and PAK4 in synergistic induction of *MUC5AC* mucin by bacterium NTHi and EGF

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Abstract

Mucin, a major component of mucus, plays a critical role in host mucosal defense response by participating in mucociliary clearance. However, if overproduced, overproduced mucus leads to airway mucus obstruction and conductive hearing loss. Despite extensive studies that focus on investigating how *MUC5AC* mucin is regulated by one inducer at a time, how *MUC5AC* is synergistically regulated by multiple factors remains unknown. Here we provide direct evidence for the first time that bacterial pathogen NTHi and human growth factor EGF synergize with each other to potentially up-regulate *MUC5AC* mucin transcription. Moreover, activation of both p38 and ERK is required for synergistic induction of *MUC5AC* by NTHi and EGF. Finally, PAK2 and PAK4 are differentially involved in this synergistic induction of *MUC5AC* by acting upstream of p38 and ERK. Our studies bring novel insights into our understanding of synergistic regulation of *MUC5AC* mucin by both pathological and physiological inducers.

Keywords

PAK2; PAK4; *MUC5AC*; NTHi; EGF; p38; ERK

INTRODUCTION

In the innate immune system, epithelial cells represent the first line of host defense against invading microbes at mammalian mucosal surfaces [1]. They elaborate diverse molecules involved in efficient pathogen clearance. Mucins, the major component of mucus secretions, are high-molecular weight and heavily glycosylated proteins synthesized by the mucosal

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epithelial cells. Under normal conditions, they protect and lubricate the mucosal surface and trap the inhaled microbial pathogens in the upper respiratory tract for mucociliary clearance. However, under diseased conditions such as chronic obstructive pulmonary disease (COPD) and otitis media (OM), the mucociliary clearance mechanism becomes defective. Overproduction of mucin will lead to airway mucus obstruction in COPD and conductive hearing loss in OM [2]. Thus, tight regulation of mucin production plays a critical role in both host mucosal innate defense response and the pathogenesis of respiratory diseases. To date, 20 human mucin genes have been identified [2]. Among these, at least *MUC2*, *MUC5AC* and *MUC5B* have been shown to play an important role in the pathogenesis of respiratory infectious diseases [3,4,5,6,7,8].

The molecular mechanisms underlying regulation of mucin under pathological and physiological conditions still remain largely unknown. Recent studies have suggested that both host- and pathogen-derived factors play critical roles in regulating mucin production. For instance, epidermal growth factor (EGF), a ligand for EGFR, has been shown to regulate mucin production in airways via EGFR/Ras/Raf/ERK pathway [9]. Moreover, Gram-negative bacterium Nontypeable *Haemophilus influenzae* (NTHi) has also been shown to potentially regulate mucin transcription. NTHi is an important human pathogen in both children and adults [10]. In children, it causes OM, one of the most common childhood infections and the leading cause of conductive hearing loss in the United States [11,12], whereas in adults, it exacerbates COPD, the fourth leading cause of patient deaths in the United States [13,14]. A hallmark of both OM and COPD is mucus overproduction that mainly results from up-regulation of mucin [15,16]. Given that EGF and NTHi are coexisting under diseased conditions, how *MUC5AC* mucin is regulated by both EGF and NTHi has yet to be determined.

Mammalian p21-activated kinases (PAKs) are effector proteins of the Rho small GTPases, Rac and Cdc-42 that have been implicated in the regulation of a number of cellular responses, including regulation of MAP kinase signaling pathways, apoptosis, cell cycle and cytoskeletal dynamics [17]. This family of serine/threonine kinases are divided into distinct subgroups based on architectural similarities: group A, comprising PAK1, PAK2 and PAK3, and group B, comprising PAK4, PAK5 and PAK6 [18]. Group B PAKs are quite different from Group A in their structures, implying that Group A and Group B PAKs may be regulated differently and have different downstream effectors.

Based on recent studies that NTHi and EGF are involved in regulating *MUC5AC* transcription, we hypothesized that NTHi and EGF may induce *MUC5AC* mucin transcription in a synergistic manner. Here, we show that NTHi and EGF indeed synergistically induce *MUC5AC* mucin transcription. PAK2 and PAK4 are differentially involved in this synergistic induction of *MUC5AC* by acting upstream of p38 and ERK.

MATERIALS AND METHODS

Reagents

PD98059 and SB203580 were purchased from Calbiochem (La Jolla, CA), Recombinant human EGF was purchased from R&D System.

Bacterial strains and culture condition

NTHi strain 12, a clinical isolate, was used in this study [7,8,19,20]. Bacteria were grown on chocolate agar at 37 °C in an atmosphere of 5% CO₂. For making NTHi crude extract, NTHi were harvested from a plate of chocolate agar after overnight incubation and incubated in 30 ml of brain heart infusion broth supplemented with NAD (3.5 ug/ml). After overnight incubation, NTHi were centrifuged at 10,000 × g for 10 min, and the supernatant was

discarded. The resulting pellet of NTHi was suspended in 10 ml of phosphate-buffered saline and sonicated. Subsequently, the lysate was collected and stored at -70°C . We chose to use NTHi lysates because of the following reasons. First, NTHi has been shown to be highly fragile and undergoes spontaneous autolysis. Its autolysis can also be triggered in vivo under various conditions, including antibiotic treatment. Therefore, using lysates of NTHi represents a common clinical condition in vivo, especially after antibiotic treatment.

Cell culture

HeLa (Human cervix epithelial) cells were maintained in minimal essential medium (ATCC, Manassas, VA), HM3 (Human colon epithelial) cells were cultured in DME H21 (University of California Cell culture Facility, San Francisco, CA), All media received additions of 10% fetal bovin serum (Invitrogen), 100 units/ml penicillin and 0.1 mg/ml streptomycin. HMEEC-1 (human middle ear epithelial) cells were maintained as described [21].

Real-time quantitative PCR analysis of *MUC5AC*, *PAK2* and *PAK4*

Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's instructions. For the reverse transcription reaction, TaqMan reverse transcription reagents (Applied Biosystems) were used. Briefly, the reverse transcription reaction was performed for 60min at 37°C , followed by 60 min at 42°C by using oligo (dT) and random hexamers. PCR amplification was performed by using TaqMan Universal Master Mix for *MUC5AC* or by using SYBR Green Universal Master Mix for human *PAK2* or *PAK4*. In brief, reactions were performed in duplicate containing 2xUniversal Master Mix, 1 μl of template cDNA, 100 nM primers, and 100 nM probe in a final volume of 12.5 μl , and they were analyzed in a 96-well optical reaction plate (Applied Biosystems). Primers and probes for human *MUC5AC* have been previously described [4]. The sequence information is as follows: human *PAK2* forward primer (GTGGACTGAATCACTAGCCTTAGGT), reverse primer (AGCAAAGTAAAATGCAATCTCAC); human *PAK4* forward primer (TCCCCCTGAGCCATTGTG), reverse primer (TGACCTGTCTCCCCATCCA). Reactions were amplified and quantified using an ABI 7500 Real-Time PCR system and the manufacturer's corresponding software (Applied Biosystems). Relative quantity of mRNAs were calculated using the comparative cycle threshold method and normalized by cyclophilin for the amount of RNA used in each reaction (Applied Biosystems).

Plasmids, transfection and luciferase activity assays

The expression plasmids of fp38 α (AF), fp38 β 2(AF), ERK1 DN, ERK2 DN, *PAK2* DN, *PAK4* DN, *PAK2* CA and *PAK4* CA were described previously[8,19,21]. The reporter construct *MUC5AC*-luc was generated as described [22]. It contains 3.7kb 5'-flanking region of the human *MUC5AC* mucin gene in a luciferase reporter vector pGL3. All of the transient transfections were carried out in triplicate using TransIT-LT1 reagent (Mirus, Madison, WI) following the manufacturer's instructions [19,20,21]. In all co-transfections, an empty vector was used as a control. 42h after transfection, the cells were treated with NTHi for 5 h and then harvested for luciferase assay. For experiments with inhibitors, HM3 cells or HeLa cells stably transfected with *MUC5AC*-luciferase plasmid were pretreated with inhibitors for 1h, then treated with NTHi and EGF for 5h, and harvested for luciferase assays.

RNA-mediated Interference

For down-regulating *PAK2* or *PAK4* expression, the *PAK2* and *PAK4* small interfering RNA oligonucleotides were purchased from Dharmacon. A final concentration of 100nM siRNA was transfected into 40–50% confluent HeLa cells using Lipofectamine 2000 Reagent (Invitrogen) by following the manufacturer's instructions. 40h after transfection,

cells were treated with or without NTHi and EGF for 3h before being harvested for Q-PCR analysis of *MUC5AC* mRNA. For measuring the efficiency of siRNA knockdown, Q-PCR was also performed to detect endogenous PAK2 or PAK4.

Western Blot Analysis

To assess phosphorylation of p38, ERK1/2, PAK2 and PAK4, Western Blot Analysis was carried out using antibodies against phospho-p38, p38, phospho-ERK1/2, ERK1/2, phospho-PAK1(Ser144)/PAK2(Ser141), PAK2, phospho-PAK4(Ser474)PAK5(Ser606)PAK6(Ser560) and PAK4 (Cell Signaling Technology, Beverly, MA). Western blots were performed as described [19,21] and following the manufacturer's instructions. Briefly, Western blots were performed using whole cell extracts, separated on 10% SDS-PAGE gels, and transferred to polyvinylidene difluoride membranes (Pall Life Sciences, Pensacola, FL). The membrane was blocked with a solution of TBS containing 0.1% Tween20 (TBS-T) and 5% nonfat milk. After three washes in TBS-T, the membrane was incubated in a 1:1000 dilution of a primary antibody. After another three washes in TBS-T, the membrane was incubated with 1:2000 dilution of the corresponding secondary antibody. The membrane was reacted with ECL plus western blotting detection reagents (Amersham Biosciences) to visualize to blots. Protein concentration were measured by Bio-Rad assay (Bio-Rad, Hercules, CA).

RESULTS AND DISCUSSION

EGF synergizes with bacterium NTHi to induce expression of *MUC5AC* in several human epithelial cell lines

To determine whether NTHi and EGF synergistically induce up-regulation of *MUC5AC* in human epithelial cells, we first measured *MUC5AC* mRNA in human epithelial HM3 cells treated with NTHi and EGF by performing real-time quantitative PCR (Q-PCR) analysis. As shown in Fig. 1A, simultaneous stimulation with NTHi and EGF resulted in a synergistic effect on expression of *MUC5AC* at the mRNA level in HM3 cells. Similar results were also observed in the other human epithelial *MUC5AC*-expression cell lines including HeLa cells and middle ear epithelial HMEEC-1 cells, indicating that the synergistic induction of *MUC5AC* by NTHi and EGF may be generalizable for most human epithelial cells. To investigate whether transcriptional regulation is involved in *MUC5AC* induction, the same epithelial cell lines were transfected with a *MUC5AC* promoter-driven luciferase reporter construct and treated with NTHi and EGF. As shown in Fig. 1B, the *MUC5AC* transcriptional activity was synergistically up-regulated in all the cell lines as indicated, suggesting the involvement of transcriptional regulation. Together, these results indicate that EGF and NTHi synergistically induce *MUC5AC* up-regulation.

p38 MAPK and ERK pathways are positively involved in synergistic induction of *MUC5AC* by NTHi and EGF

Because the p38 MAPK, a major MAP kinase superfamily member, has been shown to be involved in NTHi-induced *MUC5AC* up-regulation [6] and EGF has been shown to regulate mucin transcription in airways via extracellular signal-regulated kinase (ERK) signaling pathway [9], we explored the possibility that activation of p38 and ERK are also involved in the synergistic induction of *MUC5AC*. As shown in Fig. 2A & 2D (upper panels), NTHi and EGF synergistically induced activation of p38 and ERK. We then determined whether activation of p38 or ERK is required for synergistic induction of *MUC5AC* mucin by assessing the effects of perturbing p38 or ERK signaling using either SB203580, a specific inhibitor for p38 MAPK signaling or PD98059, a specific inhibitor for ERK signaling. As shown in Fig. 2A & D (lower panels), both treatments greatly inhibited the synergistic induction of *MUC5AC* at the transcriptional level. The requirement of both signaling

pathways was also confirmed by co-expressing dominant-negative mutant forms of p38 α , p38 β , ERK1 and ERK2 (Fig. 2B & E). Likewise, perturbing p38 or ERK signaling also inhibited *MUC5AC* expression at the endogenous mRNA level (Fig. 2C&F). Together, these data suggested that both p38 and ERK are positively involved in synergistic induction of *MUC5AC* by NTHi and EGF.

PAK2 and PAK4 are differentially involved in the synergistic induction of *MUC5AC* by NTHi and EGF

To determine the role of PAK2 or PAK4 in synergistic induction of *MUC5AC* by NTHi and EGF, we first investigated whether NTHi and EGF synergistically activate PAK2 or PAK4. Phosphorylation of PAK2 and PAK4 were determined by Western blot analysis using antiphosphorylated PAK2 or PAK4 antibody. As shown in Fig. 3A, NTHi and EGF synergistically induced phosphorylation of PAK2 or PAK4 in HM3 cells. It should be noted that synergistic activity of PAK4 is more potent. We next sought to determine the involvement of PAK2 or PAK4 in this synergistic induction of *MUC5AC* by co-expressing dominant-negative mutant and constitutively active forms of PAK2 or PAK4. As shown in Fig. 3B, overexpression of dominant-negative mutant form of PAK2 enhanced synergistic induction of *MUC5AC*, whereas overexpression of dominant-negative mutant form of PAK4 reduced synergistic induction of *MUC5AC*. Consistent with these results, overexpression of constitutively active form of PAK2 reduced synergistic induction of *MUC5AC*, whereas overexpression of constitutively active form of PAK4 enhanced synergistic induction of *MUC5AC* (Fig. 3C). To confirm the requirement of PAK2 or PAK4, we next used PAK2 siRNA and PAK4 siRNA, respectively. We first confirmed the efficiency of both siRNAs in reducing the endogenous PAK2 or PAK4 expression in HeLa cells transfected with PAK2 siRNA, PAK4 siRNA or control siRNA. PAK2 or PAK4 mRNA was markedly reduced by PAK2 siRNA or PAK4 siRNA respectively (Fig. 3D and F). As expected, PAK2 knockdown enhanced the synergistic induction of *MUC5AC* (Fig. 3E), whereas PAK4 knockdown inhibited the synergistic induction of *MUC5AC* (Fig. 3G). Collectively, these data demonstrate the opposing roles of PAK2 and PAK4 in the synergistic induction of *MUC5AC* by NTHi and EGF.

PAK2 and PAK4 are differentially involved in mediating synergistic induction of *MUC5AC* by acting upstream of p38 and ERK signaling pathways

We have demonstrated that PAK2 and PAK4 are differentially involved in synergistic induction of *MUC5AC*. Still unknown is whether both PAK2 and PAK4 act upstream of p38 and ERK signaling pathway in mediating synergistic induction of *MUC5AC* by NTHi and EGF. Because PAKs contribute to a variety of transcriptional signaling cascades, such as p38 and ERK pathways [23,24,25], we hypothesized that PAK2 and PAK4 may act upstream of p38 and ERK signaling pathway in mediating synergistic induction of *MUC5AC*. As shown in Fig. 4A & B, overexpression of dominant-negative mutant form of PAK2 enhanced synergistic activation of p38 or ERK in HM3 cells treated with NTHi and EGF, whereas overexpression of dominant-negative mutant form of PAK4 reduced synergistic activation of p38 or ERK. This data suggest that PAK2 and PAK4 indeed act upstream of p38 and ERK signaling pathway.

In summary, our data demonstrate that NTHi, a major human bacterial pathogen of OM and COPD, synergizes with human growth factor EGF to induce up-regulation of *MUC5AC* in a variety of human epithelial cells (Fig. 4C). Moreover, activation of both p38 and ERK is required for synergistic induction of *MUC5AC* by NTHi and EGF. Finally, PAK2 and PAK4 are differentially involved in this synergistic induction of *MUC5AC* by acting upstream of p38 and ERK. In view of the previous studies in *MUC5AC* mucin regulation, a majority has focused on investigating how *MUC5AC* is regulated by either a pathological or

physiological inducer. It is still unclear whether *MUC5AC* is synergistically regulated by both pathological and physiological inducers. In the present study, we provided direct evidence for the first time that a bacterial pathogen NTHi and human growth factor EGF can actually synergize with each other to potentially up-regulate *MUC5AC* mucin transcription. These results imply that the molecular mechanism underlying up-regulation of mucin by multiple factors may be quite different from that by a single inducer. These studies may help us to design more feasible therapeutic strategy for inhibiting mucus overproduction in the pathogenesis of COPD and OM.

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Figure 1. EGF synergizes with bacterium NTHi to induce expression of *MUC5AC* in several human epithelial cell lines

(A) EGF synergistically enhanced NTHi-induced expression of *MUC5AC* at mRNA level in human HM3, HeLa and middle ear HMEEC-1 cells, as assessed by real time Q-PCR analysis. (B) EGF synergistically enhanced NTHi-induced expression of *MUC5AC* at transcriptional level in human HM3, HeLa and HMEEC-1 cells, as assessed by *MUC5AC*-dependent promoter luciferase assays. Values are means $\pm$ S.D.(n=3). Data are representative of three or more independent experiments.

Figure 2. p38 MAPK and ERK pathways are positively involved in synergistic induction of *MUC5AC* by NTHi and EGF

(A) Synergistic induction of phosphorylation of p38 was observed in HM3 cells treated with NTHi and EGF (upper panel). SB203580 (1 μ M), a specific inhibitor for p38 MAP kinase, inhibited synergistic induction of *MUC5AC* at both transcription level (A.lower panel) and (C) at mRNA level in heLa cells. (B) Overexpressing dominant-negative mutant forms of p38 α or p38 β also inhibited synergistic induction of *MUC5AC* at transcription level by NTHi and EGF in HM3 cells. (D) Synergistic induction of phosphorylation of ERK was observed in HM3 cells treated with NTHi and EGF (upper panel). PD98059 (5 μ M), a specific inhibitor for MEK kinase, inhibited synergistic induction of *MUC5AC* at both transcription level (D.lower panel) and (F) at mRNA level in HM3 cells. (E) Overexpressing dominant-negative mutant forms of ERK1 or ERK2 also inhibited synergistic induction of *MUC5AC* at transcription level by NTHi and EGF in HM3 cells. Values are means $\pm$ S.D. (n=3). Data are representative of three or more independent experiments.

Figure 3. PAK2 and PAK4 are differentially involved in the synergistic induction of *MUC5AC* by NTHi and EGF

(A) Synergistic induction of phosphorylation of PAK2 and PAK4 are observed in HM3 cells treated with NTHi and EGF. (B) Overexpression of dominant-negative mutant form of PAK2 enhanced synergistic induction of *MUC5AC*, whereas overexpression of dominant-negative mutant form of PAK4 reduced it at transcriptional level in HM3 cells. (C). Overexpression of constitutively active form of PAK2 reduced synergistic induction of *MUC5AC*, whereas overexpression of constitutively active form of PAK4 enhanced it at transcriptional level in HM3 cells. (D). The efficiency of siRNA-PAK2 in reducing endogenous PAK2 mRNA was confirmed by real-time Q-PCR analysis. (E). PAK2 knockdown by using siRNA-PAK2 (100nM) enhanced synergistic induction of *MUC5AC* at mRNA level by NTHi and EGF in HeLa cells. (F) The efficiency of siRNA-PAK4 in reducing endogenous PAK4 mRNA was confirmed by real-time Q-PCR analysis. (G). PAK4 knockdown by using siRNA-PAK4 (100nM) inhibited synergistic induction of *MUC5AC* at mRNA level by NTHi and EGF in HeLa cells. Values are means $\pm$ S.D. (n=3). Data are representative of three or more independent experiments.

Figure 4. PAK2 and PAK4 act upstream of p38 and ERK signaling pathway in mediating the synergistic induction of *MUC5AC* by NTHi and EGF

(A) Overexpression of dominant-negative mutant form of PAK2 enhanced synergistic induction of phosphorylation of p38 or ERK in HM3 cells treated with NTHi and EGF. (B) Overexpression of dominant-negative mutant form of PAK4 reduced synergistic induction of phosphorylation of p38 or ERK in HM3 cells treated with NTHi and EGF. Data(A and B) are representative of three or more independent experiments. (C). Schematic representation depicting how NTHi and EGF synergistically induce *MUC5AC* transcription in human epithelial cells.