Notch Signaling Downregulates MUC5AC Expression in Airway Epithelial Cells through Hes1-Dependent Mechanisms

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Key Words

MUC5AC · Notch · Airway mucus hypersecretion · Transcription

Abstract

Background: Mucus overproduction is one of the major pathological features of asthma, and MUC5AC is the major mucin component of airway mucus. However, whether Notch signaling is implicated in the regulation of MUC5AC expression in airway secretory cells is still undetermined. **Objective:** The aim of this study is to examine whether Notch signaling can regulate MUC5AC expression and explore the molecular mechanisms. **Methods:** Mouse mtCC1–2 cells and human NCI-H292 cells were transfected with NIC, and MUC5AC expression was examined. Using gene reporter assays, site-directed mutagenesis, and ChIP assays, the activity of both mouse and human MUC5AC promoter was analyzed. **Results:** Notch signaling regulated MUC5AC expression both in mouse mtCC1–2 cells and in human NCI-H292 cells. Several Hes-binding site N-boxes were identified in the 5' region of both mouse and human MUC5AC promoters. Overexpression of NIC resulted in activation of the MUC5AC promoter. Site-directed mutagenesis report assays revealed that Hes proteins might repress both mouse and human MUC5AC promoter activity. Furthermore, ChIP assays confirmed that Hes1 binds to the MUC5AC promoter both in mouse mtCC1–2 cells and in human NCI-H292 cells. **Conclusions:** Notch sig-

naling can directly downregulate MUC5AC promoter activity through Hes1-dependent mechanisms, which may be identified as possible targets for pharmacotherapy of airway mucus hypersecretion in asthma.

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Introduction

Mucus overproduction is one of the major pathological features of asthma, which is generally thought to be responsible for small-airway obstruction and lung dysfunction that is closely linked to morbidity and mortality in asthma [1]. However, there is no specific therapy for mucus overproduction in asthma patients at present [2]. The major mucin components of airway mucus are MUC5AC and MUC5B, which contribute to the viscoelastic properties of the mucus [2]. In addition, MUC5AC has been implicated as a marker of goblet cell metaplasia in lung pathologies [3]. Though several studies have linked IL-13 and EGFR pathways to MUC5AC expression [1], more signaling pathways leading to MUC5AC expression remain to be established.

Notch signaling is a highly conserved pathway. Interaction between Notch ligands and receptors triggers a γ -secretase-mediated proteolysis of the receptors and liberation of the Notch intracellular domain (NIC) into cytoplasm. NIC then translocates into the nucleus, where it binds to and transactivates the transcription factor RBP-J.

This leads to activation of the Notch downstream target genes Hes and Hey. Hes proteins are suppressive basic helix-loop-helix (bHLH) molecules. In the lung, the best characterized Notch target is Hes1 [4].

Several studies have implicated Notch signaling in MUC5AC expression and goblet cell fate, both in development and in disease. Inhibition of Notch signaling by γ -secretase inhibitors results in an increased number of intestinal goblet cells and MUC5AC expression [5, 6]. Conversely, constitutive Notch activation impairs goblet cell differentiation and MUC5AC expression in the intestine [7, 8]. In addition, during postnatal life Notch is required to prevent Clara cells from differentiating into goblet cells [9]. However, more studies are needed to determine the exact molecular mechanisms of how Notch signaling regulates MUC5AC expression in airway epithelial cells.

In the present study, we show that Notch signaling directly targets the *MUC5AC* promoter. Hes1 can bind to the N-boxes (Hes-binding site) located in the 5' region of the *MUC5AC* gene. Hes1 is a transrepressor of the *MUC5AC* promoter in vitro. Furthermore, Hes1 binds to the *MUC5AC* promoter both in mouse mtCC1–2 cells and in human NCI-H292 cells. Taken together, our results suggest that MUC5AC expression may be directly regulated by Notch signaling in airway epithelial cells.

Materials and Methods

Cloning and Construction

The mouse *Muc5ac* promoter (from –1,450 to +132) was amplified by PCR using primers 5'-ACTCACTCATTCACAAGTCACTCCTCTA and 5'-ACTTTGAGTCATTACCTGTGCATTGGCTG. The human *MUC5AC* promoter (from –1,366 to +47) was amplified by PCR using primers 5'-GGCACCTGGGCTAGACAGGACA and 5'-TGTGTGGACGGCGGGGAAGAGTG. The amplified promoter fragments were cloned into the pGL3-basic vector (Promega Life Science, Madison, Wisc., USA) to generate pGL3-mMuc5ac and pGL3-hMUC5AC. Site-directed mutagenesis of N-boxes in the *MUC5AC* promoter was performed using a QuickChange kit (Stratagene) according to the manufacturer's instructions. Reporter constructs derived from these mutant versions of the *MUC5AC* promoter were named pGL3-mMuc5ac-*Nbox1*, pGL3-mMuc5ac-*Nbox2*, and pGL3-hMUC5AC-*Nbox* (fig. 3b, c, 4b).

Reporter Assay

mtCC1–2 cells (mouse transformed Clara cell line) (2×10^4) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine (Invitrogen). NCI-H292 cells (human mucoepidermoid cell line) were cultured in RPMI 1640 supplemented with 10% FCS, 4 mM glutamine, 1 mM sodium pyruvate, 10 mM Hepes, and antibiotics. Cells were transfected with 0.1 μ g reporter construct, 0.1 μ g pEF-

Bos-NIC-neo [10, 11], and 5 ng renilla luciferase vector (phRL-TK; Promega, Madison, Wisc., USA) using Lipofectamine 2000™ (Invitrogen). The total amount of transfected DNA was balanced with pEF-Bos-neo. Forty-eight hours after transfection, luciferase activity was assessed using a Luminoskan Ascent (Labsystems, Helsinki, Finland) and a Dual-Luciferase Reporter Assay kit (Promega) according to the manufacturer's protocol. All luciferase activity was normalized with renilla luciferase activity. Data were analyzed using t tests. $p < 0.01$ and $p < 0.05$ were considered statistically significant.

RT-PCR

Two micrograms of total RNA were reverse-transcribed with reverse transcriptase (Promega) according to the manufacturer's instructions. All PCR experiments were performed with Taq polymerase (Promega) and the primers 5'-ACTGTTACTATGCGATGTGTAGCCA and 5'-GAGGAAACACATTGCACCCGA for mouse *MUC5AC*, 5'-AGCCCGGCGCCGTGGTCTCCT and 5'-GGCGGGGCTCTT GCTGGTGTGGT for human *MUC5AC*, 5'-AACCCTAAGGCCAACCGTGAAAAG and 5'-GCAGGATGCGTGAGGGAGAG for β -actin control.

Western Blotting

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were saturated for 1 h at room temperature in TBST supplemented with 5% skim milk and immunoblotted overnight at 4°C with anti-mouse *Muc5ac* (sc-71620; Santa Cruz Biotechnology, Santa Cruz, Calif., USA), anti-human *MUC5AC* (sc-20118; Santa Cruz Biotechnology), anti-Notch1 (NIC, M-20; Santa Cruz Biotechnology), anti-Hes1 (H-140; Santa Cruz Biotechnology), or anti- β -actin antibody (I-19; Santa Cruz Biotechnology). Membranes were then washed, probed with horseradish peroxidase (HRP)-conjugated goat anti-rat, rabbit anti-goat, or goat anti-rabbit polyclonal antibodies (Zhong-Shan Biotech, Beijing, China) according to the first antibody, and revealed with enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using a ChIP assay kit following the recommendations of the supplier (Upstate Biotechnology). Anti-Hes1 antibodies (sc-13844; Santa Cruz Biotechnology) were used for immunoprecipitation of chromatin along with controls. PCR primers for ChIP assays included: mouse N-boxes, 5'-CTGTCTGTCTCTCTGTCTCT and 5'-CATTTGAACCTTCTGGTTGC, and human N-boxes, 5'-CATGGGAGGAATGGCAGGA and 5'-TGCCGGGTAGAGCCCAGTT.

Results

Ectopic Overexpression of NIC Can Regulate MUC5AC Expression

To investigate whether Notch signaling can regulate MUC5AC expression, we upregulated Notch signaling in mouse mtCC1–2 cells and detected the expression of *Muc5ac*. The results showed that the expression of *Muc5ac* significantly decreased in pEF-Bos-NIC-neo-transfected

Fig. 1. Ectopic overexpression of NIC regulated MUC5AC expression. **a** MUC5AC mRNA in the transfected mtCC1-2 cells was detected by RT-PCR with β -actin as a control (n = 5). **b** MUC5AC, NIC, and Hes1 proteins in the transfected mtCC1-2 cells were detected by Western blotting with β -actin as a control for protein

loading (n = 5). **c** MUC5AC mRNA in the transfected NCI-H292 cells was detected by RT-PCR with β -actin as a control (n = 5). **d** MUC5AC, NIC, and Hes1 proteins in the transfected NCI-H292 cells were detected by Western blotting with β -actin as a control for protein loading (n = 5).

cells both in mRNA level and in protein level compared to pEF-Bos-neo-transfected cells (fig. 1a, b; MUC5AC mRNA, $p < 0.01$; MUC5AC, $p < 0.05$). Similarly, upregulation of Notch signaling decreased MUC5AC expression in human NCI-H292 cells (fig. 1c, d; MUC5AC mRNA, $p < 0.01$; MUC5AC, $p < 0.01$). In addition, the regulatory effects of NIC are dose dependent (data not shown).

Sequence Alignment Analysis of the Human and Mouse MUC5AC Promoters

To determine whether Notch signaling directly regulated the MUC5AC promoter, we analyzed the genomic sequence of the mouse (NT_039437.8) and human (NT_009237.18) MUC5AC promoters. An alignment of the mouse Muc5ac promoter (−1,864 to +316) with the human counterpart is depicted in figure 2a, showing the consensus transcriptional start site (TSS) and TATA box of the MUC5AC promoters. In the 5' region of both mouse and human MUC5AC promoters, we found several Hes consensus binding motifs, defined as N-box (CACNAG) (fig. 2b).

Effects of the Hes-Binding Sites on the Mouse Muc5ac Promoter

To observe whether these putative transcription factor-binding sites modulated transcriptional activity, we amplified the mouse Muc5ac promoter (−1,450/+312) and constructed a reporter plasmid pGL3-mMuc5ac. Co-transfection of mtCC1-2 cells with the reporter and increasing doses of NIC expression vector pEF-Bos-NIC gradually decreased Muc5ac promoter activity (fig. 3a).

To look at the function of the N-boxes in the Muc5ac promoter, we disrupted these N-boxes by site-directed mutagenesis and examined the consequence using the reporter assay (fig. 3b). As shown in figure 3c, mutation of the N-box at position −893 resulted in an increase in promoter activity. However, disruption of the N-box located at −1,439 had no influence on the activity of the promoter. These results suggested that Hes proteins might repress mouse Muc5ac promoter activity.

Because Hes1 plays a critical role in regulating airway epithelial development, we next investigated whether

Fig. 2. a Sequence alignment of the human and mouse *MUC5AC* promoters. TATA box, TSS, and N-boxes are indicated. **b** Scheme of the 5' region of the mouse and human *MUC5AC* promoter, indicating the potential Hes recognition sites.

Fig. 3. Effects of the Hes-binding sites on the mouse *MUC5AC* promoter. a Reporter assay. mtCC1–2 cells were transfected with pGL3-*MUC5AC* (100 ng) and gradient doses of NIC (0, 60, 80, and 100 ng) as well as other plasmids as indicated. The relative luciferase activity (firefly luciferase/renilla luciferase) was analyzed 48 h later. The results are presented as means $\pm$ SD. ** $p < 0.01$ ($n = 3$). **b** Scheme of the site-directed mutant of the mouse *MUC5AC* in which the N-boxes were disrupted. **c** Reporter assay. mtCC1–2 cells were transfected independently with the wild type and deletion mutant of the *MUC5AC* reporter constructs and were assayed as in figure 3a. **d** ChIP. Chromatin preparations from mtCC1–2 cells were immunoprecipitated using anti-Hes1, and co-precipitated DNA fragments were amplified by primers specific for the N-box fragment of the *MUC5AC* promoter, respectively. Normal goat serum was used as a control. Luc = Luciferase.

Hes1 could bind to the mouse *Muc5ac* promoter in mtCC1–2 cells using the ChIP assay using anti-Hes1. The co-precipitated chromatin DNA fragments were amplified by PCR using primers for the *Muc5ac* promoter region harboring the N-box (–1,057 to –857). The results

showed that the fragment with the Hes1-binding site was co-precipitated by the anti-Hes1 antibody (fig. 3d). The result further supported that the mouse *Muc5ac* promoter might be regulated by Notch signaling through Hes1-dependent mechanisms.

Fig. 4. Effects of the Hes-binding sites on the human *MUC5AC* promoter. **a** Reporter assay. NCI-H292 cells were transfected with pGL3-hMUC5AC, and they were assayed as in figure 3a. **b** Scheme of the site-directed mutant of the human *MUC5AC* in which the N-boxes were disrupted. NCI-H292 cells were transfected independently with the wild type and mutant of the *MUC5AC* reporter constructs, and they were assayed as in figure 3a. **c** ChIP. Chromatin preparations were collected from NCI-H292 cells and were done as in figure 3d. Luc = Luciferase.

Effects of the Hes-Binding Sites on the Human *MUC5AC* Promoter

We also amplified the human *MUC5AC* promoter (–1,366/+47) and constructed a reporter plasmid pGL3-hMUC5AC. Gene report assays revealed that co-transfection of NCI-H292 cells with pEF-Bos-NIC gradually decreased human *MUC5AC* promoter activity (fig. 4a). Mutation of the N-box at position –293 increased the activity of the human *MUC5AC* promoter (fig. 4b). Similarly, ChIP assays showed that the human *MUC5AC* fragment with the Hes1-binding site (–404 to –168) could be co-precipitated by the anti-Hes1 antibody (fig. 4c). So, the human *MUC5AC* promoter might also be regulated by Notch signaling through Hes1-dependent mechanisms.

Discussion

In the present study, we described that Notch signaling regulated the expression of *MUC5AC* both in mouse mtCC1–2 cells and in human NCI-H292 cells. In vivo, a similar phenomenon is also observed in that *MUC5AC* expression increases in airway epithelial cells in a conditional inactivation of the essential Notch pathway component *Pofut1* mouse model [9]. So, Notch signaling was implicated in the regulation of *MUC5AC* expression in airway secretory cells.

Then, we identified several Hes-binding site N-boxes in the 5' region of both mouse and human *MUC5AC* promoters. This phenomenon suggested that these mo-

tifs might have some regulatory role in the regulation of *MUC5AC* expression. Indeed, using a luciferase reporter assay, we showed that overexpression of NIC in both mtCC1–2 cells and NCI-H292 cells increased the transactivation of the *MUC5AC* promoter. Site-directed mutagenesis reporter assays revealed that Hes proteins might repress both mouse and human *MUC5AC* promoter activity. ChIP assays confirmed the binding of Hes1 to the *MUC5AC* promoter regions. Thus, we believe that Notch signaling could regulate *MUC5AC* expression through an Hes1-dependent mechanism.

However, a recent study suggested that co-transfection with Hes5 full-length cDNA in LA4 cells also decreased the transcriptional activity of the *MUC5AC* promoter [9]. So, we speculate that the inhibitory effect of Notch on *MUC5AC* promoter activity might be shared by several bHLH factor downstream targets of Notch, including Hes1 and Hes5. The exact regulatory pattern may depend on the cell type, stimulatory factors, and the cellular surrounding environment, which remains to be further investigated. In addition, Notch signaling can regulate some posttranscriptional processes. Whether some posttranscriptional mechanisms are also involved in the regulatory process is still undetermined.

Kang et al. [12] showed that Notch signaling can induce EGFR and ERK phosphorylation and then upregulate *Muc5ac* expression. On the other hand, epidermal growth factor (EGF) stimulated generation of the Notch intracellular domain in an RBP-J-dependent manner

[12]. Based on our results, we speculate that a bidirectional regulatory mechanisms may exist in the expression of MUC5AC through cross talk between Notch signaling and EGFR signaling.

The regulation of MUC5AC expression by Notch through Hes1-dependent mechanisms described here is likely to be relevant in the context of human disease. Failure of this mechanism may be related to airway epithelium malfunction that leads to mucus hypersecretion in conditions such as asthma. In asthmatic patients, mucus plugging of the airway lumen has been reported as one of the major contributing factors of fatal asthma [13, 14]. Even in mild and moderate asthma, an increase in MUC5AC expression has been reported [15, 16]. A recent study reported that stimulation of NCI-H292 cells with grass pollen allergen, which is a most common allergen that can lead to allergic rhinitis and asthma, results in the alteration of gene expression levels in 38 genes out of 74 genes that are involved in Notch signaling [17]. Meanwhile, expression of Notch1 and Hes1 mostly decreased, i.e. -2-fold and -3.3-fold respectively. However, no change was observed in the expression of Hes5 [17].

This is consistent with our findings that Hes1 binds to the MUC5AC promoter regions. Notch signaling plays an important role in the pathogenesis of asthma by controlling T cell differentiation. However, only very limited data is available about whether Notch signaling can regulate the function of airway structural cells and then participate in the pathogenesis of asthma. Future experiments may further examine the effect of regulation of Notch signaling in airway epithelial cells on the phenotype of asthma in vivo. In conclusion, this study showed that Notch signaling can directly regulate MUC5AC promoter activity through Hes1-dependent mechanisms both in mouse mtCC1-2 cells and in human NCI-H292 cells, which may be identified as possible targets for pharmacotherapy of airway mucus hypersecretion in asthma.

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