IL-13–induced airway mucus production is attenuated by MAPK13 inhibition

Yael G. Alevy,1 Anand C. Patel,1,2 Arthur G. Romero,1 Dhara A. Patel,1 Jennifer Tucker,1 William T. Roswit,1 Chantel A. Miller,1 Richard F. Heier,1 Derek E. Byers,1 Tom J. Brett,1,3,4 and Michael J. Holtzman1,3

1Drug Discovery Program, Pulmonary and Critical Care Medicine, Department of Medicine, 2Department of Pediatrics, 3Department of Cell Biology, and 4Department of Biochemistry, Washington University School of Medicine, St. Louis, Missouri, USA.

Increased mucus production is a common cause of morbidity and mortality in inflammatory airway diseases, including asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis. However, the precise molecular mechanisms for pathogenic mucus production are largely undetermined. Accordingly, there are no specific and effective anti-mucus therapeutics. Here, we define a signaling pathway from chloride channel calcium-activated 1 (CLCA1) to MAPK13 that is responsible for IL-13–driven mucus production in human airway epithelial cells. The same pathway was also highly activated in the lungs of humans with excess mucus production due to COPD. We further validated the pathway by using structure-based drug design to develop a series of novel MAPK13 inhibitors with nanomolar potency that effectively reduced mucus production in human airway epithelial cells. These results uncover and validate a new pathway for regulating mucus production as well as a corresponding therapeutic approach to mucus overproduction in inflammatory airway diseases.
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Figure 1
CLCA1 is necessary for IL-13–stimulated mucus production in human airway epithelial cells. (A) hTECs were incubated with IL-13 (50 ng/ml) under submerged conditions for 2 days and then air-liquid interface conditions for up to 21 days, and cell lysates were analyzed for CLCA1, CLCA2, and CLCA4 mRNA levels by real-time qPCR assay. CLCA3 mRNA was undetectable under these conditions (data not shown). (B) Corresponding levels of MUC5AC mRNA. (C) Corresponding levels of CLCA1 in cell lysate and apical cell supernatant determined by ELISA. (D) Corresponding levels of MUC5AC determined by ELISA. (E) Corresponding immunocytochemistry for DAPI, CLCA1, and MUC5AC using confocal microscopy. Scale bar: 50 μm. (F) Corresponding immunocytochemistry at a more apical (high z axis) and subjacent (low z axis) cellular location. Arrows indicate the same reference cells for high and low z axis. Scale bar: 50 μm. (G) Levels of CLCA1 and MUC5AC mRNA in hTECs that were transduced with lentivirus encoding CLCA1 or control shRNA. (H) For conditions in E, corresponding cellular levels of CLCA1 and MUC5AC by ELISA. All values represent mean ± SEM. *P < 0.05, increase from corresponding no IL-13 treatment control or decrease from no shRNA treatment.

increased activity against MAPK13 and found that these novel compounds effectively block IL-13–stimulated mucus production in human airway epithelial cells. The results thereby validate a novel therapeutic approach to hypersecretory diseases of the pulmonary airways and perhaps other sites as well.

Results
CLCA1 controls mucin gene expression. The mouse Clca gene family contains at least 7 members, and studies of the predominantly upregulated member (mouse Clca3) provide evidence for redundancy, since Clca3–/– mice still develop IL-13–dependent mucus production after viral infection and exhibit upregulation of a functional mouse Clca5 gene (14). Moreover, when we analyzed IL-13–stimulated mucus production in mouse airway epithelial cells in primary culture using more sensitive, specific, and quantitative real-time PCR assays than were used previously (20), we found induction of mouse Clca5 and newly discovered mouse Clca7 in addition to the predominant mouse Clca3 gene expression (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI64896DS1). These findings raise the possibility that similar redundancy (and therefore difficulty in assigning function) might also occur in humans (16).

However, the human CLCA gene family contains only 4 members, and, in contrast with the mouse system, we found that human CLCA1 but not CLCA2, CLCA3, or CLCA4 gene expression was increased in concert with IL-13 induction of mucous cell metaplasia in well-differentiated human airway epithelial cells, in this case using human tracheal and bronchial epithelial cells (hTECs) maintained under air-liquid interface conditions (Figure 1, A and B). Levels of CLCA2 and CLCA4 were increased during cell differentiation under air-liquid interface conditions, but neither was increased by IL-13 treatment. Levels of CLCA3 mRNA were undetectable under these conditions. Moreover, whole-genome microarray analysis (and corresponding quantitative PCR [qPCR] assays) of gene expression showed marked upregulation of CLCA1 and MUC5AC mRNA levels relative to all other genes in IL-13–stimulated hTECs (Supplemental Figure 2). Mucus production was significantly increased in MUC5AC mRNA levels without significant changes in MUC5B gene expression (Supplemental Figure 3), consistent with a previous report (21) and indicative of other controls or cellular sources for MUC5B expression (22, 23). The increases in CLCA1 and MUC5AC mRNA resulted in corresponding increases in CLCA1 and MUC5AC protein levels, with significant detection of these proteins in cell lysate and apical cell supernatant (Figure 1, C and D). Confocal microscopy of IL-13–stimulated hTECs immunostained for CLCA1 and MUC5AC showed colocalization in a spatial pattern of apical MUC5AC coupled with subjacent CLCA1 (Figure 1, E and F, and Supplemental Video 1). Immunostaining was specific for CLCA1 and MUC5AC since there was no staining above background in the absence of primary antibody (Supplemental Figure 3). These findings are consistent with coordinated packaging of CLCA1 and MUC5AC within mucous granules as described previously (24, 25) and, based on the present findings, concerted synthesis and secretion of CLCA1 and MUC5AC to the apical cell surface in response to IL-13–stimulation as well.

To define the functional role of CLCA1 in regulating mucus production, we next analyzed the effect of specific siRNA-mediated knockdown of CLCA1 gene expression. We found that stably transducing hTECs with siRNA directed against CLCA1 resulted in quantitative inhibition of IL-13–driven mucus production (Figure 1, G and H). In this system, lentiviral transfection efficiency ranged from 50% to 60% (Supplemental Figure 3) and resulted in a similar quantitative decrease in MUC5AC mRNA and protein levels. The inhibitory effect was limited to IL-13–inducible mucus production, with no significant effect on basal mucus production. In addition, the inhibitory effect was specific to CLCA1 since siRNA-mediated knockdown did not significantly affect CLCA2 or CLCA4 mRNA levels, and CLCA3 was undetectable under these conditions (Supplemental Figure 3). Together, the results indicate that CLCA1 is a master regulator of the IL-13 to mucin gene expression signaling pathway and, in contrast to the mouse system (14), appears to function without redundancy from other CLCA gene products. Thus, further studies were performed in the human system.

CLCA1-MAPK signaling in cultured cells. To determine the signaling mechanism for CLCA1-dependent induction of mucin gene expression, we next used NCI-H292 cells (derived from a lung mucoepidermoid carcinoma) to establish a cell line with stable expression of the CLCA1 gene under the control of a doxycycline-inducible promoter system (referred to herein as NCI-H292-rtTA-CLCA1 cells) (as diagrammed in Figure 2A). Analysis of NCI-H292-rtTA-CLCA1 cells indicated that overexpression of CLCA1 was sufficient for mucin gene expression based on dose-dependent induction of CLCA1 and MUC5AC gene expression (Figure 2, B and C). As we found in primary-culture hTECs, the increases in CLCA1 and MUC5AC mRNA levels were accompanied by concomitant increases in CLCA1 and MUC5AC protein (Figure 2, D–F). The capacity of CLCA1 expression to be sufficient for mucin gene expression is consistent with our previous observations in mice, wherein gene transfer using a viral vector encoding the mouse Clca3 gene was also sufficient for mucin gene expression in vivo (14).

We next assessed downstream signaling from the CLCA1 gene product using phospho-MAPK antibody arrays to screen for any possible MAPK activation subsequent to doxycycline activation of CLCA1 expression. We observed that doxycycline treatment of NCI-H292-rtTA-CLCA1 cells caused time-dependent activation of 12 MAPKs (Figure 3A and data not shown). The largest doxycycline-induced increase in MAPK phosphorylation (doxycycline treatment compared with no treatment) was found for MAPK14 (Figure 3A), consistent with MAPK14 inhibitor blockade of cytok-
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Clara Cell 16 Expression Is Sufficient for Mucus Production in Lung Epithelial Cells. (A) Diagram for constructs used to establish an NCI-H292 cell line carrying rtTA- and CLCA1-expression cassettes. (B) Doxycycline dose response for mucin gene expression in the NCI-H292-rtTA-CLCA1 cell line. Cells were treated with doxycycline (0–10 μg/ml) for 48 hours, and CLCA1 and MUC5AC mRNA levels were determined by real-time qPCR assay. (C) Time course for mucin gene expression in NCI-H292-rtTA-CLCA1 cells. Cells were treated with doxycycline (7.5 μg/ml) for 0 to 72 hours. (D) Dose response for mucin protein levels in NCI-H292-rtTA-CLCA1 cells. Cells were treated with doxycycline for 48 hours, and MUC5AC levels were determined by cell-based ELISA. (E) Time course for mucin protein levels in NCI-H292-rtTA-CLCA1 cells. Cells were treated with doxycycline (75 μg/ml for 0 to 72 hours), and MUC5AC levels were determined by ELISA. (F) Representative immunocytochemistry for NCI-H292-rtTA-CLCA1 cells treated with doxycycline (75 μg/ml for 48 hours) and then incubated with Sytox nuclear stain and immunostained for CLCA1 and MUC5AC. Scale bar: 100 μm. (B–E) Values represent mean ± SEM. *P < 0.05, increase from corresponding no doxycycline treatment control.

Figure 2

Clara cell 16 expression is sufficient for mucus production in lung epithelial cells. (A) Diagram for constructs used to establish an NCI-H292 cell line carrying rtTA- and CLCA1-expression cassettes. (B) Doxycycline dose response for mucin gene expression in the NCI-H292-rtTA-CLCA1 cell line. Cells were treated with doxycycline (0–10 μg/ml) for 48 hours, and CLCA1 and MUC5AC mRNA levels were determined by real-time qPCR assay. (C) Time course for mucin gene expression in NCI-H292-rtTA-CLCA1 cells. Cells were treated with doxycycline (7.5 μg/ml) for 0 to 72 hours. (D) Dose response for mucin protein levels in NCI-H292-rtTA-CLCA1 cells. Cells were treated with doxycycline for 48 hours, and MUC5AC levels were determined by cell-based ELISA. (E) Time course for mucin protein levels in NCI-H292-rtTA-CLCA1 cells. Cells were treated with doxycycline (75 μg/ml for 0 to 72 hours), and MUC5AC levels were determined by ELISA. (F) Representative immunocytochemistry for NCI-H292-rtTA-CLCA1 cells treated with doxycycline (75 μg/ml for 48 hours) and then incubated with Sytox nuclear stain and immunostained for CLCA1 and MUC5AC. Scale bar: 100 μm. (B–E) Values represent mean ± SEM. *P < 0.05, increase from corresponding no doxycycline treatment control.
MAPK13 is necessary for CLCA1-driven mucus production in lung epithelial cells. (A) NCI-H292-rTA-CLCA1 cells were treated with doxycycline (7.5 μg/ml for 18 hours) with or without BIRB-796 (10 μM), and cell lysates were analyzed by phospho-MAPK antibody array. The box indicates results for MAPK values. Values represent the percentage of positive control (mean ± SEM). *P < 0.05, increase from corresponding no doxycycline control. (B) Cells were treated as in A at indicated concentrations of BIRB-796, SB-203580, and vehicle control, and levels of CLCA1 and MUC5AC mRNA were determined by real-time qPCR assay. (C) Corresponding MUC5AC levels determined by cell-based ELISA. (D) Cells were transfected with or without control or MAPK13 or MAPK14 siRNA, (25 nM) and treated with or without doxycycline (7.5 μg/ml for 48 hours), and levels of MAPK13, MAPK14, and CLCA1 mRNA were determined by real-time qPCR. (E) Corresponding levels of MUC5AC mRNA for conditions in D. (F) Corresponding MUC5AC levels determined by cell-based ELISA for conditions in D. (B–F) Values represent mean ± SEM. *P < 0.05, decrease from corresponding no drug treatment or no siRNA control.
We subsequently found that BIRB-796 markedly inhibited doxycycline-induced MUC5AC mRNA and MUC5AC protein production, whereas SB-203580, which more selectively blocks MAPK14 (30), did not significantly inhibit MUC5AC synthesis (Figure 3, B and C). We found no effect of MAPK inhibition on CLCA1 expression. We next used MAPK13 RNAi to suppress expression and thereby define loss of function for MAPK13 in the regulation of mucin gene expression. We found that suppression of MAPK13 expression did not influence doxycycline-induced expression of CLCA1 but completely blocked expression of MUC5AC gene expression (Figure 3, D and E). By contrast, treatment with MAPK14 RNAi did not block doxycycline-induced MUC5AC synthesis or influence CLCA1 mRNA levels (Figure 3, D and E). Blockade of MUC5AC mRNA induction was reflected

Figure 4
MAPK13 is necessary for IL-13–driven mucus production in human airway epithelial cells. (A) hTECs were incubated with IL-13 for 21 days, as described in Figure 1, with or without BIRB-796 or SB-203580, and levels of CLCA1 and MUC5AC mRNA were determined by real-time qPCR assay. (B) Corresponding MUC5AC levels determined by cell-based ELISA for conditions in A. (C) hTECs were transduced with lentivirus encoding MAPK13, MAPK14, or control shRNA and then were treated with IL-13 for 21 days, as described in Figure 1. Cell lysates were analyzed for CLCA1, MAPK13, and MAPK14 mRNA levels using real-time qPCR assay. (D) Corresponding levels of MUC5AC mRNA. (E) Corresponding MUC5AC levels determined by cell-based ELISA. (A–E) Values represent mean ± SEM. *P < 0.05, decrease from corresponding no drug treatment or shRNA control condition.
inhibition of corresponding MUC5AC protein levels (Figure 3F). These results placed MAPK13 downstream of CLCA1 and upstream of MUC5AC gene expression in NCI-H292 cells.

A similar influence of MAPK13 over mucin gene expression was found in primary-culture hTECs incubated with IL-13. Thus, p38-MAPK blockade with BIRB-796 markedly suppressed IL-13–induced MUC5AC gene expression, without affecting corresponding CLCA1 mRNA levels, whereas treatment with SB-203580 had no significant effect on CLCA1 or MUC5AC gene expression (Figure 4, A and B). Furthermore, suppression of MAPK13 expression (using 2 different shRNA sequences delivered with a lentiviral vector) did not influence IL-13–induced expression of CLCA1 but significantly blocked the expected increase in MUC5AC mRNA levels (Figure 4, C and D). In this system, lentiviral transfection efficiencies of 50%...
The increases in CLCA1 and MAPK13 gene expression (consistent with our previous observations in a smaller group of subjects; refs. 6, 8) as well as increased CLCA1 and MAPK13 gene expression (Figure 5A). The increases in CLCA1 and MUC5AC mRNA were also reflected in increased levels of the corresponding proteins (Figure 5B). Immunostaining of lung tissue samples demonstrated colocalization of CLCA1 and MUC5AC in mucosal mucous cells (Figure 5C). Furthermore, increases in CLCA1 and MAPK13 gene expression were accompanied by significant increases in MAPK13 activation that were relatively prominent compared with those of other MAPKs (Figure 5D). Thus, IL-13 stimulation of a CLCA1/ MAPK13 signaling pathway to mucin genes found in human airway epithelial cells in culture appears to also be activated in human lungs in vivo during chronic obstructive lung disease.

We also investigated whether the homologous Clca/Mapk signaling pathway could be found in whole lungs of mice. However, we recognized that the observed differences between the mouse Clca and human CLCA gene loci and the apparent redundancy in mouse Clca responsiveness to IL-13 signaling might indicate a corresponding difference in downstream human CLCA versus mouse Clca signaling as well. Nonetheless, the availability of Mapk13-deficient mice (32) provided a suitable genetic approach for testing whether a comparable signaling system might be present in a potentially useful animal model. In fact, similar to that in the human system, we found an increase in mouse Clca mRNA levels in response to IL-13 administration in the lungs of Mapk13−/− mice and same-strain wild-type control mice. However, we found no difference in levels of Muc5ac mRNA in the lung or corresponding Muc5ac immunostaining in the airways of Mapk13−/− mice compared with those of wild-type mice (Supplemental Figure 5).

Similarly, we found the usual increase in mouse Clca3 but no difference in Muc5ac mRNA response to IL-13 in primary-culture mouse tracheal epithelial cells from Mapk13−/− and wild-type control mice (Supplemental Figure 5). Together, these findings reinforced the position that the mouse model was not representative of the human for IL-13–dependent control of airway mucus production and that further studies to inhibit excess mucus production might be best performed in humans or human subject materials.

**Design and validation of MAPK13 inhibitors.** Taken together, our results in human cells and lungs pointed to a central role for MAPK13 in regulating airway mucus production and therefore suggested the utility of a more potent MAPK13 inhibitor. To develop such a compound, we used a drug discovery strategy known as scaffold hopping that starts with a known active compound and ends with a novel chemical entity by modifying the central core structure of the molecule (33, 34). In the present case, the strategy was designed to take advantage of MAPK13 and MAPK4 homology (60% sequence identity) and thereby provide new analogs of MAPK4 inhibitors with improved affinity for MAPK13. We recognized that BIRB-796 exhibited potent inhibition of MAPK4 (the original target for this compound) but relatively weak activity for MAPK13 (29). The selectivity of BIRB-796 for MAPK4 is based largely on incorporating a naphthalene group into part of the molecule (i.e., a naphthalene moiety) that can readily occupy a distinct binding site (designated the gatekeeper pocket) in MAPK4 but not the more confined pocket in MAPK13. The difference in access to this pocket appeared to be under the control of the adjacent gatekeeper residue, which is a small threonine in MAPK4 compared with a large methionine in MAPK13. To initiate our structure-based drug design approach, we first crystallized and determined the structure of inactive uncomplexed MAPK13 at high resolution. Indeed, superposition of the MAPK14–BIRB-796 cocystal structure onto MAPK13 revealed interference (i.e., a steric clash) between the gatekeeper methionine and the inhibitor naphthalene moiety (Figure 6A and Supplemental Figure 6).
Within our chemical series, the most active DFG-out compounds (Figure 6G). This analysis further confirmed that, concentration-response curves were performed for representative compounds (e.g., compound 124) with otherwise similar structure but exhibit greater potency for MAPK13. We were particularly interested in BIRB-796 because it binds to MAPK14 in the Asp-Phe-Gly-out mode (DFG-out mode) (35). This binding mechanism results in rotation of the conserved DFG motif out of the Phe-binding pocket (i.e., DFG-out) and thereby produces a prolonged dissociation rate that might offer a pharmacological advantage (35–37). We were also interested in the gatekeeper methionine (Supplemental Figure 7). Analysis of analog-MAPK13 cocrystals confirmed successful binding that preserved contacts and hydrogen bonds with the same MAPK13 residues (Figure 6, B and C; Supplemental Figure 8; and Supplemental Table 2). However, while some analogs (e.g., compound 61) demonstrated the expected DFG-out binding mode, other analogs (e.g., compound 124) adopted DFG-in binding due to conformational constraints imposed by the fit to the binding pocket. In concert with these results, analogs with DFG-out binding (e.g., compound 61) maintained slow dissociation kinetics, while those exhibiting DFG-in binding (e.g., compound 124) formed short-lived complexes (Figure 6, D and E). We anticipated that these structural and kinetic differences might translate into differences in compound potencies in enzyme and cellular assays of MAPK13 activity.

All analogs were tested for inhibition of MAPK13 enzyme activity using an immobilized metal affinity polarization (IMAP) assay that detected fluorescence polarization as a function of remaining MAPK13 activity. As expected, DFG-out binding compounds (e.g., compound 61 and, a similar compound, compound 62) displayed highly potent MAPK13 inhibition, whereas DFG-in binding compounds (e.g., compound 124) with otherwise similar structure displayed 65-fold to 120-fold lower inhibitory activity (Figure 6F). Structurally distinct AMG-548 and SB-203580 also exhibited weak inhibitory activity consistent with lower affinity for MAPK13 compared with MAPK14 (30, 38). More detailed 16-point inhibitor concentration-response curves were performed for representative compounds (Figure 6G). This analysis further confirmed that, within our chemical series, the most active DFG-out compounds (i.e., compounds 61 and 62) were more potent than a representative DFG-in compound (i.e., compound 124) and compared favorably with less selective inhibitors designed to inhibit MAPK14, i.e., AMG-548 and SB-203580 (Figure 6G and Supplemental Table 3). As expected, all of these compounds continued to show inhibitory activity against MAPK14 as well (Supplemental Table 4 and Supplemental Figure 9), since the compounds should continue to fit into the MAPK14 gatekeeper pocket and no changes were made to interfere with binding at other sites in the MAPK molecules.

Nonetheless, the greater potency of newly designed compounds for blocking MAPK13 suggested that these compounds would have greater potency for blocking IL-13–dependent mucus production. Accordingly, we next tested the effect of representative analogs on mucus production in comparison to the effects of compounds designed to inhibit MAPK14. We found that potency for MAPK13 inhibition correlated closely with blockade of mucus production. Thus, the most potent MAPK13 inhibitors (compounds 61 and 62) suppressed IL-13–induced MUC5AC production in primary-culture human airway epithelial cells with 50% inhibition at 10⁻⁶ M, whereas treatment with less potent MAPK13 inhibitors (compound 124 and AMG-548) had no significant effect on MUC5AC level until treatment with 10⁻⁶ M (Figure 6H). None of the tested compounds resulted in significant changes in transepithelial electrical resistance, even at 10⁻⁶ M, indicating a lack of cytotoxic effect despite treatment for 21 days (Supplemental Figure 10). Taken together, the findings reveal a potent and effective action of rationally designed MAPK13 inhibitors in blocking IL-13–induced mucus production in primary-culture human airway epithelial cells under culture conditions that maintain the features of a well-differentiated airway mucosa found in vivo.

**Discussion**

In this report, we establish the critical role of CLCA1 and MAPK13 in controlling excess mucus production that is typical of inflammatory airway disease. We use genetic and chemical tools to define a signaling pathway that begins with IL-13 induction of CLCA1 gene expression and proceeds to CLCA1-mediated activation of MAPK13 and consequent MUC5AC mucin gene expression (Figure 7). Pathway function was established by gain and loss of function for CLCA1 and MAPK13 in primary-culture human airway epithelial cells and representative cell lines and in lung explants from patients with excess mucus due to COPD. This approach to...
incorporate human subjects and materials was essential due to the substantial differences in CLCA protein families between humans and other species (including mice) (14, 16) and the consequence divergence in downstream CLCA signaling as well.

Indeed, the first reason for the present advance in understanding CLCA1 function was the shift from mouse to human model systems. Thus, the initial discovery of the role of CLCA proteins in airway disease was derived from studies of inbred and intercrossed mice in which whole-genome assessment revealed linkage between airway disease and mucus production after respiratory viral infection (14). However, this model also showed that loss of Clca3 function (using newly developed Mapk13–/– mice) did not influence IL-13–driven mucus production and led to the initial concern that CLCA proteins were not involved in regulating excess mucus production during inflammatory airway disease. However, further genetic analysis also revealed that the mouse Clc gene family contained at least 7 members, so that gene duplication and functional redundancy could account for preservation of the postviral mucus phenotype if only a single Clc gene was inactivated. In fact, additional analysis of this mouse model showed that other Clc family members were also sufficient for driving mucus production (14, 15). Similarly, in this study, we found IL-13 upregulation of mouse Muc5ac and Clca7 as well as predominant Clca3 gene expression in mouse airway epithelial cells. In addition, we found that the connection between CLCA expression and MAPK13 activation present in humans was not apparent in mice. Thus, IL-13 induction of Muc5ac gene expression was not blocked by Mapk13 deficiency (using Mapk13–/– mice compared with wild-type control mice) either in vivo (using mice treated with inhaled IL-13) or in vitro (using primary-culture mouse tracheal epithelial cells treated with IL-13). It was therefore critical to assess the epithelial mucus system in humans to establish selective induction of CLCA1 and activation of MAPK13 as key events for regulating IL-13–inducible mucus production and secretion in isolated cells and whole lungs.

A second reason for the current progress on CLCA1 function relates to the role of CLCA1 as a signaling molecule compared with the original assignment (based on sequence homology) as an integral membrane protein that functioned as a calcium-activated chloride channel (CaCC). In that regard, even the initial bioinformatic (16) and experimental data (17, 18, 39) indicated that CLCA proteins generally lack the structural features to form ion channels by themselves, since they either contain only a single transmembrane anchor or exhibit secretion from the cell in soluble form. Moreover, subsequent findings were more consistent with the proposal that CLCA1 effects on chloride transport may be based on increasing conductance of endogenous CaCCs (40). It further appears (based on analysis of crude membrane preparations) that secreted CLCA proteins are first processed intracellularly into N-terminal and C-terminal peptide fragments (18, 39, 41–44). The present findings support this alternative proposal as well, in that CLCA1 is colocalized within the cell along with MUC5AC in secretory granules and is secreted to the apical cell surface in response to IL-13. Our initial structural work with purified human CLCA1 protein suggests that a novel form of self-cleavage is required for secretion and that the consequent production of the N-terminal fragment of CLCA1 is sufficient for stimulating CaCC activity (45). The present connection between CLCA1 and MAPK13 activation and downstream mucin gene expression further support the proposal that CLCA1 functions as a signaling molecule, since MAPKs in general and p38-MAPKs in particular have always been acknowledged to transmit extracellular signals to intracellular targets via upstream receptors (46, 47). The nature of a putative CLCA1 receptor that might be linked to MAPK13 activation is currently under study. Similarly, at least some CaCCs may also trigger MAPK signaling (e.g., TMEM16A stimulates ERK1/2 activation) (48), but the CaCC signaling pathway to activate chloride transport still needs to be defined. Nonetheless, the combination of previous and present work serves to position CLCA1 as a central signaling protein for controlling both the glycoprotein and fluid components of mucus production.

In regard to CLCA1 control of mucus production, a third reason for the present advance was the need for an unbiased approach to CLCA1 signaling targets and the particular advantage of antibody arrays to screen for these targets to find MAPK13. The relatively unstudied MAPK13 was originally described as responsive to inflammatory cytokines and cellular stress but downstream targets were uncertain (49). Subsequent reports suggested a role for MAPK13 in keratinocyte differentiation and survival (50, 51), microtubule assembly and neurogeneration (52), attenuation of insulin secretion (53), and cell proliferation in skin cancer and cholangiocarcinoma (54, 55); however, no complete scheme has emerged for MAPK13 activity in human disease. Instead, most previous work has concentrated on other MAPK family members, primarily MAPK14, as contributing to inflammation in the airway and other sites (56) as well as driving mucus production (12, 26). Based on these observations, drug development activity was generally directed at MAPK14, yielding inhibitors with increased kinase selectivity and antiinflammatory activity (19, 57). The present results assign a new function for MAPK13 and should turn the field to a different strategy for the study of MAPK biology and blockade. Though here again we caution an approach that uses the mouse model, in which MAPK13 has recently been shown to regulate other types of kinase-dependent responses (e.g., TLR4-driven ERK1/2 activation in immune cells) (58).

Finally, a key reason for the lack of previous progress on MAPK13 function was the absence of potent chemical inhibitors of MAPK13. Indeed, this project benefited greatly from the identification of more potent MAPK13 inhibitors. We developed these inhibitors guided by structural analysis of MAPK13-inhibitor co-crystals and scaffold hopping from compounds that were designed previously to bind selectively to the homologous MAPK14. Our approach allowed for the discovery of potent MAPK13 inhibitors that better fit the MAPK13 gatekeeper pocket and maintained a DFG-out mode with slow dissociation kinetics (35, 36). Compounds that achieved these end points (e.g., compounds 61 and 62) provided for an approximate 100-fold increase in MAPK13-inhibitor complex half-life and a 100-fold increase in mucus inhibition. These properties should translate into more sustained and potent effects for use as tool compounds and for further drug development. It is also possible that the combination of gatekeeper- and Phe-pocket binding will improve kinase selectivity. However, the present inhibitors maintain their activity against MAPK14. Thus, the chemical approach to MAPK13 inhibition was scientifically complemented and validated by the concomitant genetic approach, and additional work will have to be done to fully characterize the kinase specificity of the present compounds and perhaps to develop others with greater specificity. Recent success in developing extremely selective MAPK14 inhibitors (59, 60) suggests that this is an achievable step. Although compounds with combined MAPK13 and MAPK14 activity may
offer a clinical advantage for diseases such as COPD that manifest both mucus and inflammatory components in the disease process. Indeed, there is ongoing clinical development of MAPK14 inhibitors to block inflammation in COPD and asthma (61, 62). In either case, the present identification and validation of the CLCA1 to MAPK13 signaling pathway to excess mucus production offers an opportunity to better define the regulation of a major disease end point and to respond to the unmet need for an anti-mucus therapeutic for inflammatory airway disease.

Methods
See the Supplemental Methods for more details.

Primary culture of human airway epithelial cells. hTECs were isolated by enzymatic digestion, seeded onto permeable filter supports, and grown as described previously (6). For the present experiments, cells were cultured in DMEM-Ham’s F12 medium with 2% NuSerum (BD), Primocin (100 μg/ml, InvivoGen), and retinoic acid (1 μM) in DMEM-Ham’s F12 medium with 2% NuSerum (BD), Primocin (100 μg/ml, InvivoGen), and retinoic acid (1 μM) with or without IL-13 (50 ng/ml, Peprotech) under submerged conditions for 2 days and then air-liquid interface conditions for 3 weeks. Cells were also cultured in the presence or absence of inhibitors that were added 1 day before addition of IL-13 and were readied with each IL-13 treatment (twice a week). Transepithelial electrical resistance of cell cultures was monitored as described previously (63).

Real-time qPCR assay. Target mRNA levels were quantified with real-time qPCR using fluorogenic probe/primer combinations specific for CLCA1, CLCA2, CLCA4, MUC5AC, MAPK13, MAPK14, and GAPDH (Supplemental Table 5). All PCR assays were quantitative and used plasmids containing the target gene sequences as standards. All probes were designed to span an intron and did not react with genomic DNA. All real-time PCR data were normalized to the level of GAPDH (1 × 10^6 copies) to correct for variation between samples as described previously (8).

Immunostaining. Cells were fixed, permeabilized, treated for epitope retrieval, and then were incubated with mouse anti-MUC5AC biotin-conjugated mAb and rabbit anti-CLCA1 antibody followed by Alexa Fluor 488- or 555–conjugated streptavidin or Alexa Fluor 555– or 633–conjugated goat anti-rabbit secondary antibody, counterstained with DAPI or Sytox Green and then imaged by confocal or conventional microscopy. Lung sections were incubated with citrate-based Antigen Unmasking Solution (Vector Laboratories) for antigen retrieval, and then were incubated with mouse anti-MUC5AC biotin-conjugated antibody and rabbit anti-CLCA1 antibody, followed by Alexa Fluor 488–conjugated streptavidin and Alexa Fluor 555– or 633–conjugated goat anti-rabbit IgG.

Generation of shRNA-expressing cells. Lentiviral vectors expressing siRNA from shRNA were obtained from the MISSION shRNA library (Sigma-Aldrich). A set of shRNA clones (constructed within the lentiviral vector) were obtained from the MISSION shRNA library for antigen retrieval, and were treated for epitope retrieval, and then were incubated with mouse anti-MUC5AC mAb and anti-CLCA1 antibody, followed by Alexa Fluor 488–conjugated streptavidin and Alexa Fluor 555–conjugated goat anti-rabbit IgG.

Generation of shRNA-expressing cells. Lentiviral vectors expressing siRNA from shRNA were obtained from the MISSION shRNA library (Sigma-Aldrich). A set of shRNA clones (constructed within the lentivirus plasmid pLKO.1-Puro) was obtained for each of the following mRNA targets: CLCA1 (NM_001285), MAPK13 (NM_002754), and MAPK14 (NM_001315). Lentiviral particles for each of the clones were generated by transfecting HEK 293T cells with shRNA-pLKO.1-Puro plasmid, pHR8.2deltaR packaging plasmid, and pCMV-VSV-G envelope plasmid using Fugene6 (Roche Applied Science) as the transfection agent (64). The shRNA lentiviral particles were added to the apical and basal sides of hTEC cultures for 24 hours at MOI 1, and 10 days later, cells were treated with or without IL13 as described above.

MUC5AC ELISA. For cell-based MUC5AC ELISA, cells were fixed and incubated with blocking buffer followed by mouse anti-MUC5AC mAb and then NeutrAvidin-HRP (Thermo Pierce Biotechnology) for absorbance at 450 nm. Cell number was determined with 0.5% crystal violet staining and absorbance at 595 nm. Values for MUC5AC levels were expressed as ratio of absorbance at 450 nm to 595 nm. For standard-based MUC5AC ELISA, the plasmid encoding the 45M1 epitope of MUC5AC was obtained from G.C. Hansson (Goteburg University, Gothenburg, Sweden), and the epitope was expressed in 293F cells and purified as described previously (65). Protein cell and tissue lysates were plated on Nunc Maxisorp ELISA Plates (Thermo Nalgé Nunc) in carbonate-bicarbonate buffer and incubated at 37°C overnight. The plates were then washed with PBS-0.05% Tween-20 (Sigma-Aldrich) and blocked overnight with Sea Block blocking buffer (Thermo Pierce Biotechnology). The reaction was developed using the 45M1 biotin-conjugated mAb antibody and avidin-HRP using the 1-Step UltraTMB-ELISA substrate (Thermo Pierce Biotechnology) and assessed for absorbance relative to standard 45M1 concentration.

CLCA1 ELISA. For determinations of CLCA1 in cell lysates, the CLCA1 ELISA was based on rabbit anti-CLCA1 polyclonal antibody raised against N-terminal amino acids 681–693 for detection and purified recombinant CLCA1-3xFLAG containing these residues as standard. For determinations of CLCA1 in cell lysate versus supernatant and in lung tissue samples, the CLCA1 ELISA was based on anti-CLCA1 mAb (clone 8D3) raised against N-terminal CLCA1 amino acids 22–694 and purified N-terminal CLCA1 as standard.

CLCA1-expressing cell lines. To generate lung cell lines that stably expressed CLCA1 driven by the tetracycline-inducible gene promoter system, human lung mucoepidermoid carcinoma NCI-H292 cells were transfected with pCMV-rTA (rTetR-VP16) (Clontech), and clones were selected with G418 (200 μg/ml). Clones with low background and high induction of luciferase gene expression when transfected with pTRE-Tight-luciferase (Clontech) and treated with doxycycline (10 μg/ml) were selected for a second transfection with a TRE-driven CLCA1-expressing plasmid. Cell lines (NCI-H292-rTATLCA1 lines) with stable expression of pCMV-rTA and pTRE-CMV-CLCA1 were selected with G418 and hygromycin (20 μg/ml). The CLCA1 gene was cloned from hTEC RNA and was inserted into pTRE-Tight using 5′-NotI and 3′-EcoV restriction enzyme sites. All constructs were verified by DNA sequencing.

MAPK phospho-arrays. MAPK activation was assessed using a human phospho-MAPK antibody array (Proteome Profiler MAPK Array, R&D Systems) according to the manufacturer’s instructions. The chemiluminescent signal from the arrays was captured on film, digitized with a PowerLook 1120 scanner (UMAX) using SilverFast Ai software (LaserSoft Imaging), and quantified with ArrayVision 8.0 software (GE Healthcare Life Sciences).

Gene knockdown with siRNA. MAPK13 or MAPK14 Stealth RNAi (Life Technologies) was transfected into NCI-H292-rTATLCA1 cells using Lipofectamine RNAiMax (Life Technologies). Cells were then incubated in the RNAi-Lipofectamine mixture for 24 hours at 37°C and then with doxycycline for an additional 24 hours. Cellular RNA was isolated and reversed transcribed using the High-Capacity cDNA Archive Kit (Life Technologies), and target mRNA levels were determined by qPCR assay.

Human lung samples. Human tissue was obtained from lung explants harvested at the time of lung transplantation from recipients (n = 20) with very severe COPD (GOLD stage IV) during the period from 2005–2009 at Barnes-Jewish Hospital. Control samples were obtained from donors without COPD (n = 11) using both lung explants that were otherwise not useable for transplantation and excess lung tissue that was resected for down sizing. For airway-specific analyses of gene and protein expression, computed tomography imaging and gross histologic examination was used to select airway-containing tissue cores in COPD explants (n = 11) and frozen lung tissues from donors without COPD (n = 7) as described previously (31). Each analysis consisted of samples from recipients with COPD with the same clinical characteristics (as summarized in Supplemental Table 1). All human studies were conducted with protocols approved by the Washington University School of Medicine Institutional Review Board.
MAPK inhibitor assay. The MAPK binding activity test of 30 compounds was determined using an IMAP assay containing activated MAPK13, FITC-labeled substrate, and test compound. IMAP assays were performed employing the linear phase of the rate kinetics. Assay reactions contained 0–100 μM test compound, 5–35 mM (EC50) activated MAPK13, 3 μM (Km,app) ATP, and 100 nM FITC-labeled EGFR peptide substrate (FITC-KRELEVCLTPS-GEAPNQALLR-NH2). The IC50 values for each compound were determined from the compound concentration versus fluorescence polarization (mP) plot using nonlinear curve fitting.

MAPK crystallization and structure determination. Human MAPK13 (amino acids 1–352) was expressed and purified for crystallization as described in the Supplemental Methods. Crystals were obtained by mixing protein solution (at 10 mg/ml) with reservoir solution (50 mM ammonium tartrate, 18% PEG 3350) in a 4:1 (protein/reservoir) ratio. These crystals were used to obtain complex structures with compounds by soaking. Briefly, compounds dissolved in DMSO at a concentration of 100 mM were added to crystallization drops at one-tenth volume for a final concentration of 10 mM compound in the drop and allowed to soak for 2 to 4 hours. X-ray diffraction data were collected at Advanced Photon Source beamline 19ID (nonphosphorylated) and Advanced Light Source (ALS) beamline 4.2.2 (compounds 61 and 124). Data were processed using HKL-2000 software (66). The phase problem was solved by molecular replacement using Phaser crystallographic software (67). For nonphosphorylated MAPK13, the probe structure was a deposited structure of MAPK13 from a structural genomics project (PDB code: 3COI). Although our structure displayed similar crystal packing to the previously deposited one, there were noticeable large differences in the C-terminal region revealed in our high-resolution structure (Supplemental Figure 6). The structures of MAPK13 with compounds were isomorphous to the nonphosphorylated structure, so they were determined by rigid body refinement. Compounds were clearly visible in F0 – F difference maps following rigid body refinement (Supplemental Figure 8). Compounds were fit to electron density maps manually using Coot software (68), and refinement was carried out in PHENIX software (69). Ramachandran analysis was as follows (% favored/percent outliers): nonphosphorylated, 95.6/0.6; compound 61, 96.1/0.3; and compound 124, 95.5/0.9. Final coordinates and experimental statistics are provided in Supplemental Table 2. All molecular graphics figures were produced using PyMOL software.

MAPK13 small molecule kinetic assay. Kinetics of MAPK13 binding to small molecules was assessed using BioLayer interferometry with an Octet (FortelBio). Super-streptavidin-coated biosensors from ForteBio were used to capture biotinylated MAPK13 onto the surface of the sensor. After reaching baseline, sensors were moved to association step for 300 seconds and then dissociated for 300 seconds. Curves were corrected by a double-refining technique, using both biotin-coated pins dipped into the experimental wells and a buffer-only reference. MAPK13-inhibitor complex half-lives were calculated from dissociation constants determined from global kinetic analysis.

Statistics. Values for gene expression were analyzed using a 1-way or 2-way ANOVA for a factorial experimental design. PCR data were compared by unpaired Student’s t test with Welch’s correction for unequal variances when appropriate. Inhibitor effects were analyzed using 2-way ANOVA. P values of less than 0.05 were considered significant.

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Address correspondence to: Michael J. Holtzman, Washington University School of Medicine, Campus Box 8052, 660 South Euclid Avenue, St. Louis, Missouri 63110, USA. Phone: 314.362.8970; Fax: 314.362.9009; E-mail: holtzmann@wustl.edu.


