Activation of NF- κ B via a Src-dependent Ras-MAPK-pp90rsk pathway is required for *Pseudomonas aeruginosa*-induced mucin overproduction in epithelial cells

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ABSTRACT Cystic fibrosis (CF) is an autosomal recessive disorder, the most common lethal genetic disease in Caucasians. Respiratory disease is the major cause of morbidity and mortality. Indeed, 95% of CF patients die of respiratory failure. *Pseudomonas aeruginosa*, an opportunistic pathogen, chronically infects the lungs of over 85% of CF patients. It is ineradicable by antibiotics and responsible for airway mucus overproduction that contributes to airway obstruction and death. The molecular mechanisms underlying this pathology are unknown. Here we show that *P. aeruginosa* activates a c-Src-Ras-MEK1/2-MAPK-pp90rsk signaling pathway that leads to activation of nuclear factor NF- κ B (p65/p50). Activated NF- κ B binds to a κ B site in the 5'-flanking region of the *MUC2* gene and activates *MUC2* mucin transcription. These studies bring new insight into bacterial-epithelial interactions and more specifically into the molecular pathogenesis of cystic fibrosis. Understanding these signaling and gene regulatory mechanisms opens up new therapeutic targets for cystic fibrosis.

Cystic fibrosis (CF) is the most common lethal genetic disease in the Caucasian population (1). The mutation responsible for the disease is in the gene encoding the cystic fibrosis transmembrane regulator (CFTR), a chloride channel. This mutation gives rise to a chain of events involving chronic bacterial lung infection and mucus overproduction. Although there have been significant clues regarding the link between the mutation and chronic infection (2–4), the nature of the link between the mutation and mucus overproduction is completely unknown.

We recently showed that bacterial exoproducts up-regulate epithelial *MUC2* mucin transcription (5). This suggests that CF-associated mucin overproduction occurs secondary to lung infection, a *sine qua non* of advanced disease. The molecular mechanisms underlying mucin induction are unknown. In the present study, we performed experiments to examine these mechanisms. Results showed that *Pseudomonas aeruginosa*, a common CF pathogen, activates the *MUC2* mucin gene transcription by activation of a Src-dependent Ras-MEK1/2-ERK1/2-pp90rsk-NF- κ B pathway. These studies provide insight into bacterial-host epithelial interactions and open up new therapeutic targets for CF.

MATERIALS AND METHODS

Reagents. *P. aeruginosa* lipopolysaccharide (LPS) from serotype 10 was purchased from Sigma. PP1, PD98059, and

caffeic acid phenethyl ester (CAPE) were purchased from Calbiochem.

Bacterial Strains and Culture Conditions. The *P. aeruginosa* strains used in these studies were grown in M9 medium with aeration at 37°C to late logarithmic phase. The broth cultures were then centrifuged at 10,000 rpm in a Sorvall RC5C for 50 min. The supernatants containing bacterial exoproducts were sterilized by passage through a 0.22- μ m polymer filter (Corning) and were then kept at –80°C until use. Bacterial culture supernatants were added to epithelial cell culture medium at a 1:4 dilution ratio.

Cell Culture. Two *MUC2*-expressing epithelial cell lines were used in these studies. NCIH292 cells (a *MUC2*-expressing airway epithelial cell line) were maintained in RPMI 1640 medium. HM3 cells (a *MUC2*-expressing colon epithelial cell line) were maintained in Dulbecco's modified Eagle's medium. All media received additions of 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin.

RNAse Protection Assay (RPA). The experiments were carried out with an RNA probe containing *MUC2*-specific sequence as described previously (5).

Cloning and Sequencing of the 5'-Flanking Region of Human *MUC2* Gene, Plasmid Construction, Transfection, and Luciferase Assay. The 5'-flanking region of the human *MUC2* gene was cloned by screening a human placental 1FIXII genomic library, using the 5' region of human *MUC2* cDNA as the probe as described (5–7). The 5'-flanking region was sequenced by dideoxynucleotide sequencing. Deletional mutants of the 5'-flanking region DNA were obtained by combining restriction digestion of the upstream region of the gene and PCR amplification. The restriction DNA fragments or PCR-amplified fragments were ligated into a luciferase reporter gene. All junctions and identifications of the DNA sequences in the chimeric constructs were confirmed by DNA sequencing. The expression plasmid of a dominant-negative mutant form (pp90rsk Δ C) of the 90-kDa ribosomal S6 kinase (pp90rsk) was a gift from Warner Greene (Univ. of California, San Francisco). v-Src was a gift from J. Michael Bishop (University of California, San Francisco). SrcRF was a gift from Joan Brugge (Harvard Medical School, Boston). RasN17 was a gift from G. Cooper (Harvard Medical School). HMEK1

Abbreviations: CAPE, caffeic acid phenethyl ester; CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; C/EBP, CCAAT-enhancer binding protein; EMSA, electrophoretic mobility-shift assay; ERK, extracellular signal-regulated kinase; JNK, c-Jun amino-terminal kinase; JNKK, c-Jun amino-terminal kinase kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NF- κ B, nuclear factor κ B; pp90rsk, the 90-kDa ribosomal S6 kinase; RPA, RNAse-protection assay; SEK, SAPK/ERK kinase.

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FIG. 1. Characterization of NF- κ B site and NF- κ B in mucin *MUC2* induction by *P. aeruginosa*. (A) Human *MUC2* regulatory regions (base pairs -2864 to +14, -1627 to +14, and -1308 to +14) were subcloned upstream of a luciferase reporter gene in pGL2 basic vector and transfected into HM3 cells. (B) Human *MUC2* regulatory regions (base pairs -1628 to -1307, -1528 to -1307, and -1430 to -1307) were subcloned upstream of a TK-32 promoter luciferase vector (named as -1.6/-1.3TK, -1.5/-1.3TK, and -1.4/-1.3TK, respectively) and transfected into HM3 cells. In both A and B, transfected cells were treated with either *P. aeruginosa* culture supernatants or vehicle for 6 hr prior to cell lysis. Luciferase activity was then assessed in *P. aeruginosa*-treated and nontreated cells. Nuclear proteins were incubated with a probe consisting of the 32 P-labeled double-stranded oligonucleotides corresponding to the human *MUC2* regulatory region base pairs -1458/-1430 in the absence (C) or presence of various mutant unradilabeled oligonucleotides (M1 to M4) as indicated (D), or preincubated with antibodies to NF- κ B p65, NF- κ B p50, c-Rel, or C/EBP (E), and were subjected to EMSA. Probe incubation was with nuclear extracts (10 μ g) from HM3 cells either treated or untreated with *P. aeruginosa* culture supernatant as indicated at the top. (F) Human *MUC2* regulatory regions (base pairs -1528 to -1430) containing the wild type (WT) or various mutated sites within the region -1458/-1430 as indicated above were subcloned upstream of the TK-32 promoter in a luciferase vector (named as M1 to M4) and transfected into HM3 cells. Transfected cells were treated with either *P. aeruginosa* culture supernatants or vehicle for 6 hr prior to cell lysis. Luciferase activity was then assessed in *P. aeruginosa*-treated and untreated cells. (G) Effect of NF- κ B inhibitor CAPE. A human *MUC2* construct p-2864luc was transfected into HM3 cells. Transfected cells were pretreated with or without CAPE (15 μ g/ml) for 1 hr before *P. aeruginosa* treatment as indicated. In all the experiments shown above, transfections were carried out in triplicate. Values are the means $\pm$ SD; $n = 3$. Similar results were observed in another *MUC2*-expressing epithelial cell line, NCIH292.

and HMEK1(K97R) were gifts from Alan Saltiel (Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI). JNKK(K116R) was a gift from Michael Karin (Univ. of California, San Francisco). Transfection was performed by a standard electroporation method as described (5). *P. aeruginosa* culture supernatant and LPS were added to the transfected cells 42 hr after transfection. After 6 hr, the cells were harvested for luciferase assay. All transfections were carried out in triplicate. Luciferase activity was normalized with respect to β -galactosidase activity.

Electrophoretic Mobility-Shift Assay (EMSA). Nuclear extracts from HM3 and NCIH292 cells were prepared according to ref. 8. Prior to extraction, cells were treated with *P. aeruginosa* culture supernatant for 6 hr. The protein concen-

tration of the cell extract was determined using a bicinchoninic acid protein assay kit (Pharmacia LKB Biotechnology) using bovine albumin as standard. Various double-stranded oligonucleotide probes as indicated in each experiment were synthesized on the basis of the results of luciferase assay experiments described in Results. The oligonucleotide was labeled by [γ - 32 P]ATP and T4 polynucleotide kinase and purified on a nondenaturing 6% polyacrylamide gel. The probe was incubated at 37°C for 20 min with nuclear extract (5–10 μ g of protein) in a solution containing 3 μ g of poly(dI-dC) in 10 mM Hepes-KOH at pH 7.9, 210 mM NaCl, 0.75 mM MgCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, and 12.5% (vol/vol) glycerol. For antibody interaction, antibodies specific for CCATT-enhancer-binding protein (C/EBP), NF- κ B p65,

FIG. 2. Involvement of Ras, MEK1/2, and pp90rsk in *MUC2* mucin induction by *P. aeruginosa*. (A) Effect of coexpressing a dominant-negative mutant form of pp90rsk (pp90rskΔC) on *P. aeruginosa*-induced *MUC2* up-regulation. p-2864luc (15 μg) was cotransfected with a dominant-negative mutant form of pp90rsk into HM3 cells. Forty hours later, HM3 cells were exposed to PAO1 culture supernatant (CS) 6 hr before harvesting. Luciferase activity was measured as described in the text. (B) Effect of PD98059 coexpressing a dominant-negative mutant form and wild-type form of MEK1/2 and a dominant-negative mutant form of SEK [JNKK9(K116R)] on *P. aeruginosa*-induced *MUC2* up-regulation. HM3 cells were cotransfected with p-2864luc (15 μg) and a dominant-negative mutant form (3 μg) or a wild-type form (3 μg) of MEK1/2. Forty hours after transfection, HM3 cells transfected with only p-2864luc were pretreated with PD98059 (37 μM) (Calbiochem) for 30 min. All the cells were then exposed to PAO1 culture supernatant (CS) 6 hr before harvesting. (C) Effect of coexpressing a dominant-negative mutant form of Ras (RasN17) on *P. aeruginosa*-induced *MUC2* up-regulation.

NF-κB p50, and Rel-C were added to the reaction during a 30-min preincubation on ice. Samples were applied to polyacrylamide gels under native conditions in high ionic strength buffer. The dried gel was exposed to x-ray film at -70°C for 2–12 hr with double intensifying screens.

RESULTS AND DISCUSSION

Activation of NF-κB Is Required for *MUC2* Mucin Induction by *P. aeruginosa*. Although we previously showed that *P. aeruginosa* up-regulates *MUC2* mucin gene transcription (5), the signal transduction pathways and transcriptional regulatory mechanisms are still unknown. Our first objective was to define *P. aeruginosa*-response elements in the 5'-flanking region of the *MUC2* mucin gene and related transcription factors. Analysis of luciferase activity from a panel of *MUC2*-luciferase reporter gene deletion mutants revealed a response element between base pairs -1627 and -1308 (Fig. 1A). To more precisely define the *P. aeruginosa*-response element, we made heterologous constructs in which human *MUC2* promoter base pair $-1628/-1307$, $-1528/-1307$, and $-1430/-1307$ regions were subcloned upstream of the thymidine kinase (TK) promoter. As shown in Fig. 1B, this suggested that the response element resides in the base pair $-1528/-1430$ region. To more accurately define the response element, we next performed EMSAs, using as probes ^{32}P -labeled synthesized oligonucleotides corresponding to base pairs $-1528/-1486$ (A1) and $-1485/-1430$ (A2) and nuclear proteins from *P. aeruginosa*-treated and untreated HM3 and NCIH292 epithelial cells. A new protein complex was formed with probe A2, but not with A1 (data not shown). When we further performed EMSAs using oligonucleotides corresponding to base pairs $-1485/-1459$ (B1) and $-1458/-1430$ (B2), this protein complex was formed with probe B2, but not with B1 (Fig. 1C). Sequence analysis showed that this region contains overlapping C/EBP and NF-κB sites. Selective mutagenesis of these sites in nonradiolabeled probes disrupted competition for protein binding to radiolabeled wild-type probe, suggesting that NF-κB and/or C/EBP binding is involved in *MUC2* mucin gene activation (Fig. 1D).

Pursuing the possibility that NF-κB is indeed involved in *MUC2* induction, we performed supershift assays using anti-NF-κB (p65, p50, and c-Rel) antibodies and an anti-C/EBPβ antibody reactive with α, β, δ, and CRP1. The NF-κB p65 and p50 antibodies supershifted the protein complex but the c-Rel and C/EBP antibodies did not (Fig. 1E), indicating that a NF-κB p65/p50 heterodimer accounts for the observed protein binding. That mutation of the NF-κB site (Fig. 1F) and that CAPE, an inhibitor of NF-κB-DNA binding (Fig. 1G) (9), abolished responsiveness of the wild-type *MUC2* luciferase construct confirmed that NF-κB binding to the κB site in the regulatory region of *MUC2* gene was critical for the up-regulation of the transcriptional activity of *MUC2* induced by *P. aeruginosa*.

pp90rsk Mediates the Activation of NF-κB by *P. aeruginosa*. Despite extensive analysis of NF-κB by previous investigators, the signaling events upstream of its activation are poorly understood. We took advantage of the fact that the identity of some of the kinases responsible for phosphorylating the NF-κB-tethering protein IκB has recently been identified (10–15). Cotransfecting HM3 and NCIH292 cells with a dominant-negative mutant expression plasmid for the IκBα kinase

HM3 cells were cotransfected with p-2864luc (15 μg) and a dominant-negative mutant form of Ras. The cells were then treated with PAO1 culture supernatant and lysed for luciferase assay. Similar results were observed in another *MUC2*-expressing epithelial cell line, NCIH292. In the experiments described above, all transfections were carried out in triplicate. Values are the means $\pm$ SD; $n = 3$.

Fig. 3. Involvement of Src in *MUC2* mucin induction by *P. aeruginosa*. (A and B) Inhibition of *P. aeruginosa*-induced *MUC2* up-regulation by coexpressing a dominant-negative mutant form of c-Src (A) and Src inhibitor PP1 (B). p-2864luc was transfected into HM3 cells with (A) or without (B) a dominant-negative mutant form of c-Src. Forty hours later, HM3 cells were pretreated with (B) or without PP1 (A) (5, 14, and 28 μ M) (Calbiochem) for 30 min and then exposed to PAO1 culture supernatant (CS) for 6 hr before harvesting. Luciferase activity was measured as described in the text. (C) Effect of coexpressing p-2864luc and v-Src. p-2864luc (15 μ g) was cotransfected with v-Src (0.3, 1, 3, and 5 μ g) into HM3 cells. Forty-eight hours after being transfected, HM3 cells were lysed for assessing luciferase activity. (D) Effect of PD98059, CAPE, and coexpressing a dominant-negative mutant form of Ras and MEK1/2 on v-Src-induced *MUC2* up-regulation. p-2864luc (15 μ g) was cotransfected with v-Src (3 μ g) and a dominant-negative mutant form of either Ras or MEK1/2 into HM3 cells. Forty hours after being transfected, the cells transfected with only p-2864luc were pretreated with either PD98059 (37 μ M, 30 min) or CAPE (15 μ g/ml, 1 hr). All the cells were then exposed to *P. aeruginosa* for 6 hr. Forty hours after being transfected, HM3 cells were either exposed or not exposed to PAO1 culture supernatant (CS) 6 hr before harvesting for assessing luciferase activity. Similar results were observed in another *MUC2*-expressing epithelial cell line, NCIH292. In all the experiments described above, all transfections were carried out in triplicate. Values are the means $\pm$ SD; $n = 3$.

pp90rsk (pp90rsk Δ C) prior to *P. aeruginosa* exposure reduced the response by approximately 70% (Fig. 2A). This finding indicates that *P. aeruginosa* up-regulation of *MUC2* transcription is pp90rsk dependent.

The MEK1/2-ERK1/2 Pathway, but Not the SEK-JNK Pathway, Is Involved in *MUC2* Mucin Induction by *P. aeruginosa*. The signaling molecules upstream of the I κ B kinases, predicted to vary with the nature of the activating stimulus, are still unknown. pp90rsk had previously been recognized as a mitogen-activated, serine/threonine ribosomal S6 kinase involved in the transduction of signals induced by stimuli that activate the Ras-Raf1-MEK1/2-ERK1/2 cascade (16, 17). Consistent with this, our earlier results had shown response sensitivity to the ERK1/2 inhibitor tyrphostin AG126 (5). Together, the data strongly suggested involvement of one or more MAP kinase pathways and led us to test the effect of inhibitors of MEK1/2, the kinase immediately upstream of ERK1/2, and SEK/JNKK, the kinase immediately upstream of c-Jun N-terminal kinase (JNK). As shown in Fig. 2B,

cotransfection of HM3 and NCIH292 cells with a dominant-negative mutant expression plasmid of MEK1/2 [HMEK1 (K97R)] but not a dominant-negative mutant form of SEK [JNKK(K116R)] (18) reduced the response by approximately 60%. Consistent with this, preincubation of the cells with PD98059 (19), a specific chemical inhibitor of MEK1/2, abolished the response, and overexpression of wild-type MEK1 greatly enhanced the response. Together these results indicate that *P. aeruginosa* up-regulates *MUC2* transcription by activation of the classical MEK1/2-ERK1/2 pathway in *MUC2*-expressing epithelial cells.

The Src-Dependent Ras Pathway Upstream of MEK1/2-ERK1/2-NF- κ B Is Involved in *P. aeruginosa*-Induced *MUC2* Up-Regulation. A key issue is how *P. aeruginosa* activates MEK1/2. On the basis of evidence that Ras is upstream of Raf-1-MEK1/2-ERK1/2, that c-Src is upstream of Ras (20–24), and that Src mediates bacterial-triggered signaling in macrophages, we investigated the involvement of Ras and Src in the *P. aeruginosa* response in epithelial cells. Cotransfection

with a dominant-negative mutant form of Ras (RasN17) (25) reduced the response by 80% (Fig. 2C), and cotransfection with a dominant-negative mutant form of c-Src (SrcRF) inhibited the response by 45% (Fig. 3A) (26). Consistent with these results, the Src-specific chemical inhibitor PP1 reduced the response by 80% (Fig. 3B) (27), and cotransfection of HM3 and NCIH292 cells with v-Src greatly increased the response (Fig. 3C). We conclude from these data that the mucin induction by *P. aeruginosa* is dependent on both Ras and Src.

To assess the overall similarity between *P. aeruginosa*-triggered and v-Src-triggered signaling pathways, we asked whether signaling downstream of Src was sensitive to inhibitors already known to inhibit the *P. aeruginosa*-activated signal transduction pathway. We found that cotransfection of dominant-negative mutant forms of Ras (RasN17) and MEK1/2 [HMEK1(K97R)] or treatment with the MEK1/2 and NF- κ B chemical inhibitors PD98059 and CAPE greatly reduced v-Src-activated responses (Fig. 3D). Consistent with this finding, there was no additive effect when *P. aeruginosa* supernatant was applied to HM3 and NCIH292 cells overexpressing v-Src (data not shown). These data strongly suggest overlap between the *P. aeruginosa* and v-Src signaling pathways.

The Endogenous *MUC2* Gene and *MUC2* Promoter-Driven Luciferase Reporter Gene Are Regulated Similarly. To confirm that the endogenous *MUC2* gene and the *MUC2* promoter-driven luciferase reporter gene are regulated similarly, we assessed the effects of the Src inhibitor PP1, the MEK1/2 inhibitor PD98059, and the NF- κ B inhibitor CAPE on endogenous *MUC2* mRNA induction by using RPAs. All these inhibitors strongly inhibited *P. aeruginosa*-induced *MUC2* mRNA induction, confirming that the c-Src-activated signaling pathway revealed by luciferase reporter gene assays is indeed responsible for induction of the endogenous *MUC2* gene (Fig. 4).

***P. aeruginosa* Culture Supernatant and LPS Up-Regulate *MUC2* Transcription by Activation of the Same Src-Dependent MEK1/2-ERK1/2-NF- κ B Pathway.** Despite the relatively detailed picture of the *P. aeruginosa* signaling pathway emerging

FIG. 4. Effects of inhibitors of Src, MEK1/2, and NF- κ B on up-regulation of *MUC2* mRNA induced by *P. aeruginosa*. HM3 cells were pretreated with PP1 (14 μ M, 30 min), PD98059 (37 μ M, 30 min), or CAPE (15 μ g/ml, 60 min) and then exposed to PAO1 culture supernatant (CS) for 6 hr before harvesting for RNA extraction. RPA was then performed with the *MUC2* gene-specific probe HAM1. A cyclophilin probe was included as a control to assess amount of RNA used in each hybridization reaction. Similar results were observed in another *MUC2*-expressing epithelial cell line, NCIH292.

FIG. 5. Effects of inhibitors of Src, MEK1/2, and NF- κ B on up-regulation of *MUC2* transcription induced by *P. aeruginosa* LPS. HM3 cells were transfected with a *MUC2* base pair -1,600/-1,300 TK promoter construct. Forty hours later, cells were pretreated with PP1 (14 μ M, 30 min), PD98059 (37 μ M, 30 min), or CAPE (15 μ g/ml, 60 min) and then exposed to *P. aeruginosa* LPS (15 μ g/ml) for 6 hr before harvesting for assessing luciferase activity. Similar results were observed in another *MUC2*-expressing epithelial cell line, NCIH292.

from this study, the most upstream elements in the pathway have not yet been identified. With respect to the identity of the active factor in *P. aeruginosa* supernatant, our data indicate major if not exclusive involvement of LPS (Fig. 5). This raises additional questions, as details of LPS interaction with epithelial cell surfaces are not available. Unlike macrophages, most epithelial cells do not express CD14, the membrane-bound receptor that ligates LPS/LPS-binding protein (LBP) (28). Although many epithelial cells are known to ligate soluble (s)CD14 from serum, and epithelial responses to LPS are

FIG. 6. Schematic diagram showing steps in the signaling pathway by which *P. aeruginosa* up-regulates human *MUC2* mucin gene transcription. As indicated, *P. aeruginosa* releases LPS, thereby activating a c-Src-Ras-Raf-1-MEK1/2-MAPK (ERK1/2)-pp90rsk pathway, which in turn leads to the activation of NF- κ B and triggers *MUC2* mucin transcription. LBP, LPS-binding protein. The overproduced mucin, in concert with abnormal airway lining fluid secondary to CFTR mutation, leads to airway mucus obstruction and lung failure.

stronger in the presence than in the absence of serum, the *P. aeruginosa* response we observed was not inhibited by a neutralizing anti-CD14 antibody (data not shown), suggesting the presence of an alternate, as-yet-unidentified, receptor.

The chronology of pathogenic events leading to CF lung disease has been contested for decades. Although the data presented here cannot in themselves resolve the issue, they do provide an explanation for why CF lung disease is exacerbated by infection. In this respect they dovetail with observations of mice carrying mutations in the CFTR gene. The mouse data show that airway mucus transport velocity is reduced in mutant mice prior to infection (29). Davidson *et al.* (30) also showed that mutant mice had impaired mucociliary clearance from airways but no mucous cell metaplasia or mucus retention before infection. Once infection occurred, however, the mucus changes followed. Taken together, the *in vivo* mouse data and the *in vitro* findings reported here suggest a two-stage model of pathogenesis of CF lung disease.

In stage 1, the CFTR mutation leads to *P. aeruginosa* retention and infection of the airways. In stage 2, the up-regulation of mucin transcription by *P. aeruginosa* via activation of the Src-Ras-MEK1/2-ERK1/2-pp90rsk-NF- κ B pathway reported here causes airways to become plugged with mucin. The evolutionary forces responsible for the development of mucociliary clearance as a defense mechanism did not take into account that mutations in the CFTR gene of CF patients would transform a "mucociliary escalator" into a stagnant pool of mucus. Opportunistic pathogens such as *P. aeruginosa* find a niche in the stagnant mucus. Mucus, unable to execute its intended clearance function, paradoxically acts to nourish, support, and protect bacteria from antimicrobial agents (Fig. 6). It appears that this may be the means by which mucus, normally a substance protective to the host, interacts with bacteria in a vicious cycle leading to irreversible airway obstruction. A more extensive use of systems such as that described here should elucidate other facets of bacterial-epithelial interactions and open up new targets for therapeutic intervention.

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