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*J Immunol* 2004; 172:418-425;
doi: 10.4049/jimmunol.172.1.418
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Localization of Cystic Fibrosis Transmembrane Conductance Regulator to Lipid Rafts of Epithelial Cells Is Required for Pseudomonas aeruginosa-Induced Cellular Activation

Michael P. Kowalski and Gerald B. Pier

The cystic fibrosis (CF) transmembrane conductance regulator (CFTR) protein is an epithelial cell receptor for the outer core oligosaccharide of the Pseudomonas aeruginosa LPS. Bacterial binding leads to CFTR-dependent bacterial internalization, initiation of NF-κB nuclear translocation, cellular desquamation, and eventual apoptosis of the infected cells, all of which are critical for innate immune resistance to infection with this pathogen. Lack of this reaction in CF patients underlies their hypersusceptibility to chronic P. aeruginosa infection. In this study we tested whether these epithelial cell responses are dependent upon the localization of CFTR to lipid rafts. Confocal microscopy showed that green fluorescent protein-tagged CFTR (GFP-CFTR) and the lipid raft marker ganglioside GM1 colocalized at sites of P. aeruginosa contact and internalization. GFP-CFTR localized to low density Triton X-100-insoluble fractions in lysates of Madin-Darby canine kidney GFP-CFTR cells, and P. aeruginosa infection increased the levels of GFP-CFTR in these fractions as determined by Western blot. Cells expressing GFP-ΔF508-CFTR did not have rafts with detectable CFTR protein. Extraction of cell surface cholesterol via cyclodextrin treatment of the cells inhibited CFTR entry into rafts. In addition, cyclodextrin treatment of both human and canine epithelial cells inhibited cellular ingestion of P. aeruginosa, NF-κB nuclear translocation, and apoptosis. These results indicate that lipid raft localization of CFTR is required for signaling in response to P. aeruginosa infection. Such signaling is needed for the coordination of innate immunity to P. aeruginosa lung infection, a process that is defective in CF. The Journal of Immunology, 2004, 172: 418–425.

Infection. One scenario that encompasses this concept involves P. aeruginosa binding to the CFTR protein at the first extracellular loop (aa 108–117) through interaction with the outer core oligosaccharide portion of the bacterial LPS (16, 17). This binding leads to bacterial entry into the epithelial cell and NF-κB-dependent epithelial cell activation, both of which have been proposed to contribute to bacterial clearance (18–20). Over 1000 mutant CFTR alleles have been described, with the most common mutation resulting in the ΔF508 allele, which occurs at a frequency of ~70% in CF patients. The ΔF508-CFTR protein is not properly trafficked to intracellular, cytoplasmic stores and the plasma membrane. Cells expressing ΔF508-CFTR as well as cells with missense, nonsense, or stop mutations in the CFTR gene have little or no CFTR on the cell surface, preventing recognition of the presence of the bacteria, internalization, and NF-κB nuclear translocation. Interestingly, some mutant CFTR alleles associated with frequent occurrence of P. aeruginosa infection produce proteins that do traffic properly to the plasma membrane, suggesting other defects in their response to this pathogen. Once infection is established, P. aeruginosa typically switches to a mucoid phenotype in the CF lung, which is accompanied by the production of a rough LPS that lacks CFTR binding capabilities, further ensuring that the bacteria cannot adequately signal their presence via CFTR even if some mutant CFTR appears on the epithelial cell surface, as has been suggested to occur during chronic infection (21).

Previous results have shown that cultured human airway epithelial cells as well as airway epithelial cells of mice expressing wild-type CFTR were better able to internalize P. aeruginosa than cells or mice unable to express CFTR in the lung or expressing ΔF508-CFTR (16, 19). In addition, cells with wild-type CFTR produced a rapid nuclear translocation of NF-κB in response to infection (20). In the wild-type mice, internalization of P. aeruginosa and rapid NF-κB nuclear translocation are associated with more effective clearance of bacteria from the lung following infection (19). In
addition to these two responses, the epithelial cells also undergo apoptosis, whose rate is facilitated by the presence of wild-type CFTR (22–24). Other investigators have shown the importance of apoptosis in the manifestation of host resistance to *P. aeruginosa* lung infection (23, 24).

As CFTR-dependent binding of *P. aeruginosa* and subsequent nuclear signaling appear to be part of the normal host response to this pathogen, the mechanisms and pathways by which the signaling occurs need to be elucidated. Lipid rafts have been shown to be involved in numerous signaling pathways, as well as in internalization of various microorganisms (reviewed in Refs. 25–27). Lipid rafts are membrane microdomains that are typically characterized by their insolubility in 1% Triton X-100 at 4°C (28). Raft formation is dependent on the presence of cholesterol in the cell membrane (29) and associate with the ganglioside GM1 (28). Raft formation can initiate signaling either by the concentration of a low number of appropriate proteins into a high local concentration or by exclusion of certain proteins from the microenvironment (30). Therefore, the localization of a protein to lipid rafts is often an indicator that the protein is essential for some signaling process. It is hypothesized that CFTR relocates to lipid rafts upon infection with *P. aeruginosa*, and that this initiates the signaling essential for clearance of the bacteria.

In support of this idea, Grassme et al. (31) have shown that acid sphingomyelinase-mediated lipid rafts were essential for host resistance to *P. aeruginosa* infection, including epithelial cell invasion and apoptosis. In addition, they implied that CFTR was increasingly localized to lipid rafts during infection, but this was only demonstrated by confocal microscopy. To investigate further the role of lipid rafts in the interaction of *P. aeruginosa* with CFTR, we used both confocal microscopy and sucrose gradients to determine whether CFTR is found in lipid rafts in response to the organism, and we also used cholesterol extraction as a means to determine whether lipid rafts are essential for epithelial cell responses to *P. aeruginosa*, including bacterial uptake, NF-kB nuclear translocation, and apoptosis.

**Materials and Methods**

**Cell lines**

Human lung epithelial cells (CFT1) from a ΔF508 homozygous CF patient were immortalized and retrovirally infected with a wild-type copy of CFTR (22). MDCK cells (MDCK-GFP-CFTR) or a GFP-tagged *P. aeruginosa* (33). Cells were grown and maintained in F12 85% Dulbecco’s Modified Eagles Medium (CMEM) and 15% fetal bovine serum (FBS), and passaged every 4 days using 0.25% TrypLE. Human lung epithelial cells (CFT1) from a *P. aeruginosa* (34). Escherichia coli DH5α was used as a control. Bacteria were grown to stationary phase overnight at 37°C in tryptic soy broth (TSB). These cultures were diluted in fresh TSB to an OD₅₀₀ of 0.1 and were grown at 37°C in TSB for 1 h to allow bacterial entry into cell phase growth. The bacteria were centrifuged, washed once in PBS, and resuspended in cell medium at an OD₅₀₀ of 0.4 (~2 × 10⁸ bacteria/ml).

**Preparation of bacteria**

LPS smooth, nonmucoid CF clinical isolates of *P. aeruginosa* obtained early in the course of infection of patients (324, N6) were used, as was a laboratory strain (PAO1-V) of *P. aeruginosa* (34). Eight different clinical strains were used as a control. Bacteria were grown to stationary phase overnight at 37°C in tryptic soy broth (TSB). These cultures were diluted in fresh TSB to an OD₅₀₀ of 0.1 and were grown at 37°C in TSB for 1 h to allow bacterial entry into log phase growth. The bacteria were centrifuged, washed once in PBS, and resuspended in cell medium at an OD₅₀₀ of 0.4 (~2 × 10⁸ bacteria/ml).

**Cell treatment**

All MDCK cells were treated overnight with 25 mM sodium butyrate (Sigma-Aldrich, St. Louis, MO) before an experiment to increase CFTR expression. Butyrate was removed 2 h before other treatments or infection. Any cells infected in the presence of methyl-β-cyclodextrin (CD; Sigma-Aldrich) were treated for 30 min at 37°C before infection.

**Confocal microscopy**

MDCK-GFP-CFTR cells were grown in dishes containing a glass coverslip and infected with strain PAO1-V for 30 min. Cells were rinsed twice with PBS and fixed with 1% paraformaldehyde. Cells were rinsed twice again and permeabilized with PBS and 0.2% Tween 20. After additional PBS rinses, cells were stained for 1 h at room temperature with Alexa 594-conjugated cholera toxin (1/2000 dilution; Molecular Probes, Eugene, OR) diluted in 2% normal goat serum to visualize GM1 and with an Ab raised to the purified LPS of strain PAO1-V (35). Cells were rinsed twice again and stained with an Alexa 647-conjugated secondary Ab to rabbit IgG (1/250 dilution; Molecular Probes) to visualize the bacteria. The cells were rinsed a final two times and were viewed using the MRC 1024 MultiPhoton Confocal System (Bio-Rad, Hercules, CA) equipped with a krypton/argon laser and a Tsunami MultiPhoton Laser (~100 nm oil-immersion objective; Spectra Physics, Mountain View, CA). Samples were excited by the 590-xl wavelength of a 60W argon laser. Five micrometer sections were taken every 0.5 μm from the top of the sample down to the bottom. Images were collected at the following wavelengths: 520-550 nm, 570-590 nm, and 680-700 nm. To determine whether lipid rafts are essential for epithelial cell responses to *P. aeruginosa*, including bacterial uptake, NF-kB nuclear translocation, and apoptosis.

**Sucrose density gradient isolation of lipid rafts and Western blotting**

After the various treatments involving infection and/or CD treatment, MDCK-GFP-CFTR cells were lysed in 2 ml of 1% Triton X-100 at 4°C for 20 min in the presence of Complete Mini Protease Inhibitor Cocktail (Roche, Indianapolis, IN) and passed five times through a syringe and 21-gauge needle. The 2 ml of lysate was added to 2 ml of 80% sucrose (40% final concentration of sucrose), added to an ultracentrifuge tube, and overlaid with 6 ml of 30% sucrose, followed by an additional 2 ml of 5% sucrose. Samples were spun overnight at 100,000 × g in a 50.2Ti rotor (Beckman, Palo Alto, CA), and 1-ml fractions were removed from the top of the gradient, yielding fractions 1–12. The Triton-insoluble lipid rafts float to the interface of the 5 and 30% sucrose layers and were found to be in fractions 2–5. Proteins were extracted from 0.5 ml of the fractions with chloroform/methanol in a modified version of the method described by Wessel et al. (36). Briefly, 10 ml of 5% deoxycholic acid was added to 500 μl of various gradient fractions to solubilize the proteins, and the samples were incubated on ice for 10 min. For some experiments, depending on the protein concentration, various volumes of fractions 2–5 were pooled either before or after protein precipitation. Four hundred microliters of methanol and 700 μl of chloroform were then added to the 510 μl of solubilized samples and mixed well, and samples were spun at 4°C for 15 min at maximum speed in a microcentrifuge to concentrate the proteins at the aqueous/orange interface. The top (aqueous) layer was removed without disturbing the interface, 700 μl of methanol was added to the remaining lower (organic) phase and mixed well, and the pellet was resuspended at maximum speed in a microcentrifuge for 30 min at 4°C.

Pelleted proteins were resuspended in 1× lithium dodecyl sulfate buffer, including 1× reducing agent (Invitrogen, Carlsbad, CA). Sixteen microliters of the bottom gradient fraction (fraction 12) were added to 4× lithium dodecyl sulfate buffer and 10× reducing agent to yield 1× final concentrations to detect the soluble CFTR normally present in the cells. This was the most accurate way to determine and normalize relative cellular protein levels in each condition, as the presence of bacterial proteins in the lysates affected the total protein concentration. All samples were incubated at 37°C for 30 min to avoid CFTR aggregation. Samples were run on a 4–12% Bis-Tris gradient gel (Invitrogen) and transferred to an Invitrogen polyvinylidene difluoride membrane (Invitrogen). Blots were blocked in 5% skim milk in PBS and 0.05% Tween 20 (PBS-T) for 2 h at room temperature. To detect GFP-CFTR, blots were next incubated overnight at 4°C with a mouse IgG Ab to GFP (1/200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBST and 5% skim milk. Blots were washed four times with PBST and incubated with an Ab to mouse IgG conjugated to HRP (1/10,000 dilution; Southern Biotechnology Associates, Birmingham, AL) in PBST with 0.5% skim milk for 1 h at room temperature. Blots were washed four times in PBST and developed using Western Blot Luminol Reagent (Santa Cruz Biotechnology). In addition, mouse IgG Ab to the human transferrin receptor (1/200 dilution; Zymed Laboratories, South San Francisco, CA; same secondary Ab) and HRP-conjugated Ab to human caveolin-1 (1/8,000 dilution; Santa Cruz Biotechnology) were used for Western blotting, with the only difference being caveolin-1 blots were washed with 0.2% Tween 20 to reduce nonspecific binding. National Institutes of Health Image software was used to determine relative band intensities.
Bacterial internalization assays

LCFSN, ΔFS08, and MDCK-GFP-CFTR cells were infected with *P. aeruginosa* strains PAO1-V, 324, and N6 for 30 min in the presence or the absence of CD. Cells were then harvested using a nuclear extraction kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. The protein concentrations of the extracts were determined using a protein assay (Bio-Rad). Five-microgram samples of the nuclear extracts were analyzed for the presence of NF-κB using the TransAM NFκB p65 Transcription Factor Assay Kit (Active Motif) according to the manufacturer’s instructions. Briefly, nuclear extracts were added to wells coated with the consensus binding sequence for NF-κB to bind the p65 subunit and were incubated for 1 h at room temperature. Wells were washed (three times) and incubated with an Ab to human p65 for 1 h at room temperature. After additional washes, the wells were incubated with an HRP-conjugated secondary Ab for 1 h at room temperature. After additional washes, developing solution was added until a blue color appeared (~5 min). An acidic stop solution was then added to end the reaction and change the blue color to yellow. The OD550 was measured on a plate reader to quantify bound NF-κB.

NF-κB activation assays

LCFSN, ΔFS08, and MDCK-GFP-CFTR cells were infected with *P. aeruginosa* strains PAO1-V, 324, and N6 for 30 min in the presence or the absence of CD. Cells were then harvested using a nuclear extraction kit (Active Motif, Carlsbad, CA) according to the manufacturer’s instructions. The protein concentrations of the extracts were determined using a protein assay (Bio-Rad). Five-microgram samples of the nuclear extracts were analyzed for the presence of NF-κB using the TransAM NFκB p65 Transcription Factor Assay Kit (Active Motif) according to the manufacturer’s instructions. Briefly, nuclear extracts were added to wells coated with the consensus binding sequence for NF-κB to bind the p65 subunit and were incubated for 1 h at room temperature. Wells were washed (three times) and incubated with an Ab to human p65 for 1 h at room temperature. After additional washes, the wells were incubated with an HRP-conjugated secondary Ab for 1 h at room temperature. After additional washes, developing solution was added until a blue color appeared (~5 min). An acidic stop solution was then added to end the reaction and change the blue color to yellow. The OD550 was measured on a plate reader to quantify bound NF-κB.

Apoptosis assays

LCFSN cells were infected with *P. aeruginosa* strains PAO1-V (MOI, ~0.5), 324 (MOI, ~0.5), and N6 (MOI, ~10) for 6 h in the presence or the absence of CD and then were incubated overnight in 150 μg of gentamicin/ml to allow the apoptosis process to go to completion while killing bacteria to prevent further effects on the cells. Cells were fixed by incubating them in methanol at ~20°C overnight. Cells were permeabilized in PBS and 0.1% Tween 20 and were stained with FITC-tagged M30 Ab (1/250, Roche) in PBS/Tween/1% BSA. M30 binds caspase-cleaved cyto keratin 18 as a marker for apoptosis in epithelial cells (37). Cells were analyzed by FACS, using forward and side scatter gates to eliminate measurements from debris, and fluorescence was measured at ~420 nm emission for 10,000 cells/condition. Cells that stained greater than the highest 1% of the infected samples were deemed apoptotic, and this percentage of the total cells (under the M1 bar in figures) are reported as the percent apoptotic cells.

Statistical analysis

Tukey’s multiple-comparison *t* test and overall ANOVA were used to analyze bacterial uptake and NF-κB data. Statistical analyses were performed using PRISM3 for Macintosh (GraphPad, San Diego, CA).

Results

**CFTR and GM1 colocalize at the site of *P. aeruginosa* internalization into epithelial cells**

To determine whether CFTR is localized to lipid rafts during *P. aeruginosa* infection, confocal microscopy was used to search for colocalization of GFP-CFTR and the marker for lipid rafts, GM1. Fig. 1A shows the merged images of the red, green, and blue channels, and the insets show zoomed images of two locations of contact between the epithelial cell and the bacteria. The bacterium on the left appears to have been internalized and is surrounded by punctate red (GM1) staining as well as an apparent ring of CFTR (green). The bacterium on the right is attached to a thin area of the cell that appears to be highly enriched in CFTR and that also stains for larger punctate areas of GM1. Fig. 1B shows the same images, but has been pseudocolored yellow at the sites of red and green colocalization (CFTR and GM1). Both bacteria border areas of CFTR and GM1 colocalization, indicating that CFTR associates with a marker for lipid rafts sites of *P. aeruginosa* contact or internalization.

**CFTR is found in Triton-insoluble fractions**

To determine more specifically whether CFTR localizes to lipid rafts, fractionated lysates from uninfected MDCK-GFP-CFTR cells were analyzed by Western blot using an Ab to GFP. Fig. 2A shows a representative blot, indicating that CFTR can be found in the lipid raft fractions (Fr. 2–5) at the 5–30% sucrose gradient interface. GFP-CFTR also was detected as two bands in the non-raft, Triton-soluble fractions, as illustrated by the first (Fr. 8) and last (Fr. 12) samples of the soluble fractions, and in total cell lysates (pos con). Taking into account the effect of concentration of proteins in the lipid raft fractions by precipitation, which was necessary to visualize CFTR in immunoblots, only ~1–2% of the total CFTR was found in lipid rafts under normal cell culture conditions. One CFTR band has an apparent *M* subunit of ~240 kDa (combined *M* of CFTR and GFP) and corresponds to the fully glycosylated form of CFTR. A second band, with an apparent *M* subunit of ~210 kDa, corresponds to the immature/unglycosylated form of GFP-CFTR, typically associated with the endoplasmic reticulum or Golgi. In the lipid raft fractions, the upper band is predominantly observed, indicating that primarily the mature CFTR is present in rafts. No bands were seen with SDS lysates of uninfected MDCK cells (neg con), illustrating the specificity of the Ab to GFP. To ensure that we were identifying lipid rafts in the correct fractions, the same blot was probed for the presence of caveolin-1, as caveolin-1 typically partitions into lipid rafts. A majority of caveolin-1 is found in the same fractions (Fr. 2–5) as CFTR, therefore indicating that the Triton-insoluble fractions contain lipid raft components. This result gives a highly specific biochemical indication that CFTR can be found in lipid rafts.

**P. aeruginosa infection increases the amount of CFTR found in lipid rafts**

MDCK-GFP-CFTR cells were infected with strain PAO1-V for various times and lysed in 1% Triton as in the previous experiment. Equal amounts of raft fractions (2–5) were precipitated and combined before SDS-PAGE and immunoblot analysis. Fig. 2B shows that infection with strain PAO1-V results in a rapid (5 min)
increase in the amount of raft-localized CFTR, with this increase peaking at 15 min and returning to approximately basal levels by 30 min postinfection. Equal volumes of the soluble fractions were loaded to ensure that an equal amount of total CFTR was present in each condition.

In addition, the blot in Fig. 2B was probed to determine the location of the transferrin receptor, which is a cell surface protein that is typically not associated with lipid rafts. The transferrin receptor exclusively localized to the soluble fractions, indicating that the CFTR found in the Triton-insoluble fractions is not the result of contamination from nonraft-associated cell surface components. Overall, these data indicate that P. aeruginosain strain PAO1-V infection induces the localization of CFTR to lipid rafts.

FIGURE 3. Raft localization of wild-type CFTR is specifically increased by P. aeruginosain. A. The increase in raft CFTR is P. aeruginosainspecific. Both a laboratory strain (PAO1-V) and two clinical isolates of P. aeruginosain from CF patients (324 and N6) cause a substantial increase in raft-localized CFTR-GFP, whereas an E. coli strain (DH5α) has no effect on CFTR raft localization. B. ΔF508 CFTR is not found in lipid rafts. Raft fractions from P. aeruginosainfected MDCK-GFP-CFTR cell lysates had wild-type CFTR detectable in raft fractions, whereas there was no CFTR detected in raft fractions of MDCK-GFP-ΔF508 cells regardless of whether they were uninfected or infected with P. aeruginosain. Detection of caveolin-1 was used to ensure that lipid rafts were fractionated normally in each cell lysate. The soluble CFTR lanes show that the ΔF508 CFTR protein is only observed in the unglycosylated form, indicative of CFTR protein that is retained in the ER. The blots are representative of at least two experiments.

ΔF508 CFTR cannot localize to lipid rafts
As ΔF508 CFTR is the most common mutant CFTR allele found in CF patients, MDCK cells expressing a GFP-tagged ΔF508 mutant CFTR were tested to determine whether the ΔF508 CFTR protein could localize to lipid rafts in either the presence or the absence of P. aeruginosainfection. These data imply that CFTR is a specific receptor whose localization to lipid rafts is induced by P. aeruginosainfection.

CD inhibits CFTR raft localization
As the presence of cholesterol in the cell membrane is essential for lipid raft formation, the extraction of cell surface cholesterol using
CD is a common method used to inhibit raft formation. MDCK-GFP-CFTR cells were infected with *P. aeruginosa* strain PA01-V for 15 min in the presence or the absence of 5 mM CD. Fig. 4A shows that 5 mM CD reduced the basal amount of CFTR found in lipid rafts by ~30% compared with that of untreated control cells (Con) and substantially reduced the *P. aeruginosa*-induced increase in CFTR localization to lipid rafts. The relative band intensities are shown in Fig. 4B. The fact that the CFTR present in the Triton-insoluble fractions is dependent upon cholesterol adds further support to the conclusion that the protein is indeed found in lipid rafts and is not being detected in Triton-insoluble fractions due to contamination from Triton-soluble fractions.

**CD inhibits *P. aeruginosa* internalization**

As it was found that CD inhibits CFTR raft localization, the effect of CD on *P. aeruginosa*-induced epithelial cell responses, including bacterial uptake and signaling, was next investigated. Fig. 5A illustrates the effect of CD on *P. aeruginosa* entry into epithelial cells. CD treatment resulted in a significant dose-dependent inhibition of uptake of all three *P. aeruginosa* strains in both the LCFSN and MDCK-GFP-CFTR cell lines that express wild-type CFTR. The ΔF508 cell line showed reduced uptake of *P. aeruginosa* compared with uptake by the LCFSN cells (~30% of uptake by LCFSN cells in the absence of CD), as has been previously found (16, 18), indicating a dependence upon functional CFTR for *P. aeruginosa* uptake by epithelial cells. CD was able to inhibit *P. aeruginosa* internalization to levels even below those in cells expressing only ΔF508 CFTR in the absence of CD. Overall, these data show that there is a requirement for both lipid rafts and cell surface CFTR for robust *P. aeruginosa* uptake by epithelial cells.

**CD inhibits *P. aeruginosa*-induced NF-κB activation**

CD was also tested for its ability to inhibit CFTR-dependent and *P. aeruginosa*-induced activation of NF-κB in epithelial cells. Nu-
P. aeruginosa clinical strains 324 and N6 showed similar results in CFTR-dependent induction of NF-κB nuclear translocation that was inhibited by CD (data not shown). It was observed that higher concentrations of CD were required to inhibit both invasion and NF-κB activation in MDCK cells compared with LCFSN cells. In addition, TNF-α increased NF-κB translocation to between 150 and 240% of that in uninfected controls in MDCK-GFP-CFTR and LCFSN cells, respectively, and CD had no effect on this response in either cell type (data not shown), implying that raft-dependent signaling effects are related to P. aeruginosa infection.

CD inhibits P. aeruginosa-induced apoptosis

It has been shown that P. aeruginosa induces apoptosis through a CD95-dependent pathway (24). To determine whether this process was dependent upon the entry of CFTR into lipid rafts, LCFSN cells were infected with P. aeruginosa strains in the presence or the absence of CD. Fig. 6 shows that CD inhibited P. aeruginosa induction of apoptosis, as determined by staining with M30 Ab. Strains PA01-V, 324, and N6 were all able to induce apoptosis, although a greater MOI was required for a substantive effect with strain N6. CD had no effect on staurosporine-induced apoptosis (data not shown), implying that it does not inhibit caspase activation or affect Ab binding. Although CD inhibits P. aeruginosa-induced apoptosis, it is unclear whether this is directly due to inhibition of initial signaling events, inhibition of CD95 signaling, which also requires lipid rafts, or a combination of the two.

Discussion

Lipid rafts have been shown to play a major role in many signaling pathways as well as microorganism internalization. Both these cellular responses are critical for orchestrating innate immune resistance to infection (16, 18–20, 24, 38). As CFTR has been shown to be a receptor for P. aeruginosa, mediating bacterial internalization and NF-κB nuclear translocation (16–20), we analyzed whether lipid rafts were involved in these processes. Colocalization of GM1 and CFTR at the site of P. aeruginosa contact with epithelial cells shows that CFTR is localized to lipid rafts at locations where P. aeruginosa is found during initial interactions with epithelial cells. The increase in Triton-insoluble CFTR during infection gives evidence for a rapid and dynamic process of raft formation and/or CFTR recruitment to lipid rafts, implying that it is a very early event in infection and precedes internalization or other epithelial cell responses.

Inhibition of the formation of CFTR-containing lipid rafts, either by mutating CFTR or by extracting membrane cholesterol, inhibited various hallmarks of P. aeruginosa interactions with epithelial cells, including bacterial internalization, NF-κB activation, and apoptosis. This inhibition has been shown not to be due to nonspecific effects of CD, and therefore is probably related to the inhibition of P. aeruginosa-induced signaling. The fact that all these processes are linked to the presence of CFTR in lipid rafts underscores the importance of this initial signaling event in the host response to infection.

We have proposed that CFTR-dependent internalization and NK-κB-dependent epithelial cell activation are critical steps for clearance of P. aeruginosa from the lung, and the lack of functional CFTR in airway epithelia of CF patients that can mediate these reactions underlies the hypersusceptibility of these patients to P. aeruginosa infection. In addition to bacterial internalization and NF-κB nuclear translocation, we have observed a subsequent CFTR-dependent step is cellular apoptosis (22). Apoptotic cells are probably then engulfed by phagocytes or APCs in the lungs of healthy individuals. Mulvey et al. (38) showed that, in a similar fashion, uropathogenic E. coli were cleared via apoptosis and exfoliation of urothelial cells, and bacterial clearance was reduced in the presence of caspase inhibitors. In addition, Grassme et al. (24) showed that apoptosis was required for host resistance to P. aeruginosa lung infection.

Most data presented in this study are consistent with the recently published work of Grassme et al. (31). Although these investigators focused on the role of ceramide-rich signaling platforms in response to P. aeruginosa infection, inhibition of the formation of these platforms lead to changes in epithelial cell uptake of bacteria and apoptosis. In addition, the time course of raft formation in response to P. aeruginosa they reported was similar to what we observed with CFTR raft localization (maximum, ~15 min). However, one apparent difference was the effect of CD treatment of cells on their response to P. aeruginosa. In this study we showed that CD treatment during P. aeruginosa infection decreases NF-κB activation. Grassme et al. (31), however, found that CD pretreatment resulted in an increase in IL-1β release after infection compared with infection alone. As IL-1β transcription is regulated by NF-κB, our work would predict that there would be less IL-1β in CD-treated cells than in those infected without CD treatment. The
apparent difference may be due to the different treatments (pretreatment vs concurrent with infection) or different end points (measurement of transcription factor activation vs measurement of protein secretion). As Grassme et al. (31) looked at IL-1β release 45 min after infection, it is likely that the protein that was released was already formed, and that additional transcription may be inhibited by CD treatment of P. aeruginosa-infected cells. Grassme et al. (31) also observed an increase in IL-1β in mice 9 h after tracheal injection of CD and subsequent infection, but it is possible that the increase could be due to the CD effect diminishing over time or could come from other cellular sources, such as macrophages or polymorphonuclear leukocytes, which synthesize and secrete IL-1β by other mechanisms.

The use of different strains of P. aeruginosa shows that the dependence of epithelial response on CFTR and lipid rafts occurs with a variety of isolates. The levels of dependence on lipid rafts seem to vary even within the small number of isolates we studied, possibly due to differences in virulence factors or the level of expression of the LPS core oligosaccharide that binds to CFTR.

There are many different CFTR mutations that result in varying levels of susceptibility to P. aeruginosa infection (39, 40). There are even some CFTR mutant proteins that are expressed on the cell surface, such as the G551D protein (41, 42), but are still associated with numerous other proteins (11, 13, 15), it would be interesting to determine what proteins CFTR associates with when present in lipid rafts.

In summary, this work gives evidence for a model by which P. aeruginosa contact with epithelial cells leads to localization of CFTR to lipid rafts at the site of contact, and that this event is essential for the rapid initiation of the host innate immune response. These responses include bacterial internalization, which leads to elimination via cellular desquamation, NF-κB nuclear translocation, which orchestrates other aspects of innate immunity, such as polymorphonuclear leukocyte recruitment and activation, and apoptosis, which is probably involved in the proper resolution of the innate response to infection. The rapidity with which CFTR enters rafts in response to P. aeruginosa indicates the critical importance of this component of the host response to effective innate immune resistance. Coupled with the previous demonstration that CFTR-dependent, NF-κB nuclear translocation also occurs within minutes of infection (20), it appears that airway epithelial cells are able to rapidly coordinate the innate response to P. aeruginosa, and these responses are heavily dependent on functional CFTR protein. Overall, the demonstration that CFTR rapidly enters into lipid rafts as part of the host response to P. aeruginosa further supports the hypothesis that a critical role for CFTR in innate immunity to P. aeruginosa infection is to detect the organism via binding and signal its presence to the lung tissue, resulting in a coordinated and regulated inflammatory response that effectively eliminates the pathogen and restores tissue homeostasis.

**Acknowledgments**

We thank Michelle Lowe for her invaluable training at the Confocal and Multiphoton Imaging Core Facility, Brigham and Women’s Hospital. We also thank Dr. Jaime Sancho for his methanol/chloroform precipitation protocol, and Drs. Carolyn Cannon, Martin Lee, and Jeffrey Lyczak for valuable discussions.

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