**Pseudomonas Invasion of Type I Pneumocytes Is Dependent on the Expression and Phosphorylation of Caveolin-2***

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**Pseudomonas aeruginosa is a major cause of pneumonia in patients with cystic fibrosis and other immunocompromising conditions. Here we showed that P. aeruginosa invades type I pneumocytes via a lipid raft-mediated mechanism. P. aeruginosa invasion of rat primary type I-like pneumocytes as well as a murine lung epithelial cell line 12 (MLE-12) is inhibited by drugs that remove membrane cholesterol and disrupt lipid rafts. Confocal microscopy demonstrated co-localization of intracellular P. aeruginosa with lipid raft components including caveolin-1 and -2. We generated caveolin-1 and -2 knockdowns in MLE-12 cells by using RNA interference techniques. Decreased expression of caveolin-2 significantly impaired the ability of P. aeruginosa to invade MLE-12 cells. In addition, the lipid raft-dependent tyrosine phosphorylation of caveolin-2 appeared to be a critical regulator of P. aeruginosa invasion.

**Pseudomonas aeruginosa** is an important cause of nosocomial pneumonia, as well as a major pulmonary pathogen in patients with cystic fibrosis and other immunocompromising conditions (1–4). A large number of patients develop *P. aeruginosa* colonization of the upper airways; however, only a small percentage of these patients develop clinically significant *P. aeruginosa* pneumonia. The morbidity and mortality of *P. aeruginosa* respiratory infections usually result from the dissemination of *P. aeruginosa* to the alveolar space prior to the establishment of pneumonia (2, 5, 6). However, the pathogenesis of *P. aeruginosa* pneumonia is not completely understood.

The alveolar epithelium is the largest host epithelial surface exposed to the external environment with an area roughly equal to the size of a tennis court. Approximately 95% of that surface area is lined by specialized type I pneumocytes (7). Once bacteria escape the mucociliary host defenses of the upper airways and enter the alveolar space, they are likely to come in contact with type I pneumocytes. The role of type I pneumocytes in the pathogenesis of *P. aeruginosa* pneumonia is not well understood. Type I pneumocytes were initially believed to serve only simple barrier functions separating the alveolar space from the pulmonary capillaries. More recently, type I cells have been implicated in a wide range of functions including lipid metabolism, cell signaling, remodeling, and host defense (8–10). The cell membrane of type I pneumocytes has a high concentration of specialized lipid rafts and caveolae, which occupy nearly 70% of the plasma membrane (11, 12). Invasion of type I pneumocytes would protect *P. aeruginosa* from phagocytosis by alveolar macrophages and offer a protected environment for replication. *P. aeruginosa* invasion of type I cells may facilitate dissemination throughout the host because of the single cell thickness of the alveolar epithelium. We hypothesized that the virulence of *P. aeruginosa* in the development of pneumonia is at least due in part to its ability to invade type I pneumocytes.

*P. aeruginosa* is able to invade nasal and bronchial epithelial cells via a lipid raft-dependent mechanism (13, 14). Lipid rafts are specialized areas of the plasma membrane that are detergent-insoluble, low density membrane fractions that are enriched in cholesterol and sphingolipids (15). In addition, these regions contain a number of important signaling molecules and structural proteins such as caveolins (16). Lipid rafts have been implicated in a wide range of cellular functions, including lipid metabolism (17), cell signaling (18), and endocytosis (19). The pathway of lipid raft-dependent endocytosis is distinguished from other clathrin-mediated endocytosis and other endocytic pathways by its sensitivity to cholesterol depletion (20).

An increasing number of pathogens have been recognized to co-opt the mechanism of lipid raft-mediated endocytosis in order to invade host cells (21–24). *P. aeruginosa* invasion of nasal and bronchial epithelial cells induces apoptosis and shedding of the superficial layer epithelial cells (13). *P. aeruginosa* may co-opt lipid raft-mediated endocytosis to invade the alveolar epithelium during the pathogenesis of *P. aeruginosa* pneumonia. However, the single cell thickness of the alveolar epithelium may lead to markedly different consequences for the host.

Caveolin proteins are key components of lipid rafts and caveolae (16). Caveolin proteins have many important functions. In addition to serving as key structural proteins that organize caveolae platforms, caveolin proteins are important in regulating endocytosis and cell signaling (16, 25, 26).

The caveolin family consists of three 21–24-kDa integral membrane proteins (27). Caveolin-1 and -2 are co-expressed on most cell types, including type I pneumocytes (26, 28). A majority of the research on caveolin proteins have focused on the functions of caveolin-1. We have shown previously that caveolin-1 expression is an important determinant in the uptake of *Escherichia coli* by bladder epithelial cells (29). Although caveolin-1 has been extensively studied, very little is known about the function of caveolin-2. Caveolin-2 interacts with caveolin-1 to form a hetero-oligomeric complex within lipid rafts (30, 31). Unlike caveolin-1, caveolin-2 is not required for the formation of caveo-
addition, via a lipid raft-dependent mechanism. In bicarbonate, 0.3 g/liter L-glutamine, 2.5 g/liter glucose, 10 mM HEPES, and phosphate-buffered saline. Cultures were washed three times to remove nonadherent cells and to enrich the percentage of type II cells. All experiments were performed on days 6–7 to allow differentiation into the type I-like phenotype (35).

Bacterial Strains and Cell Lines—P. aeruginosa strain 27853 and strain PAO-1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). P. aeruginosa strains were grown in 5 ml of static LB broth for 18 h. Listeria monocytogenes was grown for 18 h in 5 ml of brain heart infusion broth (BD Biosciences). After static incubation for 18 h, bacterial cultures were diluted 1:50 and placed on LB agar plates. The 0.25% trypsin (Invitrogen) was added to each well for 5 min, and 70 μl of bacterial suspension was transferred to 0.65-ml tubes, and each well was washed with an addition of 105 cells/cm2. ME-12 cells were seeded into 96-well plates. Type I-like cells were seeded at a density of 6 × 105 cells/cm2 and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) and supplemented with 10% fetal bovine serum (Hyclone). At 36 h, cell cultures were washed three times to remove nonadherent cells and to enrich the percentage of type II cells. All experiments were performed by identifying bacteria within the center of cells that co-localize with caveolin proteins. Co-localization was determined using a Nikon Eclipse TE200 microscope. Confocal microscopy—Primary rat type II cells were isolated and cultured on 35-mm plastic dishes. They were then washed five times with PBS and fixed overnight in 2.0% paraformaldehyde in PBS. They were then washed four times with PBS and then postfixed with 10% glutaraldehyde in PBS for 1 h. They were then washed three times with 10% goat serum and mounted using Prolong Gold anti-fade (Molecular Probes) for examination by confocal microscopy.

Confocal Microscopy—Primary rat type II cells were isolated and grown on Permanox® plastic chamber slides for 6 days. ME-12 cells were seeded onto collagen coated 12-mm diameter glass coverslips placed into the wells of 24-well plates. Type I-like cells were cultured for 36 h in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum. After static incubation for 18 h, bacterial cultures were diluted 1:50 and placed on LB agar plates. The 0.25% trypsin (Invitrogen) was added to each well for 5 min, and 70 μl of bacterial suspension was transferred to 0.65-ml tubes, and each well was washed with an addition of 0.1% Triton X-100 in PBS was then added. Cells were then washed five times with PBS and fixed with 10% glutaraldehyde in PBS for 1 h. The lungs were lavaged 10 times with PBS/EDTA to remove any nonadherent bacteria. The lungs were inflated to total lung capacity with optimal cutting temperature (OCT) medium (Miles Scientific) and frozen at −80 °C. Ten-micron frozen sections were cut using a cryostat (Leica). Sections were fixed in 100% acetone at −20 °C for 5 min prior to labeling with viral microscopy. They were then washed with PBS to block with 10% goat serum in PBS for 1 h. The primary antibodies were diluted 1:100 in 10% goat serum and added to the sections for 1 h; rabbit polyclonal caveolin-1 (BD Biosciences), rabbit polyclonal caveolin-2 (Abcam), and RT140 (courtesy of Leland Dobbs, University of California, San Francisco). Cells were then washed three times with 10% goat serum. Goat anti-rabbit or goat anti-mouse secondary antibodies conjugated to Alexa Fluor 660 (Molecular Probes) were diluted 1:100 in 10% goat serum and added to the sections for 1 h. The infected cells were incubated at 37 °C for 45 min, washed four times with PBS (Invitrogen) to remove unbound bacteria, and fixed overnight in 2.0% paraformaldehyde in PBS. To examine caveolin-1 and caveolin-2 labeling, cells were grown and infected as above. After removing the fixative, the cells were permeabilized with 0.1% Triton X-100 (Sigma). Cells were then incubated with Alexa Fluor 660 (Molecular Probes) were used for each experiment. Coverslips were examined using a Nikon Eclipse TE200 microscope. Confocal microscopy—Primary rat type II cells were isolated and grown on 35-mm plastic dishes. They were then washed five times with PBS and fixed with glutaraldehyde, dehydrated with cold acetone, and processed using standard methods. The cells were examined by transmission electron microscopy.

Bacterial Invasion and Adherence Assays—Type I-like pneumocytes or ME-12 cells were seeded into 96-well plates. Type I-like cells were seeded at a density of 6 × 105 cells/cm2. ME-12 cells were grown to ~80% confluence for all experiments. For infections with strains of P. aeruginosa, cells were infected at a multiplicity of infection (m.o.i.) of 300–600 bacteria per host cell by the addition of 100 μl of bacteria diluted in serum-free cell medium containing 10 mg/ml bovine serum albumin (Sigma), A260 1.0. Due to the increased cytotoxicity associated with L. monocytogenes, cells were infected at a m.o.i. 50–100, A260 0.1. Plates were then incubated at 37 °C for 45 min. The medium was replaced with fresh culture medium containing 50 μg/ml of the membrane-impermeable antibiotic gentamicin (Invitrogen) to kill extracellular bacteria and incubated for 45 min. Each well was washed three times with PBS. In order to remove and lyse the cells, 30 μl of 0.25% trypsin (Invitrogen) were added to each well for 5 min, and the plate was then washed three times with PBS. The cells were scrapped and transferred to 0.65-m1 tubes, and each well was washed with an addition of 100 μl of Triton X-100 that was also transferred to the tubes. The cell lysates were diluted and plated onto LB agar plates for Pseudomonas strains or brain heart infusion agar plates for L. monocytogenes. In order to quantify the number of adherent bacteria, the cells were exposed to P. aeruginosa for 45 min at 4 °C, which inhibits endocytosis. They were then washed five times with PBS to remove nonadherent

Materials and Methods

Animals—Male Sprague-Dawley rats weighing 150–200 g were obtained from Taconic Laboratories.

Type II Cell Isolation—Isolation of primary rat type II cells was accomplished by using methods published previously (35). Approximately 20–30 million type II cells were isolated per rat with >90% viability as assessed by trypan blue exclusion. Purity was greater than 0.5% type II cells as confirmed by Papanicolaou staining. Cells were seeded on tissue culture plastic plates at a density of 5 × 105 cells/cm2 and maintained in Dulbecco’s modified Eagle’s medium (DMEM) with dexamethasone and 10% fetal bovine serum (Hyclone). At 36 h cell cultures were washed three times to remove nonadherent cells and to enrich the percentage of type II cells. All experiments were performed by identifying bacteria within the center of cells that co-localize with caveolin proteins. Co-localization was determined using a Nikon Eclipse TE200 microscope. Confocal microscopy—Primary rat type II cells were isolated and grown on 35-mm plastic dishes. They were then washed five times with PBS and fixed with glutaraldehyde, dehydrated with cold acetone, and processed using standard methods. The cells were examined by transmission electron microscopy.

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The abbreviations used are: ME-12, murine lung epithelial cell line 12; TEM, transmission electron microscopy; m.o.i., multiplicity of infection; MCD, methyl-β-cyclodextrin; OA, okadaic acid; MTI, thiazolyl blue tetrazolium; cav-1, caveolin-1; cav-2, caveolin-2; PBS, phosphate-buffered saline.
bacteria, serially diluted, and plated on LB agar, and the number of colony-forming units were counted to quantify adherent bacteria. To test the effect of pretreatment by specific lipid rafts disruptors/ 使用于细菌感染，甲壳质-环糊精 (MCD, 3 mM, Sigma), nystatin (20 μg/ml, Sigma), or filipin (0.5 μg/ml, Sigma) in serum-free medium was added to the cells for 30 min and then washed off prior to infection. To reinsert cholesterol back into the plasma membrane after MCD treatment, cells were incubated for 1 h with a cholesterol-cyclodextrin complex (0.2 mM) (36). To test the effect of altering tyrosine phosphorylation on bacterial infection, the cells were pretreated with genistein (100 μg/ml, Sigma) for 30 min prior to infection and throughout the period of infection. The effects of okadaic acid (OA, 10 nM, Sigma) were examined by having OA present throughout the infection without any pretreatment. After the preincubation step, the cells were washed, and the medium was replaced with bacteria in serum-free medium with or without the agent being tested as required. A gentamicin protection assay was performed as detailed above to quantify intracellular bacteria. The viability of the cells was not affected by any of the treatments used as determined by trypan blue exclusion.

An MTT adherence assay was performed as follows. Cells were plated 96-well plates and then incubated with drugs for 30 min as stated above and then fixed overnight with 2.0% parafomaldehyde. The monolayers were washed three times with sterile PBS and pretreated at room temperature with 2% trypsin/EDTA (Gibco). After washing, the cells were incubated with 1 ml of PBS and resuspended in 50 μl of 2% nonfat dry milk in PBS. 100 μl of A. aeruginosa, ATCC 27853 (Aa, 1.0) in PBS, was incubated with cells for 1 h at 37 °C. Nonadherent bacteria were removed by washing the cell monolayers three times with PBS. Fifty microliters of LB was applied to each monolayer and incubated for 15 min at 37 °C. Fifty microliters of 2 mg/ml MTT (Sigma) was added, and the plates were incubated for 30 min at 37 °C to allow reduction of MTT to formazan by live bacteria. Next, 150 μl of isopropyl alcohol and hydrochloric acid (24:1) was added to solubilize the formazan, and the absorbance was measured at 450 nm using a Tecan Sunrise remote microplate reader.

Western Blotting and Immunoprecipitation—Cells were grown in a 6-well plate to greater than 90% confluency. Cells were lysed with lysis buffer (1:10, Upstate Biotechnology Inc., 1:10), n-octyl-β-D-glucopyranoside (60 mM, Sigma), phenylmethylsulfonyl fluoride (1 mM), protease inhibitor mixture (1:100, Sigma). Total protein was quantified using Bradford dye (Bio-Rad) and measured at A595. Cell lysates were normalized for protein and volume. Samples were diluted in Laemmli sample buffer (Bio-Rad) with mercaptoethanol and boiled for 5 min. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Western blotting was then performed using antibodies against tubulin (1:2000, Sigma), caveolin-1 (1:5000, BD Biosciences), or caveolin-2 (1:5000, BD Biosciences), and the proteins were detected using Super Signal West Pico Chemiluminescent kit (Pierce).

Caveolin-1 and caveolin-2 were immunoprecipitated from whole cell lysates of MLE-12 cells as follows. MLE-12 cells were grown to 90% confluency in 6-well plates. The cells were washed twice with cold PBS. The cells were lysed in 500 μl of lysis buffer (lysis buffer (1:10, Upstate Biotechnology Inc., 1:10), n-octyl-β-D-glucopyranoside (60 mM, Sigma), phenylmethylsulfonyl fluoride (1 mM), protease inhibitor, phosphatase mixture (Sigma, 1:100)). The sample was precleared with 30 μl of a 50% suspension of protein G-Sepharose (Sigma) in PBS for 1 h at 4 °C. The samples were then incubated for 16 h at 4 °C with 50 μl of a 50% suspension of protein G-Sepharose in PBS plus 5 μg of rabbit polyclonal anti-caveolin-1 (BD Biosciences) or 6 μg of rabbit polyclonal caveolin-2 (Abcam) was added. The immunoprecipitates were washed three times with 1 ml of PBS and resuspended in 50 μl of Laemmli sample buffer (Bio-Rad) with mercaptoethanol and boiled for 5 min. Immunoprecipitates were assayed with antibodies for caveolin-1 (BD Biosciences), caveolin-2 (BD Biosciences and Abcam), or phosphotyrosine (Upstate Biotechnology, Inc. 4G10) by Western blotting as described above. In addition, Image J software (National Institutes of Health) was used for densitometry to quantify protein expression for statistical analysis.

Statistics—Data were compiled and analyzed using Microsoft Excel®. Data for gentamicin protection assays are reported as a mean of at least three experiments with error bars set as S.E. Significance was calculated using a two-tailed t test with significance defined and a p value of less than 0.05. Graphs were created using Graph Pad® Prism 3.0.

RESULTS

P. aeruginosa Invades Type I Pneumocytes during the Pathogenesis of Pneumonia—Although P. aeruginosa frequently colonizes the upper airways of susceptible hosts, the development of P. aeruginosa pneumonia requires dissemination to the alveolar space prior to the development of epithelial cell damage and alveolar filling. We sought to determine whether P. aeruginosa invades type I pneumocytes during the pathogenesis of pneumonia by using a rat model of P. aeruginosa pneumonia. Male Sprague-Dawley rats were intubated, and fluorescently labeled P. aeruginosa was instilled directly into the left lower lobe. Animals were sacrificed 90 min after instillation to examine the early stages of pneumonia prior to the development of severe lung injury. We examined frozen thin sections of rat lung to examine the location of P. aeruginosa within alveolar spaces. In Fig. 1A, the type I pneumocytes are labeled red with the type I cell-specific antibody, RT140. The yellow staining demonstrates areas of co-localization where P. aeruginosa is directly adherent to and possibly within type I pneumocytes. Thus, type I pneumocytes appear to be a main site of P. aeruginosa infection in a rat model of P. aeruginosa pneumonia.

P. aeruginosa Invades Type I-like Pneumocytes in an in Vitro Model of the Alveolar Epithelium—We used a common in vitro model of primary type I-like pneumocytes (35) to determine whether P. aeruginosa is able to invade type I cells. Type II cells are easily isolated and known to be the progenitor cells of the alveolar epithelium. When cultured on plastic tissue culture surface for 5–7 days, the type II cells differentiate into type I-like cells with features typical of primary type I cells. Type I-like cells have a similar membrane lipid content to primary type I pneumocytes (35) and also express type I cell-specific proteins such as aquaporin-5 (8), caveolin-1 (11, 12), and RT140 (37). This model is frequently used in vitro to study the behavior of type I cells for studies of fluid homeostasis (38), signal transduction (39), and bacterial pathogenesis (40). Western blots of whole cell lysates confirmed that after culture for 6 days, type I-like cells express type I cell-specific proteins including caveolin-1 and RT140 (data not shown).

Type I-like pneumocytes were exposed to fluorescently labeled P. aeruginosa and examined using confocal microscopy. Fig. 1B shows intracellular P. aeruginosa within the center of type I-like pneumocytes. Next, we used transmission electron microscopy (TEM) to confirm the intracellular location of P. aeruginosa within type I-like pneumocytes. P. aeruginosa was identified within type I-like cells as shown in Fig. 1C. The bacteria appeared intact and were located within vacuolar membranes. We next sought to quantify the number of adherent and intracellular P. aeruginosa after exposure to type I-like cells in vitro. After exposure to P. aeruginosa at a m.o.i. of 500, 8.6% of P. aeruginosa are able to adhere to the cell surface, and 4.5% of adherent P. aeruginosa invade type I-like cells (Fig. 1D). Thus, it appears that during the initial stages of pneumonia, P. aeruginosa is able to enter the alveolar space and adhere to and invade type I pneumocytes.

MLE-12 Cells Are Good Models for P. aeruginosa Invasion of Type I Pneumocytes—Primary type I-like cells have several limitations that make them unsuitable for studying the molecular mechanisms of P. aeruginosa invasion. These primary cells are terminally differentiated and cannot be transfected to examine the role of specific proteins in P. aeruginosa invasion. Therefore, we chose to use a murine lung epithelial cell line (MLE-12) as a model for P. aeruginosa invasion of type I cells. MLE-12 cells were initially described as a model of type II pneumocytes because of their capacity to produce surfactant proteins (41). However, they are likely immortalized in the process of differentiation into type I pneumocytes, and they possess many type I cell-specific features. MLE-12 cells lack lamellar bodies that are characteristics of type II cells (41), have visible caveolae on their cell surface typical of type I cells.
(39), and also have high levels of type I cell-specific proteins including aquaporin-5 (42) and caveolin-1 (39). MLE-12 cells were exposed to fluorescently labeled \textit{P. aeruginosa} and examined with confocal microscopy. Similar to type I-like cells, \textit{P. aeruginosa} was clearly visualized intracellularly (data not shown). In addition, MLE-12 cells were exposed to \textit{P. aeruginosa} and the number of adherent and intracellular bacteria was quantified (Fig. 2E). The frequency of adherence and invasion was nearly identical between type I-like cells and MLE-12 cells (data are presented as mean ± S.E.).

**P. aeruginosa Invasion of Type I-like Cells Is Dependent on Lipid Rafts**—We next sought to determine the mechanism by which \textit{P. aeruginosa} invades type I-like pneumocytes. To determine whether \textit{P. aeruginosa} invasion of type I-like pneumocytes occurs via lipid rafts, the cells were pretreated with cholesterol-disrupting agents prior to quantifying invasion with a gentamicin protection assay. Drugs that disrupt membrane cholesterol are known to specifically inhibit lipid raft-mediated endocytosis (20). Fig. 2A shows that \textit{P. aeruginosa} invasion of type I-like cells is significantly inhibited by low doses of nystatin, filipin, and cyclodextrin. To show that the inhibition of \textit{P. aeruginosa} invasion was due to lipid raft disruption and not any other unintended effect of these drugs, cells were treated with cyclodextrin to remove membrane cholesterol, and the cholesterol was then reinserted back into the plasma membrane by using a cholesterol-cyclodextrin complex (36). As shown in Fig. 2A, reinsertion of cholesterol back into the cell membrane restores the ability of \textit{P. aeruginosa} to invade type I-like cells. Although disruption of lipid rafts impairs the entry of \textit{P. aeruginosa} into type I-like cells, it has no effect on the invasion of type I-like cells by \textit{L. monocytogenes}, which is known to invade host cells in a lipid raft independent manner (43) (Fig. 2A). To ensure that the decreased invasion was not due to decreased bacterial adherence to the cell surface, an adherence assay was performed to quantify extracellular adherent bacteria. As shown in Fig. 2B, pretreatment with nystatin, filipin, cyclodextrin, or the cholesterol-cyclodextrin complex had no effect on bacterial adherence to type I-like cells. Similar to the invasion of type I-like pneumocytes, \textit{P. aeruginosa} invades MLE-12 cells in a lipid raft-dependent manner (Fig. 2C). Finally, the disruption of lipid rafts does not alter the adherence of \textit{P. aeruginosa} to the MLE-12 cells (Fig. 2D). Because the majority of experiments are performed with the ATCC 27853 isolate of \textit{P. aeruginosa}, we wanted to ensure that lipid raft-dependent invasion was not limited to this strain. PAO-1, a common lab isolate of \textit{P. aeruginosa}, was also found to invade MLE-12 cells in a lipid raft-dependent manner (data not shown). Therefore, removal of membrane cholesterol to inhibit lipid raft-mediated endocytosis causes a significant reduction in the number of intracellular \textit{P. aeruginosa} after infection of both type I-like cells and MLE-12 cells.

**Intracellular P. aeruginosa Co-localizes with Lipid Raft Components in Type I-like Cells**—A number of proteins and glycolipids are predominantly located within lipid rafts including the caveolin proteins, GM1, and flotillin. Confocal microscopy of permeabilized type I-like cells was used to determine whether the vacuolar membranes that surrounded intracellular...
P. aeruginosa were enriched in lipid raft components. Fig. 3 shows that fluorescently labeled P. aeruginosa co-localizes with GM1, caveolin-1, and caveolin-2. Similar co-localization of intracellular P. aeruginosa with lipid raft components was also observed in MLE-12 cells (data not shown). The co-localization of P. aeruginosa with lipid raft components such as caveolin proteins led us to question what role these proteins play in the lipid raft-mediated uptake of P. aeruginosa.

The Expression of Caveolin Proteins Is Required for Optimal P. aeruginosa Invasion of MLE-12 Cells—Because type I pneumocytes express high levels of both caveolin-1 and -2 (Fig. 4, A and B), we sought to determine whether caveolin-1 and -2 play a role in the lipid raft-mediated uptake of P. aeruginosa. Caveolin-1 has been identified as a determinant of lipid raft-mediated uptake of E. coli by bladder epithelial cells (29). The role of caveolin-2 in bacterial invasion has not been studied previously. MLE-12 cells were used to create stable knockdowns of caveolin-1 (pQC-cav-1) and caveolin-2 (pQC-cav-2) by using RNA interference. Western blotting after several passages confirmed the generation of stable knockdowns of both caveolin proteins (Fig. 4 C). The adherence and invasion of P. aeruginosa to MLE-12 cells (data are presented as mean ± S.E., *, p < 0.05).

![Figure 2](http://www.jbc.org/)
After fixation, the cells were labeled with antibodies directed against caveolin-1 (C,) and caveolin-2 (2). Coverslips and infected with fluorescently labeled bacteria similar to pQC-cav-1 cells with decreased expression of both caveolin-1 and -2 was resistant to 

P. aeruginosa invasion with 50% less intracellular localization of caveolin-2. Similar co-localization of P. aeruginosa with lipid raft components was also seen after infection of MLE-12 cells (data not shown).

P. aeruginosa invasion of pQC-cav-1 and pQC-cav-2 was quantified to determine the possible role of caveolin proteins in P. aeruginosa invasion, pQC-cav-1 cells with decreased expression of both caveolin-1 and -2 were resistant to P. aeruginosa invasion with 50% less intracellular P. aeruginosa after infection (Fig. 4E). In order to determine whether the inhibition of P. aeruginosa invasion was because of decreased expression of caveolin-1 or -2, we then examined P. aeruginosa invasion in the selective caveolin-2 knockdown (pQC-cav-2). After infection with P. aeruginosa, pQC-cav-2 cells had 50% fewer intracellular bacteria similar to pQC-cav-1 cells with decreased expression of caveolin-1 and -2 (Fig. 4E). Down-regulation of caveolin-1 and -2 has no effect on P. aeruginosa adherence (Fig. 4F) or the invasion of L. monocytogenes (Fig. 4E). Thus, it appears that caveolin-2 expression is required for optimal P. aeruginosa invasion of MLE-12 cells.

P. aeruginosa Invasion of MLE-12 Cells Is Dependent on Tyrosine Phosphorylation of Caveolin-2—Because caveolin-2 expression is required for maximal P. aeruginosa invasion, we next sought to determine the mechanism by which caveolin-2 may be involved in controlling lipid raft-mediated endocytosis. Caveolin-1 and -2 have been recognized to have important sites that may be involved in controlling lipid raft-mediated endocytosis. Several authors (19, 45) have shown that caveolin-1 serves to stabilize the plasma membrane and decrease membrane fluidity. They concluded that lipid raft-mediated endocytosis is controversial. Several authors (19, 45) have shown that caveolin-1 knockdowns have been limited in their ability to use lipid raft-mediated endocytosis to invade type I pneumocytes. We believe that P. aeruginosa has co-opted the pathway of lipid raft-mediated endocytosis as a means of surviving within the protected intracellular environment. Once P. aeruginosa is intracellular, it is safe from clearance by alveolar macrophages and may be able to replicate within this protected environment. Alternatively, P. aeruginosa may leave type I cells via egress from the basolateral surface. This penetration of the alveolar capillary barrier would allow dissemination into the bloodstream and throughout the host. P. aeruginosa invasion of type I pneumocytes via lipid rafts may be a critical event in the pathogenesis of P. aeruginosa pneumonia.

The role of caveolin proteins in the regulation of lipid raft-mediated endocytosis is controversial. Several authors (19, 45) have shown that caveolin-1 serves to stabilize the plasma membrane and decrease membrane fluidity. They concluded that decreased caveolin-1 expression led to increased caveolae-mediated uptake. On the other hand, previous work from our laboratory has shown that decreased expression of caveolin-1 impairs E. coli invasion of bladder epithelial cells through lipid rafts (29). It is possible that the role of caveolin proteins differs depending on the specific cell type and also the endocytic cargo. In addition, studies of caveolin-1 knockdowns have been lim-

**FIG. 3. Intracellular P. aeruginosa co-localizes with lipid raft components.** Type I-like cells were cultured on Permanox® plastic coverslips and infected with fluorescently labeled P. aeruginosa (red). After fixation, the cells were labeled with antibodies directed against typical lipid raft components (green) and examined using confocal microscopy. Confocal sections through the center of type I-like cells reveals that intracellular P. aeruginosa co-localizes (yellow) with GM1 (A), caveolin-1 (B), and caveolin-2 (C). Using confocal microscopy to quantify the percent of co-localization with lipid raft proteins, we found that 80% of intracellular P. aeruginosa co-localized with a caveolin-1, and 84% of intracellular P. aeruginosa co-localized with caveolin-2. Similar co-localization of P. aeruginosa with lipid raft components was also seen after infection of MLE-12 cells (data not shown).
edited due to the concomitant decrease in caveolin-2 expression because caveolin-1 is required as a chaperone for the transport of caveolin-2 (33, 34). It is possible that many of the functions previously attributed to caveolin-1 may be due to caveolin-2. For example, the caveolin-2 knock-out mice have a similar pulmonary phenotype to caveolin-1-deficient mice despite ade-
quate expression of caveolin-1 and the formation of visible caveolae on the cell membrane (46).

In our model of *P. aeruginosa* invasion, decreased expression of caveolin-1 and -2 significantly inhibits *P. aeruginosa* invasion of MLE-12 cells. Our data suggest that caveolin-2 may be a more important determinant of *P. aeruginosa* invasion than caveolin-1 because a selective knockdown of caveolin-2 expression has the identical susceptibility to *P. aeruginosa* invasion as the combined caveolin-1 and -2 knockdown. Although we cannot rule out a significant contribution of caveolin-1 to the regulation of *P. aeruginosa* invasion, we can conclude for the first time that caveolin-2 plays a critical role in mediating this endocytic process.

Lipid rafts have been implicated in a wide range of cellular functions including endocytosis and cell signaling. We have shown that the endocytosis of *P. aeruginosa* not only requires the integrity of lipid raft platforms and expression of caveolin proteins but also the tyrosine phosphorylation of key lipid raft proteins including caveolin-2. In our model, tyrosine phosphorylation of caveolin-2 appears to be a key regulator of lipid raft-mediated endocytosis. Caveolin-2 has a recognized conserved site for tyrosine phosphorylation at its N terminus on FIG. 5. Lipid raft-dependent tyrosine phosphorylation of caveolin-2 appears to regulate *P. aeruginosa* invasion of MLE-12 cells.

*Fig. 5.* Lipid raft-dependent tyrosine phosphorylation of caveolin-2 appears to regulate *P. aeruginosa* invasion of MLE-12 cells. A, gentamicin protection assays were used to compare the number of intracellular *P. aeruginosa* after manipulating phosphorylation signaling pathways. Treatment with the tyrosine kinase inhibitor genistein (100 μg/ml) for 30 min prior to infection and during infection significantly decreased the number of intracellular *P. aeruginosa*. In contrast, the number of intracellular *P. aeruginosa* bacteria was significantly increased when MLE-12 cells were infected in presence of OA (10 mM). Most interestingly, the combination of OA and genistein had no effect on *P. aeruginosa* invasion. Pretreatment with methyl-β-cyclodextrin (3 mM) for 30 min prior to *P. aeruginosa* infection in the presence of OA negated the previously noted increased invasion associated with OA. Neither OA nor genistein affected the invasion of *L. monocytogenes*. B, OA and genistein had no effect on the adherence of *P. aeruginosa*. C, immunoprecipitation of caveolin-2 was performed to examine the effects of *P. aeruginosa* infection on the tyrosine phosphorylation of caveolin-2. The bar graph shows the densitometry measured from Western blots obtained from three separate experiments. *P. aeruginosa* infection significantly increases cav-2 tyrosine phosphorylation. Treatment with genistein before and during *P. aeruginosa* infection decreased caveolin-2 phosphorylation. On the other hand, Cav-2 tyrosine phosphorylation was significantly increased by infection in the presence of OA. Finally, tyrosine phosphorylation of caveolin-2 is dependent on lipid raft integrity as pretreatment with MCD prior to *P. aeruginosa* infection with OA negated the effects of OA (data are presented as mean ± S.E., *, *p < 0.05).
tyrosine 19 (QLFMADDApY, where pY is phosphotyrosine) and can be phosphorylated by c-Src, which is located within lipid rafts (47). We are currently trying to identify the site and mechanism of caveolin-2 phosphorylation after P. aeruginosa infection. Multiple pathogens have been recognized to induce phosphorylation of host proteins and alter host cell signaling during infection (44). It has been recognized that P. aeruginosa infection of airway epithelial cells is able to induce phosphorylation of other host proteins including MUC1 and also activate mitogen-activated protein kinase signaling pathways (48). Bacterial pathogens may have evolved mechanisms of altering host cell signaling pathways in order to invade host cells and develop a survival advantage. In addition, it is not known how caveolin-2 phosphorylation may mediate the downstream events that appear to regulate lipid raft-mediated uptake of P. aeruginosa. Phosphorylation of caveolin-2 causes the dissociation of caveolin-2 from caveolin-1 hetero-oligomers. After phosphorylation caveolin-2 remains with the lipid rafts and is now able to bind Src homology 2 domain containing proteins, including RAS-gap (47). Therefore, the phosphorylation of caveolin-2 may activate host cell signaling pathways that regulate lipid raft-mediated endocytosis. Alternatively, caveolin-2 may interact directly with important cytoskeletal proteins depending on its phosphorylation state. Although endocytosis and signaling are often considered distinct functions of lipid rafts, we have shown here that they are interdependent. The integrity of lipid rafts brings together key signaling molecules that coordinate the complex cellular process of endocytosis of P. aeruginosa by alveolar epithelial cells.

In summary we have shown that P. aeruginosa has evolved the ability to invade type I pneumocytes by co-opting the pathway of lipid raft-mediated endocytosis. The uptake of P. aeruginosa by alveolar epithelial cells is dependent on the lipid raft integrity as well as tyrosine phosphorylation. Specifically, P. aeruginosa invasion requires the adequate expression and phosphorylation of caveolin-2. Although P. aeruginosa has evolved the ability to invade type I pneumocytes through lipid rafts, the evolutionary response of the host is still unknown. Previous studies (13) have shown that nasal and bronchial epithelial cells undergo apoptosis after P. aeruginosa invasion. Similarly, bladder epithelial cells undergo apoptosis after E. coli invasion via lipid rafts (49). This host defense mechanism allows these epithelial cells to shed infected cells and clear bacterial pathogens. The bronchial, nasal, and bladder epithelia are several layers thick and able to tolerate superficial apoptosis. The alveolar epithelium is a single layer thick and an important regulator of permeability within the lung. Apoptosis may not be protective in this compartment but rather may have detrimental effects on alveolar permeability leading to alveolar filling and worsening oxygenation. This process may contribute to the observation that invasive P. aeruginosa infections in the alveolar space frequently lead to pneumonia, sepsis, and death (2). Future studies to examine the role of lipid rafts and caveolin in an animal model will help to elucidate the host response in the ongoing evolutionary battle.

Acknowledgments—We thank Kathy Evans for help with transmission electron microscopy and Jaret Malloy for help with isolation of primary rat type II pneumocytes.

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