β -Arrestin 1 Participates in Platelet-Activating Factor Receptor-Mediated Endocytosis of *Streptococcus pneumoniae*†

Jana N. Radin,^{1,2} Carlos J. Orihuela,¹ Gopal Murti,³ Christopher Guglielmo,¹
Peter J. Murray,¹ and Elaine I. Tuomanen^{1,2*}

Department of Infectious Diseases¹ and Department of Molecular Biotechnology,³ St. Jude Children's Research Hospital, Memphis, Tennessee 38105, and Department of Molecular Sciences, University of Tennessee Health Science Center, Memphis, Tennessee 38163²

Received 26 April 2005/Returned for modification 10 June 2005/Accepted 1 September 2005

Pneumococci traverse eukaryotic cells within vacuoles without intracytoplasmic multiplication. The platelet-activating factor receptor (PAFr) has been suggested as a portal of entry. Pneumococci colocalized with PAFr on endothelial cells and PAFr^{-/-} mice showed a substantially impaired ability to support bacterial translocation, particularly from blood to brain. Pneumococci-induced colocalization of PAFr and β -arrestin 1 at the plasma membrane of endothelial cells and PAFr-mediated pneumococcal uptake in transfected COS cells were greatly increased by cotransfection with the scaffold/adaptor protein β -arrestin 1. Activation of extracellular signal-regulated kinase kinases was required for uptake and was limited to the cytoplasmic compartment, consistent with activation by β -arrestin rather than PAFr. Uptake of the pneumococcal vacuole involved clathrin, and half the bacteria proceeded into vacuoles marked by Rab5 and later Rab7, the classical route to the lysosome. Overexpression of β -arrestin in endothelial cells decreased colocalization with Rab7. We conclude that the association of β -arrestin with the PAFr contributes to successful translocation of pneumococci.

The integrity of body compartments is maintained in part by the boundaries of epithelial and endothelial cells. Invasive pathogens have developed strategies to traverse these boundaries by passing either between or through cells. Most bacteria enter cells by subverting host cell membrane trafficking and signal transduction pathways to create a cytoplasmic vacuolar compartment suited for bacterial multiplication (12). However, a few significant human pathogens do not multiply intracellularly but rather translocate efficiently through cells in vacuoles. One example is *Streptococcus pneumoniae*, the leading gram-positive pathogen causing pneumonia, sepsis, and meningitis (26). Pneumococci translocate across human endothelial cells without intracellular multiplication (18). The mechanism of this intracellular routing is unknown.

Pneumococcal infection is initiated by asymptomatic colonization of the nasopharynx followed by dissemination into the lungs, blood, and cerebrospinal fluid during invasive disease. To cross into these compartments, pneumococci engage the platelet-activating factor (PAF) receptor (PAFr), a G-protein-coupled receptor (GPCR) for the circulating lipid chemokine PAF (5, 18). Pneumococci bind to the PAFr via cell wall phosphorylcholine (5), a moiety that mimics the bioactive determinant of PAF. Utilization of PAFr as a mechanism of bacterial entry into human cells was first described for pneumococci (5) but is now recognized as a portal of invasion for

many respiratory pathogens that externally display phosphorylcholine on their surfaces (22, 24, 25). Cundell et al. showed that PAFr-transfected cells enabled adherence, whereas adherence was absent in untransfected epithelial cell lines (5). Ring et al. extended this model to brain microvascular endothelial cells (18). Transparent colony variants bearing more phosphorylcholine interacted with brain microvascular endothelial cells three- to fivefold better than opaque variants.

Typical of GPCRs, PAF bound to PAFr is taken up into a vacuole that can then either recycle back to the cell surface or fuse with lysosomes to terminate signaling (3, 4). When pneumococci bind PAFr, trafficking of the vacuole has been shown not only to recycle to the apical surface but also to translocate to the basolateral surface of the cell, with resultant delivery of viable bacteria across endothelia and epithelia (18). How the bacteria alter the destination of the PAFr vacuole to circumvent lysosomal fusion is unknown.

It is reasonable to hypothesize that the adapter proteins, the β -arrestins, would play a role in the direction of vacuolar movement, since they tether ligand-occupied GPCRs to the vesicular trafficking system. β -Arrestin 1 and 2 bind to clathrin and β 2 adaptin and target GPCRs to clathrin-coated pits and endocytosis (15). This process also uncouples G-protein signaling and down regulates the response to ligand-bound GPCRs (4). Pneumococcal uptake is consistent with this concept, since classical G-protein signaling is absent upon ligation of PAFr by pneumococci (5, 9). Once β -arrestins target the GPCR to endocytosis and uncouple G-protein signaling, they recruit and activate the extracellular signal-regulated kinase 1 (ERK-1)/ERK-2 and Jun N-terminal protein kinase 3 mitogen-activated protein (MAP) kinase pathways (14). In accordance with this sequence of events, exposure of cells to pneumococci

* Corresponding author. Mailing address: St. Jude Children's Research Hospital, Mailstop 320 IRC 8057, 332 N. Lauderdale Rd., Memphis, TN 38105. Phone: (901) 495-3486. Fax: (901) 495-3099. E-mail: elaine.tuomanen@stjude.org.

† Supplemental material for this article may be found at <http://iai.asm.org/>.

activates ERK-1/ERK-2 (19). Thus, circumstantial evidence suggests pneumococcal uptake has characteristics of a β -arrestin-mediated event. Here we sought to determine whether pneumococci use the same β -arrestin-mediated uptake pathway as the PAFr and, if so, what host cell mechanisms the bacteria use to alter the fate of the PAFr vacuole to transcytosis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used were T4R, an unencapsulated derivative of the clinical strain of *S. pneumoniae* Norway type 4 (10), and D39X, an encapsulated, stable bioluminescent derivative of D39 (16). *S. pneumoniae* was grown without aeration at 37°C in 5% CO₂ in a defined semisynthetic medium (13) or plated on tryptic soy agar (Difco) supplemented with 3% sheep blood. Pneumococci with integrated plasmids were grown in the presence of the appropriate antibiotics (5 μ g/ml of chloramphenicol for T4R and 400 μ g/ml of kanamycin for D39X).

Confocal laser scanning microscopy. A rat brain capillary endothelial cell line rBCEC₆ (2) (provided by J. Weber, Berlin, Germany) was grown in Dulbecco's minimal essential medium (MEM) (Sigma) containing 10% fetal calf serum (GIBCO), 2 mM L-glutamine (Cellgro), 110 μ g/ml of sodium pyruvate (Sigma), 100 μ g/ml heparin (Sigma), and 10 μ g/ml endothelial cell growth factor (Biomedical Technologies, Inc.) in 2-well chamber slides (Nalge Nuc International). Twenty-four hours later, the cells were exposed to 10⁷ CFU/ml of pneumococcus T4R for 0, 15, 30, 60, or 90 min. The cells were washed, fixed with 4% paraformaldehyde, blocked and permeabilized with 1× Tris-buffered saline, 0.5% bovine serum albumin, 0.5% Triton X-100, and 4% goat serum, and incubated with goat polyclonal antibody against either PAFr (1:250, sc-8744; Santa Cruz), Rab7 (1:200, ab15702; abcam), or rabbit polyclonal antibody against Rab 5 (1:100, ab13253; abcam) or Rab11 (1:500, ab3612; abcam), followed by Alexa 488 goat anti-rabbit antibody (1:200; Molecular Probes) or Alexa 488 rabbit anti-goat antibody (1:200; Molecular Probes). Subsequently, the cells were incubated with either monoclonal mouse antibody against TEPC-15 (1:100; Sigma), followed by Texas Red goat anti-mouse antibody (1:100; Antibodies, Inc.) or Texas Red rabbit anti-mouse antibody (1:100; Open Biosystems) to label the bacteria or mouse anti- β -arrestin antibody (1:250; BD Transduction Laboratories), followed by Alexa 594 goat anti-mouse antibody (1:200; Molecular Probes). To label the nucleus, the cells were mounted in a medium containing the DNA-specific dye TO-PRO-3 (Molecular Probes). The samples were examined in a Leica TCS NT SP confocal laser scanning microscope equipped with argon (488 nm), krypton (568 nm), and helium-neon (633 nm) lasers. The lasers permitted the imaging of green (emission, 518 nm), red (emission, 570 nm), and far red (emission, 661 nm; pseudocolored blue, TO-PRO-3) fluorochromes, respectively. Scanning was performed in the X, Y, and Z planes at a laser power of 100% on all lasers. Single optical sections (0.5 μ m) were obtained through the center of the sample, with the averaging function set at 4. Quantitation of colocalization was determined by counting at least 100 rBCEC₆ cells.

Cell transfection. COS-7 cells (ATCC) or rBCEC₆ cells were grown in Dulbecco's MEM (Sigma) supplemented with 10% fetal bovine serum (BioWhittaker) and 2 mM L-glutamine (Cellgro). Cells (5 × 10⁴ per well) were seeded in a 24-well plate. One day later, the COS-7 cells were transfected using the FuGENE6 kit (Roche) with expression vectors for PAFr-green fluorescent protein (GFP), mutant PAFr D289A-GFP, β -arrestin 1, and mutant β -arrestin V53D (0.2 to 0.4 μ g of DNA per well) as described previously (4). Alternatively, 0.2 μ g of β -arrestin 1 vector DNA per well was used for rBCEC₆ cells. The mutation of PAFr D289A-GFP uncouples G protein signaling, so PAFr cannot be internalized. The β -arrestin mutant V53D is a dominant-negative mutant and cannot target the PAFr to clathrin-coated vesicles (4). Success of transfection was monitored by fluorescence microscopy for PAFr-GFP constructs and by Western blotting for β -arrestin 1 (data not shown).

Invasion and adhesion assay. Monolayers (1 × 10⁶ cells) of transfected and control COS cells were exposed for 1 or 3 h to 2 × 10⁶ CFU/ml of *S. pneumoniae* T4R (6). The strain T4R is an unencapsulated derivative of the type 4 strain used in the *in vivo* challenge studies; although they adhere to and invade cells by the same mechanisms, unencapsulated variants adhere more, making quantitation *in vitro* easier. For adhesion assays, infected cells were washed, trypsinized, and counted, and 100 μ l was plated on blood agar plates to determine the number of adhering bacteria. For some adhesion assays, rBCEC₆ cells were treated with 50 μ g/ml of anti-PAFr antibody (sc-8744; Santa Cruz) or 1 μ M ($\pm$) *trans*-2,5-bis (3,4,5-trimethoxyphenyl)-1,3-dioxolane (active PAFr antagonist) or ($\pm$) *cis*-2,5-bis (3,4,5-trimethoxyphenyl)-1,3-dioxolane (inactive PAFr antagonist) for 1 h prior to infection. Treating cells with the PAFr antagonist did not have an effect

on pneumococcal adherence, since mean values for cells treated with active and inactive PAFr antagonist were 1.30 × 10⁶ CFU/10⁶ cells and 8.83 × 10⁵ CFU/10⁶ cells, respectively. For invasion assays, infected cells were washed and incubated in media containing 10 μ g/ml of penicillin (Fisher Biotech) and 200 μ g/ml of gentamicin (GIBCO) for 1 h to kill extracellular bacteria. Cells were counted, lysed with 0.025% Triton X-100 (Sigma), and plated on blood agar plates to determine the number of internalized bacteria. For some experiments, 50 μ M chlorpromazine (Sigma) was added for 1 h in Eagle's MEM supplemented with 20 mM HEPES, 2 mg/ml bovine serum albumin. The cytotoxicity of chlorpromazine was determined by exclusion of trypan blue (8% in MEM) at 0, 30, 60, 120, 180, and 240 min. Trypan blue exclusion was quantified by counting a minimum of 100 cells per well using an inverted phase-contrast microscope. Alternatively, the MAP kinase inhibitor PD98059 (10 μ M; Calbiochem) was added for 30 min prior to challenge of the cells with bacteria.

ERK kinase assay. For the ERK kinase assay, 2 × 10⁶ rBCEC₆ cells were plated on 60-mm tissue culture dishes for 24 h. For some experiments, the ERK kinase assay was performed on rBCEC₆ cells pretreated with 1 μ M PAFr antagonist ($\pm$) *trans*-2,5-bis (3,4,5-trimethoxyphenyl)-1,3-dioxolane (BIOMOL Research Labs) or inactive control ($\pm$) *cis*-2,5-bis (3,4,5-trimethoxyphenyl)-1,3-dioxolane for 1 h. Cells were infected with 10⁵ CFU/ml of *S. pneumoniae* T4R for between 15 min and 2 h. ERK kinase activity was detected as described previously (19) using the Phospho Plus p44/42 ERK kinase (Thr 202/Tyr 204) antibody kit (Cell Signaling). The nuclear and cytosolic fractions of rBCEC₆ cells were prepared as described previously (7). Briefly, whole cells were lysed by mechanical disruption through a narrow-gauge needle (25-gauge, 5/8 in.) in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and protease inhibitor cocktail tablet) and centrifuged to pellet the crude nuclei and membrane fraction; the supernatant was harvested as the cytosolic fraction. The pellet was resuspended in high-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and protease inhibitor), again disrupted mechanically and centrifuged at 25,000 × g to pellet nuclear debris; the supernatant was harvested as the nuclear extract. Protein concentrations were determined using the Bio-Rad protein concentration reagent as described by the manufacturer. To confirm absence of cross contamination of cytoplasmic and nuclear fractions, 20 μ g of protein from each fraction was assessed by Western blotting with anti-human histone deacetylase 1 (HDAC1) antibody (1:100; Santa Cruz Biotechnology, Inc.) and anti-human dynamin II antibody (1:100; Santa Cruz). Binding was detected by rabbit anti-goat immunoglobulin G horseradish peroxidase-conjugated secondary antibody (1:3,000) and Super West Pico chemiluminescent substrate (Pierce) (data not shown).

Generation of mice lacking the PAFr. The *PAFR* is encoded on a single exon, and the knockout strategy was designed to delete 951 of 1,023 bp of the coding region. We used a *Saccharomyces cerevisiae*-based strategy to target the PAFr gene carried on a yeast artificial chromosome (YAC) clone as described in detail previously (27). YAC clones containing the murine *PAFR* gene were isolated from the WI/MIT 850 YAC library by PCR screening. Primers were designed to insert a HIS3-*neo* cassette into the *PAFR* gene to delete the majority of the coding region. Primers complementary to the 5' and 3' regions to be deleted were used to amplify the HIS3-*neo* cassette as described. Yeast were transformed with the PCR product, and clones were isolated on histidine-deficient yeast nitrogen base plates and screened by PCR for the presence of the correct recombinant. The targeted *PAFR* locus was recovered from yeast using the recircularization technique described in detail in reference 27. BamHI was used to digest total yeast genomic DNA carrying the targeted YAC prior to recircularization. Recombinants were selected on LB plates with zeocin. Correct clones were digested with *AscI* to liberate the HIS3-*neo* cassette. This cassette was replaced with a thymidine kinase-*neo* cassette for embryonic stem (ES) selection. The targeting vector was further digested with *EcoRI* and BamHI to isolate a probe fragment suitable for Southern blotting. Bruce4 ES cells were transfected with the linearized targeting vector and selected with 150 μ g/ml G418. Genomic DNA from G418-resistant ES cells was digested with BamHI and *MluI* and screened with the probe to isolate correctly targeted cells. Five of 192 ES clones with the correct targeting event were isolated, further screened by PCR, and then karyotyped prior to injection into BALB/c blastocysts. Chimeric mice were identified by coat color and used for germ line transmission of the mutant allele. Mice were genotyped using the PCR-based strategy depicted in Fig. S1b and c in the supplemental material.

Mouse models. BALB/c wild-type (WT) and PAFr^{-/-} mice were maintained in a BSL2 facility at St. Jude Children's Research Hospital. Four-week-old mice were anesthetized with either 2.5% inhaled isoflurane (Baxter Healthcare Corp.) or 5 μ l of MKX (1 ml of 100-mg/ml ketamine [Fort Dodge Laboratories], 5 ml of xylazine [Miles Laboratories], and 21 ml of phosphate-buffered saline)/g of

FIG. 1. Colocalization of PAFr and pneumococci. rBCEC₆ cells were infected with 10^7 CFU/ml of pneumococcus T4R for 60 min, and immunostaining for the PAFr and bacteria was performed and imaged by confocal microscopy. (a) PAFr staining is shown in presence and absence of pneumococci. T4R pneumococci were detected by Texas Red TEPC-15 antibody to the choline on the cell wall. PAFr labeled with Alexa 488-tagged secondary antibody appeared green. Colocalization was indicated by yellow in the merged images. Blue staining shows the location of the nucleus. (b) A three-dimensional Z-stack of the PAFr-pneumococcus interaction (movie available at <http://www.stjuderresearch.org/data/b-arr>).

body weight. WT and PAFr^{-/-} mice (9 WT each for 24 and 48 h, 7 null for 24 h, and 5 null for 48 h; $n = 3$ experiments) were infected intratracheally with 2×10^5 to 4×10^5 CFU/ml of *S. pneumoniae* D39X, a bioluminescent strain suitable for tracking by the Xenogen Imaging System. At 24 and 48 h postinfection, mice were imaged using the Xenogen IVIS camera to document differences in the course of invasive disease. Lung and blood samples were collected at 6, 9, 12, 15,

and 18 h, and bacterial titers were determined ($n = 34$ for WT and 54 for PAFr^{-/-} mice). To model meningitis, WT or PAFr^{-/-} mice ($n = 24$ for WT and 30 for PAFr^{-/-} mice) were challenged intravenously with 10^4 CFU/ml of D39X ($n = 3$ experiments). At 21 h, blood and cerebrospinal fluid (CSF) samples were collected, and bacterial titers were determined (minimum level of detection, 1,000 CFU/ml for each site).

TABLE 1. Effect of anti-PAFr antibody and PD98059 on bacterial adhesion and invasion

rBCEC ₆ treatment	Adherence (CFU/10 ⁶ cells)	Invasion (CFU/10 ⁶ cells)	P value
None	2.22×10^6		
Anti-PAFr antibody (50 μ g)	6.92×10^5		<0.001
Isotypic antibody (50 μ g)	1.17×10^6		0.333
None		1.12×10^4	
PD98059 (10 μ M)		5.08×10^3	0.008

RESULTS

Pneumococci interact with PAFr. Previous evidence from cell culture systems has indicated that the presence of the PAFr contributes to pneumococcal attachment (5). We therefore sought evidence for physical proximity of pneumococci and PAFr within infected endothelial cells by confocal imaging (Fig. 1a and b). An overlay of the two images demonstrated colocalization of the signals consistent with close apposition of bacteria and endocytic vesicles containing the PAFr. A movie showing a three-dimensional Z-stack of the PAFr-pneumococcus complex provided further evidence of the interaction of the two (available at <http://www.stjuderresearch.org/data/b-arr>). Examination of 200 cells revealed 82% of bacteria (452 of 549 bacteria) colocalized with PAFr ($n = 3$). Interaction of pneumococci with the PAFr was further evidenced by pretreatment of rBCEC₆ cells with a PAFr antibody, resulting in a 72% $\pm$ 11% decrease of adhesion (Table 1).

To show definitively that the PAFr played a significant role in bacterial translocation in vivo, courses of pneumonia and meningitis were monitored in wild-type (WT) and PAFr^{-/-} mice. PAFr^{-/-} mice appeared healthy with normal life spans and breeding capacities. To detect translocation in the lung, animals were challenged intratracheally with pneumococci and the timing and incidence of subsequent appearance of bacteria in blood were determined. PAFr^{-/-} mice had greater bacterial growth locally in the lung at 24 h, as demonstrated by bioluminescent imaging (example shown in Fig. 2a). This was confirmed in lung homogenates by median bacterial titers of 1.16×10^7 CFU/ml for PAFr^{-/-} mice versus 3.4×10^5 for WT mice ($P = 0.010$, t test). However, at 48 h, titers in lungs of WT mice had overtaken those of PAFr^{-/-} mice (3.95×10^8 versus 4.7×10^7 , respectively). Escape of bacteria from the lung into the blood appeared to be somewhat impaired in PAFr^{-/-} mice, since for a given lung titer at 24 h, the incidence and degree of bacteremia was greater in WT mice than in PAFr^{-/-} mice (Fig. 2b). This suggested that translocation from the lung to the blood was at least partially delayed in PAFr^{-/-} animals.

To study the role of PAFr in transmigration across the endothelium of the blood brain barrier in vivo, high-titer bacteremia (10^5 CFU/ml) was created by intravenous bacterial injection. WT and PAFr^{-/-} mice were monitored for the spread of bacteria into the subarachnoid space. WT mice developed meningitis with median titers in CSF of 10^4 and 10^5 CFU/ml for titers in blood of 10^6 and 10^7 CFU/ml, respectively, while bacteria were undetectable in the CSF of 16 of 20 PAFr^{-/-} mice (Fig. 2c) ($P \leq 0.038$). A 2-log increase in bacterial titer in blood to 10^8 CFU/ml was required before a significant number of PAFr^{-/-} mice (3 of 4) developed meningitis. This indicates

that absence of the PAFr results in a substantial barrier to bacterial translocation across the cerebral capillary endothelium.

Role of β -arrestin in pneumococcal invasion. The nonvisual arrestins, β -arrestin 1 and 2, act as adapter proteins and target GPCRs to clathrin-coated vesicles. Binding of PAF to the PAFr has been shown to induce translocation of β -arrestin 1 from the cytosol to the plasma membrane, followed by movement of both PAFr and β -arrestin into intracellular vesicles (4). Since pneumococci invade by binding to the PAFr, β -arrestins and clathrin would be expected to participate in bacterial uptake. Confocal microscopy studies (Fig. 3a) showed that, in uninfected rBCEC₆ cells, expression of the PAFr was low and β -arrestin was mainly localized in the cytosol. Upon infection with *S. pneumoniae* T4R, PAFr expression increased and β -arrestin moved to the plasma membrane, colocalizing with PAFr as shown in the confocal microscopy image and the three-dimensional Z-stack movie (Fig. 3a and b; movie available at <http://www.stjuderresearch.org/data/b-arr>). Colocalization was independent of G protein signaling by PAFr, since the presence of PAFr antagonist resulted in persistent PAFr- β -arrestin colocalization (Fig. 3a).

To examine the role of clathrin, cells were treated with chlorpromazine, a specific inhibitor of the assembly and recycling of clathrin-coated vesicles. Chlorpromazine treatment was not toxic (trypan blue uptake of 3.7 to 5.4% in treated and untreated cells). Chlorpromazine pretreatment of COS cells transfected with PAFr-GFP and β -arrestin 1 resulted in a decrease in pneumococcal invasion from 18.4×10^3 ($\pm 3 \times 10^3$) CFU/10⁶ cells to 6.3×10^3 ($\pm 0.8 \times 10^3$) CFU/10⁶ cells ($P \leq 0.004$). Pneumococcal vacuoles were also shown to stain with endosomal markers EEA-1 and LAMP-1 (see Fig. S2 in the supplemental material). Taken together, the colocalization data of Fig. 1 and 3 suggest that, immediately after uptake, pneumococci traffic along the same cellular route as PAFr, β -arrestin, and clathrin.

The N-terminal region of β -arrestin harbors a GPCR binding site and a regulatory domain (14). The C-terminal region contains the phosphorylation site at S412, the clathrin binding site (LIEF motif), and the binding site for β 2-adaptin (RXR motif) (14). The two regions are separated by a domain which senses phosphorylation of the GPCR to promote β -arrestin binding. A β -arrestin mutant, V53D, a dominant-negative mutant, prevents GPCR targeting to clathrin-coated pits (4). Similarly, a PAFr mutant, D289A, which contains a mutation in the DPXXY motif of the seventh transmembrane domain, cannot bind β -arrestin, leading to a lack of PAFr internalization (4). To test whether β -arrestin 1 function promoted pneumococcal invasion, COS cells were cotransfected with combinations of constructs of WT and mutant PAFr and β -arrestin 1 and challenged with pneumococci. Adhesion assays with transfected COS cells showed that cells transfected with either PAFr-GFP or D289A-GFP equally enabled pneumococcal adherence, while transfection with β -arrestin alone did not (Fig. 3c). Despite adequate adherence, *S. pneumoniae* showed low basal levels of invasion into cells transfected with PAFr-GFP, levels equal to β -arrestin 1 alone (Fig. 3c). In contrast, cells transfected with both PAFr-GFP and β -arrestin 1 showed greatly enhanced invasion ($P \leq 0.017$). Although supportive of adherence, cotransfection with pairs of constructs bearing dys-

FIG. 2. Effect of absence of PAFr on the course of pneumonia and meningitis in vivo. (a) WT and PAFr^{-/-} mice were infected intratracheally with D39X. Disease progression was monitored at 24 and 48 h using the Xenogen IVIS camera. (b) Bacterial titers in the lung and blood were monitored from 6 to 48 h for WT (black circles) and PAFr^{-/-} (gray squares) mice infected as described for panel a. *, $P = 0.036$. Data for individual mice are shown. (c) WT (black circles) and PAFr^{-/-} (gray squares) mice were infected intravenously with D39X, and bacterial titers in blood and CSF were determined between 6 and 48 h. Data for individual mice are shown. *, $P = 0.004$; **, $P = 0.007$.

FIG. 3. β -Arrestin 1 colocalizes with the PAFr after pneumococcal infection and is required for PAFr-dependent bacterial invasion. (a) For uninfected rBCEC₆ cells or cells infected with 10^7 CFU/ml of T4R for 60 min, immunostaining for the PAFr and β -arrestin 1 was performed and imaged by confocal microscopy. For some studies, infected cells were also pretreated with 1 μ M PAFr antagonist. PAFr was labeled with Alexa 488-tagged secondary antibody (green), β -arrestin 1 was labeled with Alexa 594-tagged secondary antibody (red), and merging of the images demonstrates colocalization in yellow. Blue staining defines the nucleus. (b) A three-dimensional Z-stack of the PAFr- β -arrestin complex is shown (available at <http://www.stjude-research.org/data/b-arr>). (c) COS cells were transfected with either PAFr-GFP alone, β -arrestin 1 alone, PAFr-GFP and β -arrestin 1, PAFr-GFP and mutant β -arrestin 1, or with β -arrestin 1 and mutant PAFr-GFP. Transfected cells were infected with 2×10^6 CFU/ml of T4R for 1 or 3 h, and adhesion (black bars) or invasion (gray bars) assays were performed as described in Materials and Methods. The assays were repeated a minimum of three times for each condition. Data are depicted as percentages of the mock-transfected control cells (100% = 8.41×10^3 CFU/ 10^6 cells for the invasion assay and 4.48×10^5

functional PAFr or β -arrestin 1 failed to enable pneumococcal invasion (Fig. 3c), suggesting a significant role for an active PAFr- β -arrestin pathway in pneumococcal uptake in this reconstituted system.

MAP kinase activation during invasion. Phosphorylation of MAP kinases occurs when β -arrestin binds GPCR. Prolonged engagement of β -arrestin 1 and GPCR holds activated MAP kinases in the cytoplasmic compartment and potentially changes the course of vacuolar traffic (23). The β -arrestin-bound pool of ERK kinases localizes to the cytoplasm, while GPCR-activated ERK kinases predominate in the nucleus (23). To test whether the interaction of PAFr and β -arrestin 1 induced by pneumococci resulted in activation and phosphorylation of MAP kinases, rBCEC₆ cells were challenged with *S. pneumoniae* and phosphorylation of ERK-1/ERK-2 was monitored in the cytoplasm and nucleus. Phosphorylation was detected after 15 min (Fig. 4a). This signaling was important for bacterial translocation, since inhibition of MAP kinase phosphorylation by PD98059 resulted in no change in adherence but a 55% decrease in invasion (Table 1) ($P \leq 0.008$). Two findings were consistent with β -arrestin 1 participation in the activation. First, pretreatment of rBCEC₆ cells with active PAFr antagonist or inactive control antagonist failed to inhibit bacterium-induced ERK phosphorylation (Fig. 4b). Second, isolation of cytoplasmic and nuclear fractions showed that, although ERKs were located both in the nucleus and cytoplasm, the phosphorylation of ERK-1/ERK-2 occurred mainly in the cytoplasm (Fig. 4).

Colocalization with Rab proteins. Rab proteins belong to the group of Ras small GTPases (21). Different Rab proteins are located in specific intracellular membrane compartments where they regulate endocytotic pathways. Some of the crucial Rab proteins are Rab5, which is involved in early endocytosis, Rab7, which is found in the late endosome, and Rab11, which regulates recycling (28). Confocal microscopy studies of infected rBCEC₆ endothelial cells showed that, between 15 and 60 min, 48 to 56% of pneumococci colocalized with Rab5 and 37 to 43% with Rab7 (Fig. 5; Table 2). This was confirmed using the alternative early and late endosomal markers EEA1 and Lamp1, respectively (see Fig. S2 in the supplemental material). Few bacteria (16 to 24%) (Fig. 5; Table 2) costained with Rab11, marking the recycling endosome and the trans-Golgi network. This suggested that at least half of the entering bacteria proceeded through Rab5 to Rab7 marked endosomes toward the lysosome. If Rab7 were directing bacteria to death in the lysosome, then enhanced survival of bacteria as seen in endothelial cells overexpressing β -arrestin would be expected to decrease bacterial Rab7 interactions. Consistent with this notion, transfection of rBCEC₆ cells to overexpress β -arrestin shifted the proportion of Rab7 colocalizing bacteria down from 43 to 27% (Table 2) without changing the pattern for Rab5 or 11.

CFU/ 10^6 cells for the adhesion assay). +, present in transfection; -, absent from transfection; *, $P = 0.017$; **, $P = 0.024$; ***, $P = 0.027$.

FIG. 4. Pneumococcal activation of ERK-1/ERK-2 and effect of MAP kinase inhibition. (a) rBCEC₆ cells were infected with 10^5 CFU/ml of *S. pneumoniae* T4R for 15 min. Cytoplasmic and nuclear fractions were separated, and ERK phosphorylation (P-ERK) was measured by Western blotting. (b) rBCEC₆ cells were pretreated with 1 μ M PAFr antagonist or inactive control for 1 h. Cells were then infected as before and lysed, and ERK phosphorylation was measured by Western blotting.

DISCUSSION

In vitro *S. pneumoniae* is internalized into the eukaryotic cell in a vacuole (5, 11, 29). This process is dependent on the PAFr and involves molecular mimicry of PAF by the bacterial cell wall phosphorylcholine moiety (5). Colocalization of the bacteria with PAFr, as shown in this study by confocal microscopy, further supports direct binding of pneumococci to the PAFr. This interaction was shown to be critical to the progression of disease in vivo. Pneumococcal translocation in PAFr^{-/-} mice was delayed from the lung to blood and substantially blocked from the blood to cerebrospinal fluid. PAFr^{-/-} required blood titers 2 logs higher than the WT to detect translocation across the blood-brain barrier, so as to develop meningitis. It is noteworthy that titers in the lung and blood (10^8) required for PAFr^{-/-} mice to develop bacteremia and meningitis, respectively, were supraphysiologic and thus would likely indicate overwhelming the pulmonary and blood-brain barriers rather than a non-PAFr-dependent translocation route. The animal models indicate that the PAFr facilitates progression of disease in vivo, particularly at points of transition where the bacteria cross human cell barriers during invasive disease. This appears to be distinct from the role of PAFr in colonization, since a previous study showed that intranasally infected PAFr^{-/-} mice experienced diminished growth of pneumococci in the lungs and a delayed mortality from pneumonia compared to WT mice (17). The known targeting of pIgR rather than PAFr for translocation of pneumococci across cells of the nasopharynx (29) is consistent with these differences and suggests that, in the nasopharynx, PAFr behaves in a manner consistent with chemokine signaling rather than bacterial transcytosis.

Many eukaryotic membrane-bound GPCRs, including the PAFr, downregulate their response to ligands by undergoing endocytosis (20). Ligand binding by PAFr induces translocation of β -arrestin to the plasma membrane (4) to form a complex with the GPCR that is then sequestered in clathrin-coated vesicles (1, 14). Consistent with both of these steps, exposure of cells to pneumococci caused β -arrestin translocation to the membrane, where it colocalized with PAFr and invasion was blocked by the interruption of clathrin. Our transfection experiments showed that, while only PAFr was required for bacterial adherence, both PAFr and β -arrestin 1 were needed to

support pneumococcal uptake and, specifically, that their endocytic functions were necessary for bacterial entry. Mutations of either PAFr or β -arrestin that blocked endocytosis and prevented PAFr targeting to clathrin-coated vesicles eliminated pneumococcal invasion. A domain of β -arrestin distinct from that involved in endocytosis initiates signaling by the MAP kinase pathway. Inhibition of the MAP kinase signaling resulted in decreased bacterial invasion indicating that not only the endocytic but also the signaling effects of β -arrestin were required to serve pneumococcal endocytosis.

The pneumococcal cell wall has been shown to induce phosphorylation and activation of MAP kinases ERK-1/ERK-2 (19). Consistent with this, the pneumococcus-induced PAFr- β -arrestin interaction was associated with activation of the MAP kinases. It has been shown that the activation of ERK-1/ERK-2 by β -arrestins differs from that of the G-protein-dependent mechanism in several respects (23). A more stable association of ligand and receptor favors β -arrestin-mediated ERK activation. Such β -arrestin-induced ERK activation leads to cytoplasmic ERK-1/ERK-2 activity, while G-protein activation of the ERK-1/ERK-2 leads to nuclear translocation of the ERKs. Pneumococcal ERK activation predominated in the cytoplasmic fraction. Since (i) activation of MAP kinases was required for pneumococcal uptake, (ii) blocking PAFr-mediated MAP kinase phosphorylation did not prevent bacterium-induced MAP kinase activation, and (iii) cytoplasmic trapping of ERK activity predominated after bacterial challenge, we conclude that β -arrestin appears to be critically involved in directing the pneumococcal endosome through the cell.

Endocytosed PAFr- β -arrestin vesicles are targeted to the early endosome and are then either recycled to the cell surface or destroyed in the lysosome. Progression to both destinations requires detachment of β -arrestin from the early endosome and recruitment of pathway-specific Rab proteins (8). The Rab proteins are members of the Ras small GTPase superfamily and are involved in vesicle budding, motility, and fusion (21). Rab5 regulates the shift from clathrin-coated vesicle to the early endosome (28). Further progression through the late endosome to the lysosome involves Rab7. Alternatively, Rab11 regulates recycling through the perinuclear recycling endosome (28). Virtually all pneumococci moved into clathrin-

FIG. 5. Colocalization of pneumococci and Rab proteins. rBCEC₆ cells were infected with 10⁷ CFU/ml of T4R for 15 min to 1 h. Immunostaining for endosomal markers was performed by Alexa 488-tagged secondary antibody (green), and bacteria were detected with Texas Red-tagged secondary antibody (red) by confocal microscopy. Staining of anti-Rab11 is shown at 15 min, staining of anti-Rab5 is shown at 30 min, and staining of anti-Rab7 is shown at 60 min. Colocalization of markers is indicated by yellow. Blue staining shows the location of the nucleus.

coated vesicles and half then proceeded to colocalize with Rab5 and EEA1, consistent with entry into early endosomes by 15 min. Few vacuoles acquired Rab11, consistent with known recycling of only a small portion of the bacteria back to the apical cell surface. By 30 min, half the pneumococcal vacuoles became decorated with Rab7 and LAMP1, consistent with known targeting of a subset of bacteria to the lysosome for killing. Increased expression of β -arrestin decreased colocalization of pneumococci with Rab7 and increased intracellular survival. This pattern suggests that bacterium-containing vacuoles are diverted away from Rab7 to transcytosis. One possible explanation for greater transcytosis than intracellular killing in the lysosome could involve prolonged binding of β -arrestin to the GPCR/Rab complex, since detachment of β -arrestin is required for complete normal vacuolar trafficking (8).

TABLE 2. Colocalization of pneumococci with Rab5, Rab7, and Rab11 over time

Time (min)	% (total no. of bacteria/total no. of cells) of bacteria colocalized with ^a :		
	Rab5	Rab7	Rab11
15	48 (61/247)	42 (24/75)	21 (67/83)
30	56 (73/250)	43 (47/111)	24 (33/153)
60	54 (85/316)	37 (54/107)	16 (32/99)
Overexpression of β -arrestin ^b	54 (151/635)	21 (38/307)	17 (54/303)

^a Data are representative of the results from 3 experiments.
^b Results are for rBCEC₆ cells transfected with β -arrestin and counted at 30 min.

We conclude that β -arrestins are key determinants of the machinery subverted by pneumococci to drive PAFr-containing vacuoles away from the lysosome and across human cell barriers. Uptake and survival of bacteria required the scaffold function of the β -arrestin to recruit clathrin as well as the MAP kinase activation function to sequester ERKs in the cytoplasm. Greater expression of β -arrestin directed more bacterium-containing vacuoles away from lethal traffic to the lysosome and toward transcytosis of viable bacteria.

ACKNOWLEDGMENTS

We thank G. Gao, J. Sublett, and S. Bhuvanendran for technical assistance and J. Stankova for providing PAFr and β -arrestin 1 constructs.

This work was supported by NIH R01 AI27913 (to E.I.T.), CA21765, and the American Syrian Lebanese Associated Charities.

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