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Host Glycoprotein Gp96 and Scavenger Receptor SREC Interact with PorB of Disseminating *Neisseria gonorrhoeae* in an Epithelial Invasion Pathway

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SUMMARY

Neisseria gonorrhoeae **expresses numerous surface proteins that mediate bacterial adherence and invasion during infection. Gonococci expressing serotype A of the major outer membrane porin PorB ($PorB_{IA}$) are frequently isolated from patients with severe disseminating infections. $PorB_{IA}$ triggers efficient adherence and invasion under low phosphate conditions mimicking systemic bloodstream infections. Here, we identify the human heat shock glycoprotein Gp96 and the scavenger receptor SREC as $PorB_{IA}$-specific receptors. Gonococci expressing $PorB_{IA}$, but not those expressing PorB serotype B instead, bind to purified native or recombinant Gp96. Depletion of Gp96 from host cells prevented adherence but significantly triggered gonococcal invasion. Furthermore, such invasion was blocked by chemical inhibitors of scavenger receptors, and we identified SREC as the scavenger receptor involved in $PorB_{IA}$-dependant invasion. Thus, we establish Gp96 as an anti-invasion factor and SRECs as receptors mediating host cell entry of highly invasive disseminating gonococci.**

INTRODUCTION

Neisseria gonorrhoeae is a gram-negative obligate human-specific pathogenic bacterium that causes the sexually transmitted disease, gonorrhea. This diplococcus preferentially colonizes the mucosal surface of the male urethra and the female cervix. If left untreated in women, gonorrhea may cause pelvic inflammatory disease and salpingitis, resulting in ectopic pregnancy or infertility. In most cases, gonococci cause local inflammation but also may spread within the host to cause systemic infections, leading to serious conditions such as endocarditis, meningitis, and pneumonia.

Several factors are thought to be key players during gonococcal infection. A remarkable variety of adhesins, including the type IV pili (McGee et al., 1981; Swanson, 1973), different phase-variable colony opacity-associated (Opa) outer membrane proteins (Stern et al., 1986), as well as the principal outer membrane protein PorB (P.IA) (van Putten et al., 1998; Edwards et al., 2002) and the lipooligosaccharide (LOS) (Porat et al., 1995; Harvey et al., 2000), enable the bacteria to interact with human tissue.

The family of Opa-proteins comprises two classes that differ in the type of receptor recognized on the cell surface. The first class is represented by the Opa_{50} adhesin, which binds to surface heparan sulfate proteoglycan (HSPG) receptors (van Putten and Paul, 1995; Chen et al., 1995). The second class of Opa proteins, the $Opa_{51\text{-}60}$ adhesins, targets members of the carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs) mediating the complex interaction of gonococci with epithelial cells and phagocytes (for review see Hauck and Meyer, 2003).

Neisserial porins have been implicated in pathogenic mechanisms such as serum resistance, immune stimulation, host cell survival, and invasion (Massari et al., 2003). *N. gonorrhoeae* strains express PorB of either serotype A ($PorB_{IA}$) or B ($PorB_{IB}$), which differ in structural and immunochemical characteristics. The PorB serotypes play different roles during infection. Expression of $PorB_{IA}$ correlates with serious systemic gonococcal infection (Cannon et al., 1983; Britigan et al., 1985; Morello and Bohnhoff, 1989). Moreover, $PorB_{IA}$, but not $PorB_{IB}$, mediates the efficient uptake of gonococci in a phosphate-sensitive manner independent of Opa proteins (van Putten et al., 1998). Since the low phosphate levels found in blood and serum permit the uptake of gonococci via $PorB_{IA}$, the $PorB_{IA}$-triggered invasion is believed to be especially relevant during disseminating gonococcal infection (Kühlewein et al., 2006).

Several proteins have been shown to interact with neisserial PorB under high phosphate conditions. Meningococcal PorB interacts with TLR-2 on B cells inducing their activation (Massari et al., 2006). Complement receptor 3 (CR3) is mainly expressed in the female genital tract (Edwards et al., 2001) and binds PorB of either serotype (Edwards et al., 2002). C4b-binding protein is implicated

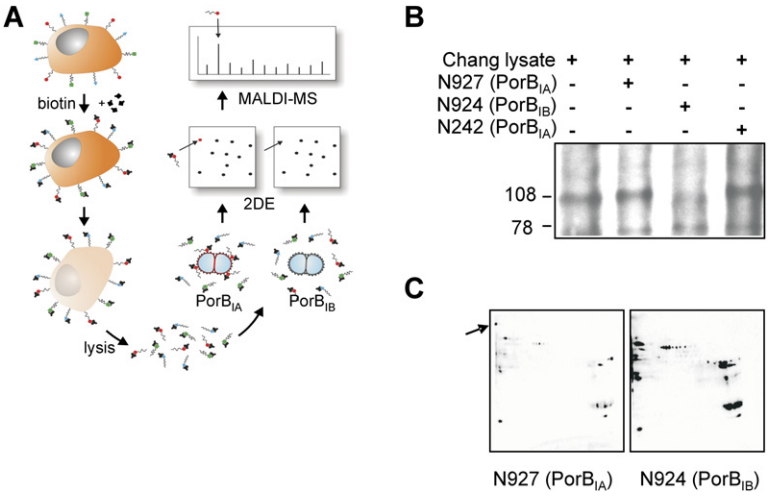

Figure 1. Identification of Gp96 as Interaction Partner of the Neisserial PorB$_{IA}$

(A) Experimental strategy to identify PorB$_{IA}$ interacting proteins.

(B) 110 kDa protein binds to PorB$_{IA}$-expressing gonococci. Chang cells (10^7) were detached and labeled with biotin for 1 hr at 37°C. Labeled cells were then lysed for 1 hr at 4°C and incubated with N927 (PorB$_{IA}$), N924 (PorB$_{IB}$), and N242 (PorB$_{IA}$) for 15 hr. Bacteria were pelleted, and biotin-labeled proteins were detected using immunoblot analysis.

(C) 2DE analysis of PorB$_{IA}$-binding proteins. N927 (PorB$_{IA}$) and N924 (PorB$_{IB}$) loaded with biotin-labeled proteins as described under (B) were subjected to 2DE analysis and immunoblotting to detect avidin-bound proteins. The arrow points to the spot that was identified as Gp96.

in serum resistance of neisserial strains and is another complement factor interacting with PorB (Ram et al., 2001). Yet another mechanism of serum resistance may depend on the interaction with factor H, a negative regulator of the alternative complement pathway. Factor H binds to loop 5 of PorB$_{IA}$, resulting in the downregulation of CR3 (Ram et al., 1998).

Despite the putative importance of the phosphate-sensitive interaction and invasion of PorB$_{IA}$-expressing gonococcal strains, the corresponding host cell receptor has not been characterized to date. Here, we identify Gp96 as a protein binding to gonococcal PorB$_{IA}$, but not PorB$_{IB}$, during infection. Unexpectedly, bacterial adherence is increased, whereas invasion into epithelial cells is inhibited when Gp96 is present. We show that the recently identified class F scavenger receptors are involved in the invasion of PorB$_{IA}$-expressing gonococci. This demonstrates that scavenger receptors can function in the uptake of pathogens by epithelial cells. Our findings suggest a two-step process initiated by the binding of Gp96 to PorB$_{IA}$, which then competes for efficient uptake of bacteria via SREC receptors.

RESULTS

Identification of Gp96 as a PorB$_{IA}$-Interacting Protein

A combined genetics and proteomics approach was designed to identify host cell receptors interacting with PorB$_{IA}$, but not PorB$_{IB}$ (Figure 1A). Defined isogenic *Neisseria* strains expressing either PorB$_{IA}$ of strain VP1 (N927) or PorB$_{IB}$ of strain MS11 (N924) were generated (Bauer et al., 1999) that did not express any Opa proteins or pili and differed only in the porin type expressed (data not shown). HeLa cells were then detached from the plate and labeled with NHS-LC-Biotin using conditions favoring the crosslinking of NHS-LC-Biotin to surface exposed proteins (Gray-Owen et al., 1997) (for details see the Supplemental Experimental Procedures in the Supplemental Data available with this article online). The labeled cells were lysed and incubated with either N927(PorB$_{IA}$) or N924(PorB$_{IB}$) isogenic gonococci. Unbound proteins were removed by extensive washing, and bacterial proteins bound to HeLa proteins were analyzed using immunoblotting to detect biotinylated proteins. A band in the range of 110 kDa was always stronger in N927 samples as compared to N924 samples (Figure 1B). Increased binding of the 110 kDa biotinylated HeLa protein was also detected with the VPI strain. The gene encoding the PorB$_{IA}$ protein of N927 was originally derived from the VPI strain (Bauer et al., 1999). In contrast, strain N934 expressing PorB$_{IB}$ and Opa$_{50}$ did not bind the 110 kDa protein (data not shown), ruling out an interaction of these proteins. In order to identify the 110 kDa protein, bacteria and bound biotinylated HeLa proteins were separated by high resolution two-dimensional electrophoresis (2-DE) prior to immunodetection. A highly acidic protein of approximately 110 kDa was detected in the bacterial pull-downs of N927, which was absent in the bacterial lysate and pull-down of N924 (Figure 1C). The 110 kDa protein was subsequently identified as glycoprotein 96 (Gp96) using MALDI-MS and peptide mass fingerprinting (Figure S1). Gp96 has a theoretical mass of 90,138 Daltons and an acidic pI of 4.83, consistent with the position in the 2-DE gel.

The binding of eukaryotic Gp96 to bacteria expressing PorB$_{IA}$ was confirmed by incubating N924 and N927 with purified, native or recombinant Gp96 (Arnold-Schild et al., 2000) followed by detection of bound protein using immunoblotting (Figure 2A) or immunofluorescence (Figure 2C). Gp96 bound to gonococci expressing PorB$_{IA}$ (N927), but not to isogenic strains expressing PorB$_{IB}$ (N924), suggesting that Gp96 binds PorB$_{IA}$ in a serotype-specific manner. Preincubation of the bacteria with PorB$_{IA}$-specific antiserum prevented Gp96 binding (Figure 2B), demonstrating that PorB$_{IA}$ is the direct binding partner of Gp96.

Gp96, also known as *g*lucose-*r*egulated *p*rotein *94* (grp94) or endoplasmin, is a major component of the lumen of the endoplasmic reticulum (ER) and belongs to the familiy of molecular chaperones (Ma and Hendershot, 2001; Li et al., 2002). However, Gp96 has also been detected on the surface of several cell types (Srivastava

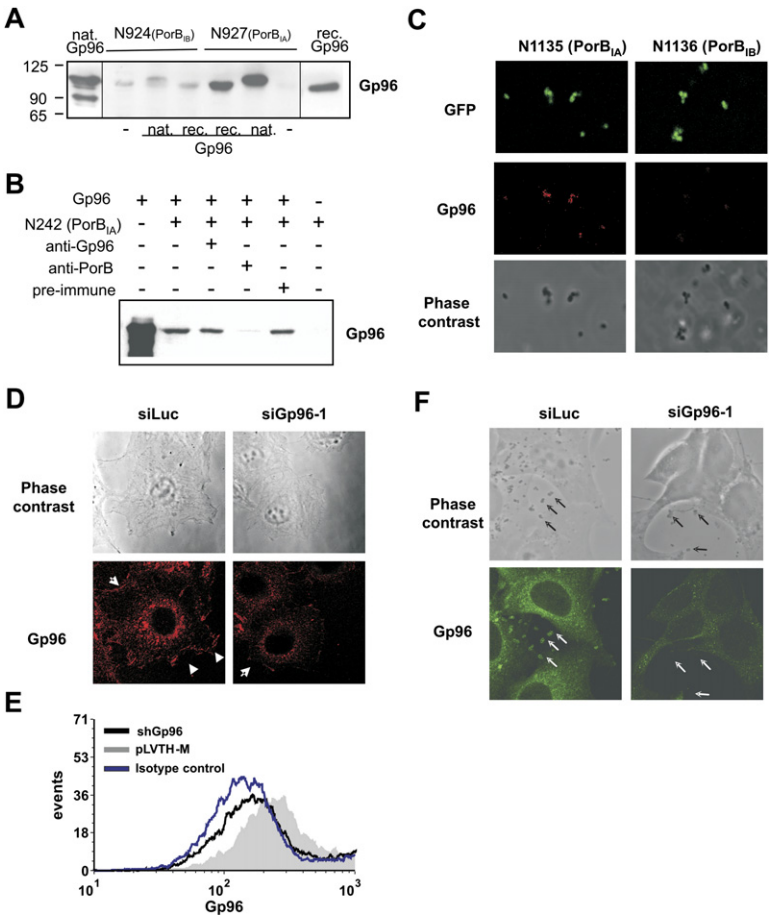

Figure 2. Gp96 Binds PorB$_{IA}$-Expressing Gonococci during Infection

(A) Native and recombinant Gp96 binds preferably to N927. Bacteria were subcultivated for 2 to 3 hr under phosphate-free conditions and incubated with native or recombinant human Gp96. Unbound proteins were removed by extensive washing. Bacterial lysates were subjected to immunoblot analysis and probed with a specific antibody against Gp96.

(B) Purified Gp96 protein binds to PorB$_{IA}$. Bacteria grown in HEPES medium under phosphate-limited conditions (Kühlewein et al., 2006) were incubated with 5 μg/ml native Gp96 protein in the presence or absence of preimmuneserum (preimmune) or an antiserum against PorB or Gp96 as indicated. Unbound Gp96 was removed by extensive washing in HEPES medium, and Gp96 bound to bacteria was detected using immunoblot analysis.

(C) PorB$_{IA}$-expressing gonococci, but not PorB$_{IB}$-expressing bacteria, bind purified Gp96 protein. N1135 (PorB$_{IA}$) or N1136 (PorB$_{IB}$) both expressing GFP protein were incubated with purified Gp96 for 30 min at 37°C, washed extensively, and stained with a polyclonal rabbit antibody against Gp96 and Cy3-conjugated goat, anti-rabbit IgG. Bacteria and bound Gp96 were detected by immunofluorescence.

(D and E) Gp96 surface expression. (D) Surface-expressed Gp96 of siLuc and siGp96-1 transfected cells were stained with an antibody against Gp96 and Cy3-coupled secondary antibody and detected by immunofluorescence microscopy. Arrowheads point to cell boarders. (E) Surface-expressed Gp96 was detected by FACS analysis on paraformaldehyde-fixed, nonpermeabilized control (pLVTH-M) and Gp96-depleted cells (shGp96) with an antibody against Gp96 and Cy3-coupled secondary antibody.

(F) Host cell Gp96 binds to gonococci during infection. Chang cells transfected with siLuc or siGp96-1 were infected with N927 in the absence of phosphate. Infected cells were stained with a polyclonal rabbit antibody against Gp96 and Cy2-conjugated goat, anti-rabbit IgG. Bacteria and bound Gp96 were detected using immunofluorescence. Arrows point to bacteria.

et al., 1986; Altmeyer et al., 1996; Takemoto et al., 1992). Using a monoclonal antibody and confocal immunofluorescence staining, we detected Gp96 in granular structures surrounding the nucleus as well as at the borders of Chang cells (Figure 2D). Similar results were obtained for the HeLa cell line (data not shown). To verify the specificity of the Gp96 antibody, Gp96 expression was silenced in Chang cells. Short hairpin RNA (shRNA) and short interfering RNAs (siRNAs) were designed, and their efficiency for silencing Gp96 expression was measured using real-time PCR and immunoblotting (Figures S2A–S2C). Cells depleted of Gp96 lacked the typical staining observed in wild-type Chang cells, confirming the specificity of the antibody (Figure 2D). FACS analyses performed on nonpermeabilized control and shGp96-expressing cells (Chang-shGp96) confirmed the surface exposure of Gp96 in Chang cells (Figure 2E). Our results show that Gp96 can be localized to the cytoplasmic membrane in Chang and HeLa cells.

To test whether Gp96 associates with bacteria during infection, Chang cells were infected with GFP-expressing N927, and Gp96 was detected using immunofluorescence. Gp96 clearly colocalized with gonococci bound to Chang cells (Figure 2F). When Gp96 expression was suppressed by transfection of siRNAs, the anti-Gp96 antibody no longer stained adhering gonococci. In summary, our results demonstrated the PorB$_{IA}$-specific association of Gp96 during infection.

Gp96 Is Involved in the Binding of PorB$_{IA}$ to Epithelial Cells

PorB$_{IA}$, but not PorB$_{IB}$, mediates the binding to and invasion into a wide variety of human and animal cells (Kühlewein et al., 2006) in a strictly phosphate-sensitive manner (van Putten et al., 1998). To test whether Gp96 is involved in PorB$_{IA}$-dependent infection, adherence and invasion were investigated in cells with depleted Gp96. When Chang cells transfected with siGp96-1 or siGp96-2 were infected with N927 under low-phosphate conditions, the number of adherent bacteria was dramatically reduced in comparison to cells transfected with control siRNAs, where bacteria still bound cells efficiently (Figures 3A and 3B). Gp96 silencing did not prevent the binding of strain N931 (Opa50) to HSPG (Figures S4A and S4B) on

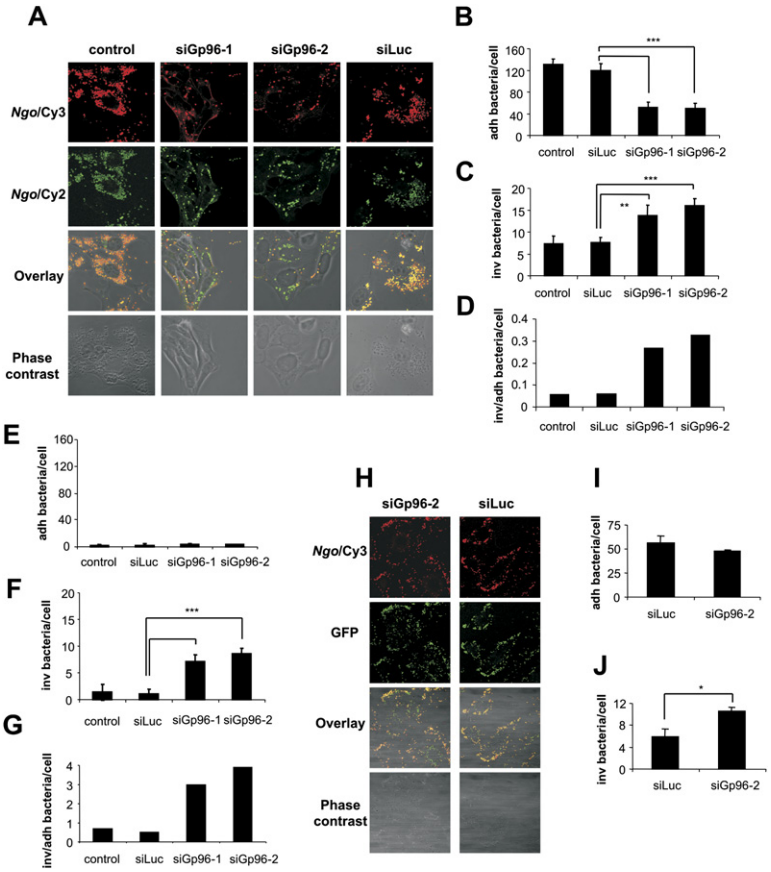

Figure 3. Gp96 Silencing Leads to Decreased Adherence and Enhanced Invasion

Chang cells either transfected with control siRNA siLuc, siGp96-1, or siGp96-2 or left untransfected (control) were infected with N927 ($PorB_{IA}$) in the absence (A–D) or presence (E–G) of phosphate. Adherent bacteria (red) were stained with polyclonal rabbit anti-gonococcal antiserum and Cy3-conjugated goat, anti-rabbit IgG. Cells were then permeabilized, and adherent and invasive (green) bacteria were stained with polyclonal rabbit anti-gonococcal antiserum and Cy2-conjugated goat, anti-rabbit IgG. In the overlay, adherent bacteria appear yellow, and invasive bacteria appear green. (H–J) HUVECs transfected with control siRNA siLuc or siGp96-2 were infected with N1135 (N927-GFP) at an MOI of 100 for 2 hr in the absence of phosphate. Adherent bacteria were stained as described for (A) and appear yellow due to GFP expression of strain N1135. Invasive bacteria appear green. (B,E, and I) Adherence (adh) and (C,F, and J) invasion (inv) was quantified by gentamicin protection assays. Data represent the mean ± SEM of five independent experiments. *$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0005$. (D and G) Data from (B) and (C) and (E) and (F) were expressed as a ratio of invasive and adherent bacteria.

the Chang cell membrane, ruling out a general impairment of gonococcal adherence in these cells. Similar results were obtained with the Chang-shGP96 line, in which Gp96 expression was permanently silenced by expression of a short hairpin RNA targeting *gp96* mRNA (Figures S5A, S5B, and S6). Our results confirm that Gp96 is required for the binding of $PorB_{IA}$-expressing gonococci.

Gp96 Protects Epithelial Cells from $PorB_{IA}$-Triggered Invasion

The low adherence of N927 to Gp96-silenced cells suggested that subsequent invasion may also have been reduced. Surprisingly, however, intracellular bacteria were still detected in Gp96-depleted cells (Figure 3A). Quantification of intra- and extracellular bacteria revealed that invasion was more efficient in the absence than in the presence of Gp96 (Figure 3C). Extending the infection periods from 2 to 7 hr did not significantly increase the number of extra- or intracellular bacteria (Figures S5B and S5C), excluding differential intracellular replication as a major reason for the high yield of intracellular bacteria in Gp96-depleted cells. The increased invasion was specific for $PorB_{IA}$-mediated invasion, since the efficiency of Opa-dependent invasion was not affected by Gp96 (Figure S4C). Similar results were obtained with Chang-shGp96 cells (Figures S5 and S6) and in primary human umbilical vein endothelial cells (HUVECs) (Figures 3H and 3J). Although silencing of Gp96 was not as effective in HUVECs (Figure S2D), invasion was clearly increased in the Gp96-depleted cells, demonstrating a similar role of GP96 in primary and in tumor cells.

We subsequently tested whether invasion of N927 into Gp96-depleted cells required low phosphate concentrations typical for $PorB_{IA}$-triggered infection. As expected, only few bacteria bound to Chang cells in the presence of phosphate, and the low adherence was not affected by Gp96 levels (Figure 3E and Figure S3). Invasion was, however, significantly increased when cells were depleted of Gp96 in phosphate-rich medium (Figure 3F and Figure S3). Intriguingly, invasion still depended on the expression of $PorB_{IA}$, since N924, which expresses $PorB_{IB}$, did not invade Chang cells irrespective of whether Gp96 was expressed or not (data not shown). Thus, the $PorB_{IA}$-triggered interaction with epithelial cells appears to be a two-step process involving Gp96 for efficient phosphate-sensitive attachment and another receptor for the phosphate-insensitive invasion.

Gp96 Mediates the Binding of Gonococci to the Surface of Epithelial Cells

To obtain final proof that silencing of Gp96 is responsible for the reduced binding and increased invasion of N927, we cotransfected cells with siGp96-2 (targeting the 3′UTR of Gp96 mRNA) and the cloned *gp96* cDNA comprising only the open reading frame. In this setting, endogenous *gp96* transcripts containing the 3′-UTR are

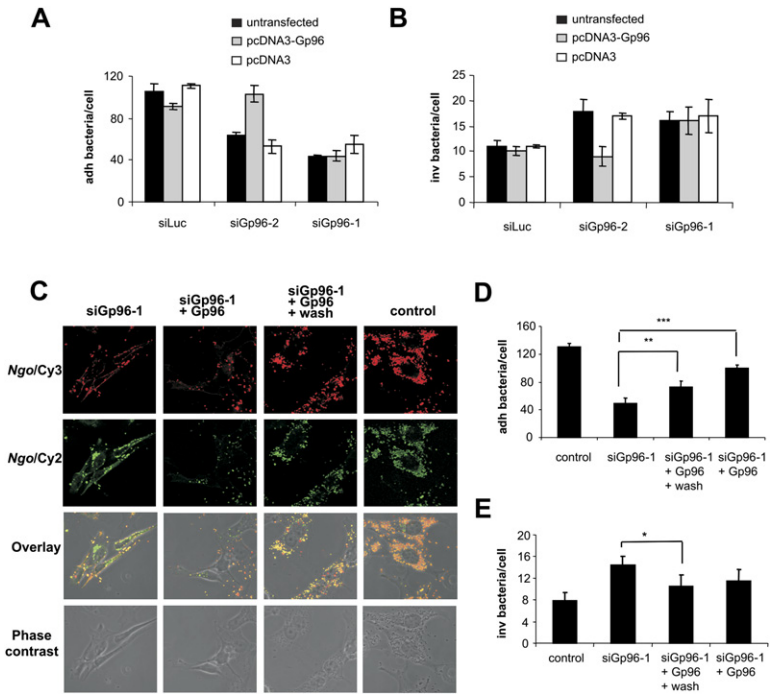

Figure 4. Genetic and Biochemical Complementation of Gp96

Expression of Myc-Gp96 coding region restored N927 adherence (adh) to (A) and inhibited invasion (inv) into cells (B) transfected with siGp96-2 (targeting the 3′UTR of Gp96 mRNA), as revealed by gentamicin protection assays. siGp96-1 (targeting the coding region of endogenous and recombinant Gp96 mRNA) transfection served as an internal control. Transfected cells were infected with N927 for 90 min at MOI 100 in HEPES medium. Data represent the mean ± SEM of three independent experiments. (C) Chang cells (2×10^5) were incubated with 10 μg/ml purified Gp96 protein (+ Gp96) for 30 min. Afterwards cells were either directly infected or washed three times (+ wash) and subsequently infected with N927 for 2 hr in the absence of phosphate with an MOI of 100. Adherent bacteria (red) were stained with polyclonal rabbit anti-gonococcal antiserum and Cy3-conjugated goat, anti-rabbit IgG. Afterwards, cells were permeabilized, and invasive bacteria (green) were stained with polyclonal rabbit anti-gonococcal antiserum and Cy2-conjugated goat, anti-rabbit IgG. In the overlay, adherent bacteria appear yellow, and invasive bacteria appear green. Adherent (D) and invasive (E) bacteria from 100 cells were counted. The mean ± SEM of three independent experiments is shown. *$p \leq 0.01$, **$p \leq 0.001$, ***$p \leq 0.0005$.

degraded, whereas transcripts of the cloned *gp96* form lack the 3′-UTR and cannot be targeted by RNAi (Figure S7). Expression of Gp96 cDNA restored the binding of N927 to cells lacking endogenous Gp96 (Figure 4A). Invasion, in contrast, was reduced when Gp96 expression was restored in these cells (Figure 4B), proving the specific effect of Gp96 silencing.

Gp96 itself has no membrane anchor, but is exposed at the cell surface by binding to cell surface receptors such as SR-A and CD91 (Binder and Srivastava, 2004; Berwin et al., 2003). Randow and Seed (Randow and Seed, 2001) found that loss of Gp96 expression in a human B cell line correlated with the loss of several receptors, including TLR2 and TLR4, as well as the CD11a, CD18, and CD49d integrins. Yang and colleagues (2007) recently demonstrated a broad defect in TLR signaling in macrophages lacking Gp96 expression. To rule out that the effect we observe for N927 adherence and invasion upon deletion of Gp96 is due to an indirect effect of Gp96 on the display or function of other receptors, the transcomplementation of Gp96-depleted cells with purified, native Gp96 was tested. Addition of purified Gp96 prior to infection strongly increased the number of adherent and reduced the number of invasive bacteria (Figures 4C–4E). The counteracting effect on invasion was strengthened if unbound Gp96 was not removed from the infection sample. The complementation of the Gp96 defect by the addition of purified protein clearly argued against an indirect effect of Gp96 depletion on the surface localization of other $PorB_{IA}$ receptors.

Identification of the $PorB_{IA}$ Invasion Receptor

Gp96 has been shown to bind to several receptors expressed on hematopoetic cells, including TLR-2 and -4 (Vabulas et al., 2002), Scavenger receptor A (SR-A) (Berwin et al., 2003), and CD91 (Binder et al., 2000). However, since $PorB_{IA}$-dependent infection was observed in many different epithelial and endothelial cell lines (Kühlewein et al., 2006), an involvement of these receptors in invasion via $PorB_{IA}$ was highly unlikely. None of these receptors was expressed in HeLa cells in significant amounts except for TLR4, which was induced 4-fold in infected cells (Figure S8B). Because TLR2 has previously been shown to interact with PorB and TLR4 was upregulated during infection, we tested their involvement by infecting primary mouse embryonic fibroblasts (MEFs) isolated from TLR2 and TLR4 knockout mice (Figure S8). No difference in bacterial binding was observed under low phosphate conditions between wild-type, TLR2-, and TLR4-knockout mice. Similar results were obtained with MEFs isolated from MyD88 knockout mice, which have a broad defect in TLR signaling (data not shown). Likewise, silencing TLR2, -4, and MyD88 in Chang cells using siRNAs had no effect on $PorB_{IA}$-triggered invasion (data not shown). These results suggest that TLRs play no major role in the interaction of N927 and target cells under low phosphate conditions. We next tested a possible role of scavenger receptors in $PorB_{IA}$-dependent infection using the well-established chemical inhibitors, Fucoidin and acetylated low density lipoprotein (acLDL) (Gough and Gordon, 2000). Adherence occurred normally in the absence and

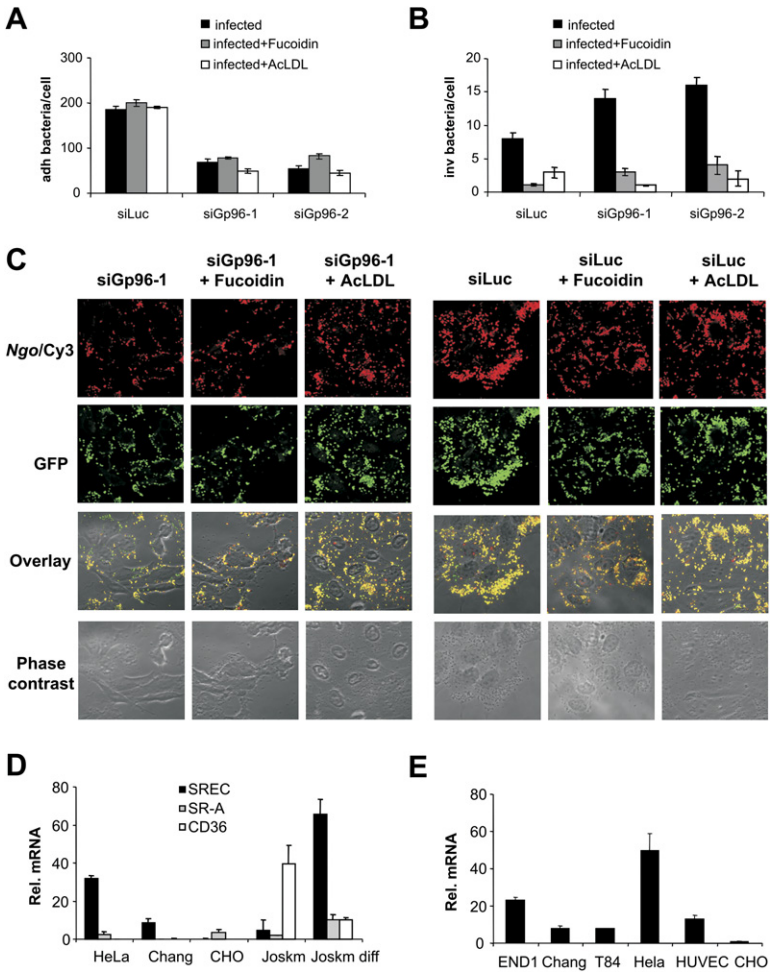

Figure 5. Scavenger Receptors Are Involved in PorB$_{IA}$-Mediated Invasion

(A–C) Chang cells transfected with siGp96-1, siGp96-2, or siLuc were incubated with either 75 μg/ml Fucoidin, 10 μg/ml AcLDL, or no inhibitor for 30 min. Then, cells were infected with N1135 (N927-GFP) in the absence of phosphate for 2 hr with an MOI of 100. (C) Adherent and invasive bacteria were stained differentially. Gonococci express GFP protein. Additionally, adherent bacteria were stained with polyclonal rabbit anti-gonococcal antiserum and Cy3-conjugated goat, anti-rabbit IgG. In the overlay, adherent bacteria appear yellow, and invasive bacteria appear green. Adherent (A) and invasive (B) bacteria were quantified by gentamicin survival assays. Data represent the mean ± SEM of three independent experiments.

(D) The Scavenger Receptor, SREC, is expressed on epithelial cells. Real-time PCR was performed to determine the levels of the scavenger receptors, SR-AI, CD36, and SREC on HeLa, Chang, CHO cells as well as on differentiated and nondifferentiated phagocytes (JOSK-M cells). Expression levels were normalized to the expression level on CHO cells. Data represent the mean ± SEM.

(E) SREC is expressed in nontransformed cells. Real-time PCR was performed as described under (D) on END1 (cervix epithelial cells), T84 (colon epithelial cells), and HUVEC (endothelial cells) mRNAs. Data represent the mean ± SEM.

presence of Fucoidin and acLDL (Figure 5A). In contrast, both inhibitors efficiently prevented the invasion of N927 into Gp96-depleted Chang cells (Figure 5B) and primary HUVECs (data not shown), confirming the involvement of scavenger receptors in PorB$_{IA}$-dependent infection. This finding was unexpected since scavenger receptors (SRs) are predominantly expressed by phagocytic cells. We, therefore, tested the expression of different members of the SR family in HeLa and Chang cells, which are both efficiently invaded by N927. The only significantly expressed receptor was *S*cavenger *r*eceptor *e*xpressed in *e*ndothelial *c*ells (SREC) (Figure 5D), previously identified to be expressed on endothelial cells (Adachi et al., 1997). As expected, SREC mRNA was detected in primary HUVECs but also in primary END1 cervix epithelial cells (Figure 5E), suggesting SREC as the prime candidate for PorB$_{IA}$-dependent infection of N927.

SREC Is the Receptor for PorB$_{IA}$-Dependent Infection

We then analyzed the cellular distribution of SREC in highly polarized T84 epithelial cells, a well-established model system for gonococcal infection (Wang et al., 1998, 2007). SREC was clearly present in the apical membrane in polarized T84 epithelial cells as was investigated by immunofluorescence labeling and confocal microscopy (Figure 6A). A possible role of SREC in the invasion of epithelial cells by strain N927 was then tested by antibody inhibition experiments. Preincubation of Chang cells with two different SREC sera significantly reduced the uptake of N927, whereas adherence was not affected (Figures 6B and 6C). In contrast, preincubation of cells with antiserum directed against SR-A neither affected adherence nor invasion of N927 (Figures 6B and 6C), suggesting a specific role of SREC in N927 invasion. To confirm these results, scavenger receptors were expressed in CHO-K1 cells, which have previously been shown not to express SREC receptors (Berwin et al., 2004). Expression constructs for SR-A1 and SREC were transfected into CHO cells, and the invasion of N927 was tested. The reconstituted SR-receptors were functional since SR-expressing CHO cells efficiently endocytosed DiI-AcLDL (Figure S9B). N927 was taken up by CHO cells expressing SREC in a phosphate independent manner, whereas CHO cells expressing SR-A1 were unable to significantly engulf N927 (Figure 6E; for quantification see Figure S9C and S9D). The uptake depended on the expression of PorB$_{IA}$, since strain N924 failed to interact and invade CHO-SREC

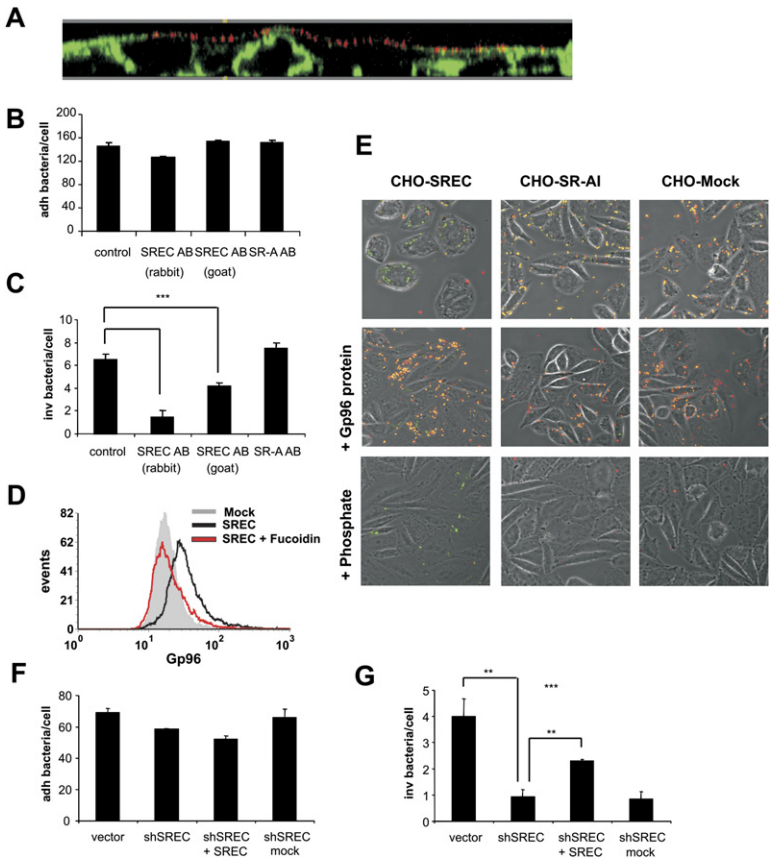

Figure 6. SREC Is the Host Cell Receptor for PorB$_{IA}$-Dependent Invasion

(A) SREC is expressed on the apical site of human epithelial cells. Polarized T84 cells were stained with rabbit polyclonal antibody against SREC and Cy3-conjugated goat, anti-rabbit IgG (red), and actin was stained with Alexa Phalloidin 488 (green). A representative x-z plane is shown from specimens analyzed by confocal laser scanning microscopy.

(B and C) Anti-SREC antiserum blocks N927 invasion. Chang cells (2×10^5) were incubated with either 2 μg/ml SR-A antibody, SREC goat polyclonal antibody, SREC rabbit polyclonal antibody, or buffer alone (control) for 30 min. Cells were then infected with N927 for 2 hr at an MOI of 100 in the absence of phosphate. Adherent (B) and invasive (C) bacteria were quantified by gentamicin protection assay. Data represent the mean ± SEM of three independent experiments. ***$p \leq 0.0005$.

(D) Gp96 binds to SREC receptors. CHO cells overexpressing SREC were incubated with FITC-labeled Gp96 protein (15 min, 37°C) in the absence (black line) or presence of 75 μg/ml Fucoidin (red line) and analyzed by FACS.

(E) Expression of SREC triggers PorB$_{IA}$-dependent invasion in CHO-K1 cells. CHO-K1 cells overexpressing SR-AI or SREC were either directly infected or preincubated with 5 μg/ml purified Gp96 protein (+ Gp96 protein) and then infected with N1135 (N927-GFP) at a MOI of 100 in the absence or presence (+ phosphate) of phosphate. Adherent (red) and invasive (green) bacteria were detected using differential immunodetection. Adherent bacteria were stained with polyclonal rabbit anti-gonococcal antiserum and Cy3-conjugated goat, anti-rabbit IgG. In the overlay, adherent bacteria appear yellow, and invasive bacteria appear green.

(F and G) HeLa cells were either transduced with the lentiviral vector pLVTH-M (vector) or pLVTH-M-shSREC to permanently silence SREC expression (shSREC). Additionally, shSREC cells were transfected with a SREC expression construct to restore SREC expression (shSREC+SREC) or with the empty vector (shSREC+mock). Recombinant cells were then infected with N927 in the absence of phosphate at a MOI of 100 for 90 min. Adherent (F) and invasive (G) bacteria were quantified by gentamicin protection assay. Data represent the mean ± SEM of three independent experiments. **$p \leq 0.001$, ***$p \leq 0.0005$.

cells (data not shown). These data demonstrate that SREC mediates the PorB$_{IA}$-dependent invasion into CHO cells.

To obtain further proof for a role of SREC in PorB$_{IA}$-dependent invasion, we established Chang cell lines with a permanent knockdown of SREC (Chang-shSREC). SREC mRNA levels were reduced by 67% in Chang-shSREC cells (Figure S10A). Consistently, surface expression of SREC was reduced by 40% as analyzed by FACS (Figure S10B). When shSREC cells were infected with N927, invasion was significantly reduced (Figure 6G). Moreover, invasion of N927 was completely restored when SREC was expressed in the Chang-shSREC cell line (Figure 6G). These data demonstrate a crucial role of SREC in N927 invasion.

We then performed FACS analysis to test whether soluble purified Gp96 interacts with SREC receptors expressed on CHO-SREC cells (Figure 6D). FITC-labeled Gp96 bound to CHO-SREC cells, but not to the cells transfected with the empty vector. In addition, Fucoidin prevented the binding of FITC-Gp96 to CHO-SREC cells (Figure 6D), suggesting that Gp96 directly interacts with SREC on the surface of cells.

If Gp96 blocks N927 invasion by interfering with its binding to SREC, the incubation of CHO-SREC with purified Gp96 should impair the invasion of N927. CHO-SREC cells were, therefore, preincubated with purified, native Gp96 prior to infection with N927. Invasion of N927 was prevented in the presence of Gp96, and only low levels of background binding similar to that in control CHO cells was observed (Figure 6E). Taken together, these data strongly support a two-step model as the basis of the bacteria-host interaction during PorB$_{IA}$-dependent infection: an adherence-promoting and invasion-inhibiting activity elicited by the interaction of Gp96 and PorB$_{IA}$, and an invasion-promoting activity brought about by the binding of PorB$_{IA}$ to SREC.

DISCUSSION

The well-recognized role of PorB$_{IA}$ in disseminating gonococcal infection has so far been mainly attributed to its role in mediating serum resistance. However, PorB$_{IA}$-dependent and independent mechanisms underlying serum

resistance have been described, including LPS sialylation, binding of factor H to $PorB_{IA}$, or binding of C4bp to either $PorB_{IA}$ or $PorB_{IB}$ (for a review see Ram et al., 1999). Therefore, activities of $PorB_{IA}$ other than those mediating serum resistance very likely contribute to its role in disseminating infection. By investigating the host cell receptors involved in $PorB_{IA}$-dependent infection, we uncovered a two-step and opposing interaction specifically for $PorB_{IA}$—the phosphate-sensitive binding of Gp96 and the phosphate-insensitive binding to SREC.

Gp96 was originally identified as a protein capable of crosspresenting peptides to the MHC class I receptor (Singh-Jasuja et al., 2000). The finding that tumor antigens presented by Gp96 elicited a strong antitumor activity in mice (Srivastava et al., 1986) together with the demonstration of Gp96 at the surface of tumor cells (Srivastava et al., 1986; Altmeyer et al., 1996; Takemoto et al., 1992) boosted a major effort to understand its function. In line with a function for Gp96 in the crosspresentation of antigens, CD91 (α_2 macroglobulin receptor) and scavenger receptor A were identified as receptors involved in the uptake of Gp96 peptide complexes and peptide presentation by MHC (Li et al., 2002; Binder and Srivastava, 2004; Berwin et al., 2002, 2003). It may well be possible that the binding of Gp96 by $PorB_{IA}$-expressing bacteria counteracts the immune response against invading gonococci by preventing the presentation of bacterial peptides by Gp96. A similar mechanism has recently been suggested for infections with the obligate intracellular bacterium, *Orientia tsutsugamushi*. These bacteria counteract the immune response by decreasing the cytosolic levels of Gp96 (Cho et al., 2004).

However, the finding that Gp96 also binds to TLR2 and TLR4, which are involved in recognition of pathogen-associated molecular patterns (PAMPS), has shifted the focus to a possible function of Gp96 in mediating so-called "danger" signals (Vabulas et al., 2002). This hypothesis is supported by the finding that Gp96 released from damaged or stressed cells primes dentritic cells for activation and thereby regulates the immune response. The anti-invasive activity demonstrated in the study presented here adds evidence for an innate immune function of Gp96 whereby Gp96 acts in a direct way to interfere with pathogen invasion. Indeed, previous reports support a role of Gp96 in directly modulating the interaction of pathogens with their host cells consistent with an invasion-promoting function of Gp96. For example, *Escherichia coli (E.coli)* Outer Membrane Protein A (OmpA) interacts with the Gp96 homolog, Ecgp, thereby supporting invasion (Prasadarao et al., 2003). A recent report identified cell surface Gp96 as the receptor for *Listeria monocytogenes vi*rulence *p*rotein (Vip), also mediating bacterial invasion (Cabanes et al., 2005). A possible explanation for the invasive and anti-invasive functions observed for Gp96, is that the function may depend on the interacting bacterial factor. Binding of $PorB_{IA}$ is phosphate-sensitive whereas phosphate does not appear to play a major role in *Listeria* or *E. coli* invasion. This suggests a difference in the mechanism of interaction or in the binding domain between the bacterial factors binding in a phosphate-sensitive or insensitive manner that may affect Gp96's function in such a dramatic way.

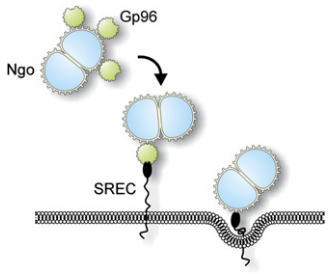

Figure 7. Model of the Interaction of $PorB_{IA}$-Expressing Gonococci with Gp96 and SREC

Low phosphate allows the binding of $PorB_{IA}$-expressing gonococci (Ngo) to Gp96. Interaction of bacteria with SREC via Gp96 leads predominantly to adherence. Invasion may require the dissociation of Gp96 from SREC.

The highly efficient invasion observed in epithelial cells depleted of Gp96 was solely dependent on Scavenger receptors (SRs) since two structurally different inhibitors for SRs efficiently block invasion. This finding was unexpected, although scavenger receptors are well known to mediate uptake of bacteria by phagocytes (Peiser et al., 2002). Originally identified in endothelial cells (Adachi et al., 1997), SREC-I has been shown to be expressed in several tissues (Ishii et al., 2002), which fits well with the broad cell type spectrum of $PorB_{IA}$-dependent infection (Kühlewein et al., 2006). Our screen revealed the expression of class F SR (SREC-I) in Chang epithelial cells, which we could demonstrate to act in the uptake of bacteria. Considering the high expression of SREC in endothelial cells and the efficient invasion associated with SREC receptors, a relevant aspect of our finding may be the invasion of the endothelial cell by disseminating invasive meningococci and gonococci, which will be followed up on in future investigations.

The interaction of $PorB_{IA}$ with SREC was highly selective, since neither $PorB_{IB}$- nor Opa_{50}-expressing bacteria infected CHO-SREC cells. On the other hand, the interaction of $PorB_{IA}$ and SREC was not phosphate-sensitive. We, therefore, hypothesize that SREC receptors are constantly masked to $PorB_{IA}$ binding in cells with surface-exposed Gp96, such as the Chang cell. Otherwise, strains expressing $PorB_{IA}$ should have invaded these cells under high phosphate conditions as in the Gp96-depleted cells (Figure 3F). One may also speculate that if Gp96 is absent from the host cell surface, invasion may be dramatically increased or occur even under high-phosphate concentrations.

The host fights pathogens with potent effector cells that are able to engulf and kill bacterial invaders. On the other hand, pathogens have developed potent evasion mechanisms that allow their survival even in such a hostile environment as that represented by the immune system. Our findings do not yet allow us to draw a clear picture of how $PorB_{IA}$ strains may support disseminating infections. However, based on the observation that both identified host factors showed a highly specific interaction with $PorB_{IA}$, the following model is proposed (Figure 7).

Gonococci entering the bloodstream encounter a low phosphate environment, which may result in the binding of released Gp96 to $PorB_{IA}$. This association inhibits the uptake of the decorated bacteria by host cells if other phase variable adhesins are not expressed. Sequestering Gp96 may prevent the presentation of gonococcal peptides by Gp96, leading to a reduced immune response. These bacteria may, however, very efficiently invade epithelial or endothelial cells in the absence of Gp96. It will be a challenge to unravel the mechanisms of these novel interactions, which may be future targets for therapy or prevention of disseminating gonococcal infections.

EXPERIMENTAL PROCEDURES

Neisseria Strains

The following *N. gonorrhoeae* strain MS11 derivatives were used in this study: N924 ($PorB_{IB}$; Opa^-), N927 ($PorB_{IA}$; Opa^-), and N934 ($PorB_{IA}$; Opa_{50}) (Kupsch et al., 1993; Bauer et al., 1999); N242 ($PorB_{IA}$; $Opa_{27;27,5;28;29;30}$ (Makino et al., 1991); N1135 ($PorB_{IA}$; Opa^-, GFP) and N1136 ($PorB_{IB}$; Opa^-, GFP) (this study; for details see Supplemental Experimental Procedures). Gonococci were routinely grown on GC agar base plates (Becton Dickinson, Difco and Remel) supplemented with Proteose Pepton Nr. 3 (Difco) and 1% vitamin mix for 14–20 hr at 37°C in 5% CO_2 in a humidified atmosphere. Opa phenotypes were monitored by colony morphology under a stereo microscope or by immunoblotting.

Cell Lines

Cell lines used in this study included Chang human conjunctiva cells (ATCC CCL20.2); HeLa human cervix carcinoma cells (ATCC CCL2); CHO-K1 chinese hamster ovary cells (ATCC CCL61); 293HEK human kidney epithelial cells (ATCC CRL-11268); primary mouse embryonic fibroblasts from TLR2, TLR4, or MyD88 knockout mice; Human Umbilical Vein Endothelial Cells (HUVEC); human colonic carcinoma T84 cells (ATCC CCL248); END1 cervix epithelial cells; and monocytic-like JOSK-M cells (DSM ACC30). For growth conditions, see Supplemental Experimental Procedures.

Generation of Scavenger Receptors Expressing CHO-K1 Cells

CHO-K1 cells (2×10^5) were transfected with 1 μg SR-AI cDNA (IRAKp961M16141Q2, rzpd) or SREC cDNA (IRATp970A0758D6, rzpd) using Lipofectamine2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. After 48 hr, cells were used for infection experiments.

Infection Experiments

Cells were grown on glass coverslips in 24-well cell culture plates to 70%–80% confluency. Infections were performed in phosphate-free HEPES medium as previously described (Kühlewein et al., 2006). The monolayers were washed four times with HEPES medium to remove phosphate before infection. Bacteria were suspended in HEPES medium and added to the cells at a multiplicity of infection (MOI) of 100. To synchronize infections, the infected monolayers were centrifuged for 3 min at 120 × g. Infections were stopped after 120 min (or 90 min in case of complementation assays) incubation at 37°C in 5% CO_2 atmosphere by washing the cells four times with HEPES medium. High phosphate control samples contained 30 mM phosphate added to the HEPES medium prior to infection. For complementation of *gp96*-silenced cells, purified Gp96 was added prior to infection at 10 μg/ml for 30 min. Scavenger Receptor inhibitors Fucoidin (75 μg/ml, Sigma) and AcLDL (10 μg/ml, Molecular Probes) were added 15 min prior to infection. For antibody inhibitor studies, cells were incubated with SREC antibody (rabbit polyclonal, Santa Cruz or goat polyclonal, R&D Systems) or SR-A antibody (rabbit polyclonal, Santa Cruz) 15 min before infection.

Quantification of Total Cell-Associated and Intracellular Colony-Forming Units

To quantify total cell-associated bacteria, washed monolayers were lysed in HEPES medium containing 1% saponin (Sigma) for 7 min at 37°C, releasing adherent and intracellular gonococci. After vigorous titration, serial dilutions of the lysates were plated on GC-agar plates, and colony forming units were determined after a 24 hr incubation period at 37°C and 5% CO_2. For quantification of intracellular viable bacteria, extracellular bacteria were selectively killed by incubating monolayers with 50 μg/ml gentamicin (Sigma) in HEPES medium for 2 hr at 37°C, 5% CO_2, prior to saponin lysis and plating. Gentamicin survival assays were performed at least three times in duplicate.

Immunocytochemistry

Cells grown on glass coverslips were infected as described above. Cells were fixed after infection with 4% paraformaldehyde (PFA) for 15 min at 37°C in a 5% CO_2 atmosphere. Extra- and intracellular bacteria were differentially labeled at room temperature in the dark after several washes with PBS and blocking of unspecific binding sites with 0.2% bovine serum albumin (BSA, Biomol) in PBS for 10 min. The coverslips were incubated for 1.5 hr with a polyclonal rabbit anti-gonococcal antiserum in 0.2% BSA for staining of the extracellular bacteria. After several washes with PBS, coverslips were incubated for 1 hr with Cy-conjugated goat anti-rabbit IgG (Jackson Immunosearch). After extensive washes with PBS, cells were permeabilized with 0.2% Triton X-100 (Calbiochem) for 15 min, and the procedure described above was repeated using a secondary antibody coupled to a different fluorochrome. If GFP-expressing bacteria were used (N1135 and N1136) for infection experiments, the permeabilization and second staining procedure was omitted. Specimens were mounted in Mowiol (Hoechst) and analyzed by confocal laser scanning microscopy using a Leica TCS NT equipped with an argon-krypton mixed gas laser. Images were taken using appropriate excitation and emission filters for the fluorescence dyes used. Overlay images of the single channels were obtained using Adope Photoshop 7.0. In general, double-positive staining (or GFP signal) indicated extra-, single-positive staining (or GFP signal) intracellular bacteria.

RNA Interference

To silence gene expression by RNA interference, 1×10^5 cells per well were seeded into a 12-well plate on the day of transfection. Freshly seeded HeLa or Chang cells were transfected with 160 nM siRNAs for the silencing of *gp96* or luciferase as negative control using RNAiFect transfection reagent (QIAGEN, Valencia, CA) according to the manufacturer's instructions. For complementation experiments, cells were transfected with 160 nM siRNA and 0.5 μg of pcDNA-Gp96-myc using the RNAiFect transfection kit as described above. HUVECs were transfected with 800 nM siRNAs using Hiperfect transfection reagent (QIAGEN, Valencia, CA) according to the manufacturer's instructions. Efficiency of gene silencing was generally validated by real-time PCR as described previously (Machuy et al., 2005) and by western blot analysis 72 hr posttransfection. Gp96 siRNAs were designed according to the sequence deposited under the accession number NM_003299.1. The following siRNAs were used: siGp96-1: 5′ AAGUUGAUGUGGAUGGUACAG 3′; siGp96-2: 5′ TGT GGAGAGGGAATGTGAAAT 3′; and siLuc: 5′ AACUUACGCUGAGUA CUUCGA 3′. The generation of shRNA clones is described in the Supplemental Experimental Procedures section.

Immunoblotting

Cell lysates were resolved by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes (Millipore) and blocked with Tris-buffered saline containing 0.1% Tween 20 (Sigma) and 3% bovine serum albumin. The membranes were then probed with antibodies for mouse

actin monoclonal antibody (Sigma), mouse lamin A/C monoclonal antibody (Chemicon), rabbit prohibitin polyclonal antibody (NeoMarkers), or rat grp94 monoclonal antibody (Stressgen). Proteins were detected with peroxidase-coupled secondary antibody using the ECL system (Amersham).

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures and 11 supplemental figures and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/2/6/393/DC1/.

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