CD46-Independent Binding of Neisserial Type IV Pili and the Major Pilus Adhesin, PilC, to Human Epithelial Cells

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***Neisseria gonorrhoeae* is a gram-negative bacterial pathogen which infects the human mucosal epithelium. An early critical event in neisserial infection is the type IV pilus-mediated adherence to the host cell. The PilC protein, located on the pilus tip, has earlier been identified as the major pilus adhesin. Previous studies suggested that the cell surface protein CD46 is a pilus receptor for *Neisseria*. We investigated the role of CD46 in pilus-mediated gonococcal infection of epithelial cells. Differences in binding efficiencies of piliated gonococci as well as purified pilus adhesin PilC2 on human epithelial cell lines did not correlate to the level of surface-expressed CD46. Additionally, no binding of piliated gonococci or PilC2 protein was observed on CD46-transfected CHO and MDCK cells. Furthermore, specific down-regulation of CD46 expression in human epithelial cell lines by RNA interference did not alter the binding efficiency of piliated gonococci or purified PilC2 protein, although other CD46-dependent processes, such as measles virus infection and C3b cleavage, were significantly reduced. These data support the notion that pilus-mediated gonococcal infection of epithelial cells can occur in a CD46-independent manner, thus questioning the function of CD46 as an essential pilus receptor for pathogenic neisseriae.**

Neisseria gonorrhoeae is an obligate human pathogen that causes the sexually transmitted disease gonorrhea. This gram-negative diplococcus colonizes not only the mucosal surface of the male urethra and the female cervix but also the pharynx, the rectum, and the conjunctiva of the eye. The initial attachment to the apical side of epithelial tissues is mediated by type IV pili, which are important for establishing infection in vivo (45). Piliated gonococci adhere typically by forming dense microcolonies on the cell surface (24, 28, 44). After adherence via pili, the bacteria use other virulence factors, such as the Opa proteins and the lipopolysaccharides, to bind more intimately via several epithelial host cell surface receptors (2, 3, 11, 33, 48, 49). These stable interactions can lead to bacterial invasion and transcytosis of the epithelial host cell (6, 25, 29, 51).

Neisserial type IV pili are composed of a major structural subunit, the pilin or PilE protein, which is assembled into a helical pilus fiber (31). In vitro data indicate that antigenic variation of this protein can affect pilus-mediated adherence to human cells (14, 22, 39). Besides pilin, several other proteins are required for type IV pilus biogenesis and function (32, 53). Among them, the PilC proteins are 110-kDa proteins that have been associated with the pili and the outer membrane (35, 38). PilC proteins have functions in pilus biogenesis and natural transformation competence (15, 37). As the major pilus adhesin, they play an essential role in infection of epithelial and endothelial cells (38, 42). Piliated PilC-null strains do not adhere to epithelial cells (30, 39). Purified PilC protein binds to epithelial and endothelial cells in vitro and inhibits competitively the adherence of *N. gonorrhoeae* and *Neisseria meningitidis* (42). Two copies of PilC, PilC1 and PilC2, are present in

gonococci and meningococci. For *N. gonorrhoeae* it was shown that the two proteins PilC1 and PilC2 have similar functions, i.e., piliation, competence, and adhesion (14, 37). In meningococcal strains both PilC proteins are capable of mediating piliation and competence, but only PilC1 promotes adhesion (27, 41). Another minor constituent of the pilus fiber, the PilV protein, was shown to be important for type IV pilus-mediated adherence to human epithelial cells (52).

Pilus-mediated neisserial infection involves complex host responses, such as cytosolic Ca^{2+} flux, exocytosis, and cortical plaque formation (1, 16, 24). Current data suggest that CD46, a member of the complement resistance protein superfamily, acts as a neisserial pilus receptor (17). CD46, also called membrane cofactor protein, undergoes alternative splicing and is expressed in four major isoforms, BC1, BC2, C1, and C2 (21). Most cell types express all isoforms, but with different ratios (40). Using Chinese hamster ovary (CHO) cells transfected with the four isoforms, Kallström and colleagues showed that piliated gonococci bind to BC1-expressing cells and, less efficiently, to BC2-expressing cells but not to cells transfected with the C1 or C2 isoforms. Gonococcal binding can be blocked with recombinant CD46 or anti-CD46 antibodies (17). Additionally, upon infection with piliated gonococci, CD46 isoforms containing the cytoplasmic tail 2 are rapidly tyrosine phosphorylated by Src kinase c-yes (20). Recently, Johansson et al. presented a CD46 transgenic mouse model that is susceptible to meningococcal disease (13). To date, however, no studies have demonstrated a direct interaction between the pilus adhesin PilC and CD46. Interestingly, Tobiasson and Seifert found that the level of pilus-mediated gonococcal adherence does not correlate with the amount of surface-expressed CD46 (47). In addition, it was repeatedly reported that preincubation with various anti-CD46 antibodies could not block pilus-mediated adherence of gonococci to epithelial cells (8, 10).

In this study, we investigated the role of CD46 in gonococcal

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adherence and binding of the neisserial pilus adhesin PilC. Using CHO and Madin-Darby canine kidney (MDCK) cell lines stably transfected with specific CD46 isoforms and RNA interference, we found that piliated gonococci do not bind to epithelial cells in a CD46-dependent manner. This correlates with data obtained from binding studies with purified PilC protein. Taken together, our data question the role of CD46 as a receptor for neisserial pili.

MATERIALS AND METHODS

Bacterial strains. *N. gonorrhoeae* MS11 strains N138 (P⁺, Opa⁻), N303 (P⁻, Opa₅₀), N302 (P⁻, Opa⁻), N557 (P⁺, PilC2, Opa⁻), and N558 (P⁺, PilC1, Opa⁻) have been described previously (19, 38, 42). Piliated Opa⁻ and nonpiliated Opa₅₀-positive variants were distinguished by colony morphology under a binocular microscope. Bacteria were grown on GC agar base with vitamin supplements at 37°C with 5% CO₂.

Cell lines and growth conditions. The cell lines used in the experiments were Me180 human cervix carcinoma cells (ATCC HTB33), Chang human conjunctiva cells (ATCC CCL20.2), Hec1B human endometrium carcinoma cells (ATCC HTHB133), HeLa human cervix carcinoma cells (ATCC CCL2), Jurkat human lymphoma T cells (ATCC TIB-152), CHO-K1 hamster ovary cells (CHO, ATCC CCL-61), MDCK cells (ATCC CCL34), and the stable transfected cell lines CHO-BC1, MDCK-BC1, and MDCK-BC2 (kindly provided by Andrea Maisner).

The Me180 cells were maintained in McCoy's 5A medium supplemented with L-glutamine and 10% fetal calf serum (FCS). Chang, Hec1B, HeLa, and Jurkat cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% FCS. All MDCK cell lines were grown in minimal essential medium with 10% FCS. CHOK-1 and CHO-BC1 cells were maintained in Ham's 12 medium with L-glutamine and 10% FCS. For transfected cell lines, 0.5 mg/ml G418 (Geneticin) was added to the medium. All cell lines were grown at 37°C with 5% CO₂.

PilC2 purification. The PilC2 protein was purified as previously described (42). Briefly, using the gonococcal strain N560 PilC_{His6}, protein expression was induced overnight on GC plates containing tetracycline (10 mg/ml) and IPTG (isopropyl-β-D-thiogalactopyranoside) (100 mg/ml). Bacteria were collected in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl and lysed by sonication. After centrifugation at 4,000 rpm, the supernatant (membrane-containing fraction) was centrifuged for 1 h at 20,000 rpm. The PilC2 protein was dissolved by incubating the membrane in 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 500 mM NaCl containing 2% lauryldimethylamino-N-oxide for 1 h at 37°C.

After centrifugation, the supernatant was loaded on an Ni-nitrilotriacetic acid column, and after washing with 50 mM imidazole, the PilC2 protein was eluted by a pH shift from 8.0 to 4.0 with 10 mM sodium citrate buffer containing 150 mM NaCl.

Infection experiments. Cells were grown in 24-well cell culture plates to 70 to 80% confluency on the day of infection. Infections with the strains N138, N303, N557, and N558 were done in medium without FCS. Opa₅₀ (N303) infection was performed in medium with 10% FCS. The monolayers were washed three times with medium before infection.

Bacteria were suspended in medium and added to the cells at a multiplicity of infection (MOI) of 100. Infected monolayers were centrifuged for 3 min at 120 × g to synchronize infection and incubated for 90 min at 37°C in 5% CO₂. To stop the infection and to remove nonadherent bacteria, the cells were washed three times with 1 ml medium.

Adherent bacteria were quantified by lysing the monolayer with 1% saponin in medium for 7 to 10 min and suspending gonococci by vigorous pipetting. CFU were determined by plating of serial dilutions. For microscopic analyses, infections were done on glass coverslips and cells were fixed in 3.7% paraformaldehyde (PFA).

PilC2 Binding. For immunoblot analyses, cells were grown in six-well cell culture plates to 70 to 80% confluency. The monolayers were washed twice with cold medium without FCS, and 1 μg PilC2 protein was added per well and incubated for 1 h at 4°C. Cells were washed four times with cold phosphate-buffered saline (PBS) to remove unbound PilC2 protein and then lysed in Laemmli buffer. For flow cytometric analyses, cells were detached with 1 mM EDTA in PBS. Cells were collected and washed with cold medium. PilC2 protein was added (1 μg protein/10⁶ cells) and incubated for 1 h at 4°C. Cells were washed three times with PBS and suspended in PBS containing 3% FCS.

Immunofluorescence microscopy. Surface-exposed CD46 was detected by using mouse monoclonal antibody J4.48 (Coulter), and cell-associated gonococci were detected by using the rabbit antiserum AK213 (raised against gonococcal lysate). All secondary antibodies were purchased from Jackson ImmunoResearch (Baltimore, MD).

Cells were grown on glass coverslips, fixed with 4% PFA for 10 min at room temperature, washed with PBS, and blocked for 30 min with 7% FCS in PBS. Coverslips were incubated with the primary antibodies for 1 h, washed in PBS, and then incubated with secondary antibody for an additional hour. After washing, slides were mounted with Mowiol (Merck, Darmstadt, Germany), dried, and analyzed by confocal laser scanning microscopy (Leica TCS NT).

Flow cytometry. For flow cytometric analyses, the monoclonal CD46 antibody J4.48 and the rabbit anti-PilC2 serum (raised against purified PilC2 protein) were used. Cells (10⁶) suspended in 100 μl PBS containing 3% FCS were incubated with 2 μg of J4.48 and 2 μl of PilC2 antiserum for 1 h at 4°C. The cells were washed three times with PBS–3% FCS and then incubated for 1 h in 100 μl of a 1:100 dilution of Cy2-conjugated anti-mouse and Cy5-conjugated anti-rabbit (Jackson ImmunoResearch, Baltimore, MD) for 1 h at 4°C. Cells were washed three times and suspended in 300 μl PBS. One microliter of propidium iodide was added to detect dead cells. The samples were analyzed on a FACScalibur (Becton Dickinson) with Cell Quest software.

Immunoblotting. For Western blot analyses the following antibodies were used: polyclonal goat CD46 antibody (N19; Santa Cruz Biotechnology), rabbit anti-PilC2 rabbit serum, mouse lamin A/C monoclonal antibody (MAb) (MAb 3211; Chemicon), mouse α-tubulin MAb (Sigma), MAb 755 (50), and MAb G-3E (12). Cell lysates were resolved by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis or as indicated and transferred to polyvinylidene difluoride membranes. After blocking with Tris-buffered saline containing 0.1% Tween 20 and 3% bovine serum albumin or 5% nonfat dry milk (for C3b cleavage analyses), membranes were probed with specific antibodies. Proteins were visualized with peroxidase-coupled secondary antibody by using the ECL system (Amersham).

RNA interference. The RNA duplex targeting all CD46 isoforms (accession no. NM_002389, 5'-CCAAACCTACTATGAGA-3') was synthesized with the Silencer small interfering RNA (siRNA) construction kit according to the instructions of the manufacturer (Ambion, Massachusetts). The control RNA duplex targeting lamin A/C (accession no. M13451, 5'-CTGGACTCCAGAA GAACA-3') was synthesized by Dharmacon Research, Inc. (Lafayette, CO). The transfection of siRNAs (160 nM) was carried out using TransMessenger transfection reagent (Qiagen) according to the manufacturer's instructions. The cells were used for experiments 72 h after transfection.

MV infection. Recombinant measles virus (MV) of the Edmonston B strain expressing enhanced green fluorescent protein (eGFP) was used for infection (9). Virus infection was performed as previously described with some modifications (36). Briefly, after 3 days of siRNA treatment, HeLa cells were seeded into 12-well-plates and virus was added immediately at an MOI of 0.5 or 5. After incubation for 3 days at 35°C, cells were analyzed by flow cytometry.

C3b cleavage. In order to study the cofactor activity of CD46, we performed C3b cleavage assay as previously described with some modifications (4). After 3 days of siRNA treatment, HeLa cells, seeded in 24-well plates, were washed twice with PBS containing Ca²⁺ and Mg²⁺ and incubated with 500 ng factor I (Calbiochem) and 2.5 μg C3b (Calbiochem) in 500 μl PBS containing Ca²⁺ and Mg²⁺ for 2 h at 37°C. Incubation with factor I or C3b alone was included as a control. The supernatants and cell lysates were collected, resolved by SDS-polyacrylamide gel electrophoresis (9%), transferred to polyvinylidene difluoride membranes, and proceed for immunoblotting.

RESULTS

Piliated gonococci do not adhere to CD46-transfected cell lines. One isoform of CD46 (BC1) has been described as the most potent pilus receptor isoform (17). We used a CHO cell line transfected with the BC1 isoform (CHO-BC1) and MDCK cell lines transfected with the BC1 (MDCK-BC1) and the BC2 (MDCK-BC2) isoforms. Epithelial cell lines of human origin (Me180 and Chang) were used as positive controls. The expression of CD46 was determined by immunoblotting. As seen in Fig. 1, the CD46 isoforms are expressed in the human epithelial cell lines Me180 and Chang and in the transfected CHO and MDCK cells but not in the nontransfected ones. To

FIG. 1. Gonococcal adherence to CD46-expressing cells. (A) Human epithelial cell lines (Me180 and Chang) and animal cell lines (CHO and MDCK) stably transfected with the BC1 or BC2 isoform of CD46 were infected with the pilated gonococcal strain N138 (P^+ , Opa^-) for 90 min, and cell-associated CFU were quantified by saponin lysis. Gonococci adhered to Me180 and Chang cells. No significant bacterial binding was observed for nontransfected and CD46-transfected animal cell lines (CHO and MDCK). The bars show the mean number of bacteria associated per cell $\pm$ the standard deviation from one representative experiment. The assay was performed in triplicate and repeated three times (upper panel). The CD46 expression of the cell lines was analyzed by Western blotting, using polyclonal goat antibody N19. Me180 cells expressed moderate level of CD46, whereas Chang cells showed strong CD46 expression. Transfected CHO and MDCK cells expressed large amounts of CD46 (bottom panel). (B) Me180, CHO, and CHO-BC1 cells were infected with the pilated gonococcal strain N558 (P^+ , PilC1, Opa^-) or N557 (P^+ , PilC2, Opa^-) for 90 min, and cell-associated CFU were quantified by saponin lysis. Both strains bind to Me180 cells but not to CHO or CHO-BC1 cells.

check whether CD46 is surface exposed in all cell lines, we performed indirect immunofluorescence staining of surface-exposed CD46 on nonpermeabilized cells. Microscopic analysis showed that CD46 could be detected on the cell surface of all cell lines tested except the nontransfected CHO and MDCK cells (Fig. 2). To study the pilus-mediated adherence, semiconfluent monolayers were infected with the pilated gonococcal strain N138 (MOI, 100) for 90 min, and cell-associated bacteria were quantified. As seen in Fig. 1A, pilated gonococci bound at highest number to the human cervix carcinoma cell line (Me180), whereas the amount of bound gonococci was significantly lower on Chang cells. Pilated bacteria did not bind to CD46-transfected CHO or MDCK cell lines.

Two copies of the pilus-associated protein PilC, PilC1 and PilC2, are present in gonococci. Although they appear to mediate similar functions in adherence, we analyzed the adherence of pilated gonococcal strains expressing one or the other PilC protein. Both the PilC1-expressing strain N558 and the PilC2-expressing strain N557 bound to Me180 cells; however, both did not adhere to CD46-transfected CHO cells (Fig. 1B).

Purified pilus adhesin PilC2 does not bind to CD46-transfected cell lines. PilC has been identified as the major pilus adhesin (38). To test whether the adhesin alone can bind to CD46-transfected cells, we performed a binding assay with purified PilC2 protein at 4°C (see Materials and Methods). Cell monolayers were incubated with and without PilC2 pro-

FIG. 2. Surface expression of CD46. Cells were grown on coverslips, fixed with 3.7% PFA, and processed for immunofluorescence staining. Monoclonal antibody J4.48 was used for CD46 detection. The human cell lines (Me180 and Chang) and the transfected cell lines (CHO-BC1, MDCK-BC1, and MDCK-BC2) exposed CD46 on the cell surface. CD46 expression was not seen on nontransfected cells (CHO and MDCK).

tein, and after lysis, the amount of cell-bound PilC2 protein was determined by immunoblotting with anti-PilC2 polyclonal mouse serum (Fig. 3). A large amount of PilC2 protein was bound to Me180 cells. Chang cell lysates contained significantly less PilC2 protein. No PilC2-specific signal was detected in the cell lysates of CD46-expressing CHO and MDCK cells or control cells. To confirm these results, we measured the amount of cell-bound PilC2 protein by flow cytometry. As seen in Fig. 4 (upper panel), HeLa cells showed the highest PilC2 binding, whereas Chang cells bound significantly less protein.

CHO-BC1 cells did not show significant binding of PilC2 compared to nontransfected CHO control cells. The results are consistent with those obtained from immunoblot analyses. Protein binding assays performed at 37°C revealed similar results (data not shown).

PilC2 binding does not correlate with CD46 expression on human cell lines. In order to quantify PilC2 binding and to compare adhesin binding to surface-exposed CD46, we performed PilC2- and CD46-specific immunostaining and flow cytometric analyses with different cell lines of human origin.

FIG. 3. Binding of purified PilC2 protein to human epithelial and CD46-transfected animal cell lines. Human epithelial cell lines (Me180 and Chang) and animal cell lines (CHO and MDCK) stably transfected with the BC1 or BC2 isoform of CD46 C were incubated or not with purified PilC2 protein for 1 h at 4°C, followed by frequent washing. Cells were collected in SDS sample buffer and analyzed by Western blotting, using rabbit anti-PilC2 serum and anti-CD46 polyclonal goat antibody N19. PilC2 bound efficiently to Me180 cells, whereas only a slight signal was seen with Chang cells. PilC2 protein did not bind to CD46-transfected and nontransfected animal cell lines. A sample with purified PilC2 protein (600 ng) was included as a positive control.

FIG. 4. Flow cytometry analysis of PilC2 binding and CD46 surface expression. Human epithelial cell lines (Me180, HeLa, and Chang), a human T-cell line (Jurkat), and the animal cell line CHO, nontransfected or transfected with CD46 isoform BC1, were incubated with PilC2 protein for 1 h at 4°C and stained with antibodies against PilC2 or CD46 (see Materials and Methods). PilC2 bound to human epithelial cell lines (Me180, Chang, and HeLa), whereas for the T-cell line (Jurkat) and the animal cell lines CHO and CHO-BC1, no PilC2 binding was detectable (upper panel). All human cell lines (Me180, HeLa, Chang, and Jurkat) expressed CD46 on the cell surface, as did the CD46-transfected animal cell line CHO-BC1. Nontransfected CHO cells did not show CD46 expression (bottom panel). The assay was performed in duplicate and repeated three times. Shown is the mean fluorescence $\pm$ standard deviation from one representative experiment.

Human cervical carcinoma cell lines (Me180 and HeLa), human conjunctiva cells (Chang), and a human T-cell line (Jurkat) were used in this experiment. The levels of surface-exposed CD46 and the amounts of cell-associated PilC2 protein were different for the cell lines tested. A correlation between CD46 expression and PilC2 binding could not be observed (Fig. 4). The highest level of PilC2 binding was detected in both cervical carcinoma cell lines (Me180 and HeLa). Chang cells bound clearly less PilC2 protein but showed the highest level of surface-exposed CD46. Although the T-cell line (Jurkat) exhibited a level of surface-exposed CD46 comparable to that of HeLa cells, PilC2 protein did not bind to these cells.

Down-regulation of CD46 by RNA interference. To confirm that CD46 does not play a role as a pilus receptor, we used siRNAs to down-regulate the expression of CD46 in human

epithelial cells. Because of higher transfection rates, Hec1B and HeLa cells were used in these experiments. In order to down-regulate all CD46 isoforms, a common sequence within the N-terminal part of the molecule was used for the siRNA design. Lamin A/C siRNA was included as a control. The down-regulation of CD46 was assessed by immunoblotting and fluorescence-activated cell sorter analysis (Fig. 5A and B). CD46 expression in CD46 siRNA-treated cells was reduced by 65 to 73% in Hec1B cells and by 67 to 74% in HeLa cells. Lamin A/C silencing had no effect on CD46 expression.

In order to demonstrate a functional consequence of CD46 down-regulation for ligand interaction, we tested two CD46-dependent processes on siRNA-treated HeLa cells. CD46 was shown to serve as a receptor for the MV Edmonston B strain (7). Upon siRNA-mediated down-regulation of CD46 in HeLa cells, MV infection was significantly reduced (Fig. 5C). Fluorescence-activated cell sorter analyses revealed that only about 19% (MOI, 0.5) and 25% (MOI, 5) of the CD46 siRNA-treated cells were infected compared to control cells. To further confirm the functional down-regulation of CD46, we analyzed the cofactor activity of CD46 in the proteolytic cleavage of the complement factor C3b (43). In the presence of factor I and CD46, the α' chain of C3b is cleaved into fragments of 67, 42, and 40 kDa. MA b 755, raised against the C-terminal part of the C3b α' chain and therefore recognizing the 40-kDa fragment, and MA b G-3E, which is specific for the 67-kDa fragment, were used for immunoblot analyses. Figure 5D shows that in the presence of factor I, C3b is cleaved. The protein levels of both cleavage products (40 and 67 kDa) are significantly reduced in CD46-down-regulated cells compared to control cells. Together, these data clearly demonstrate that in our assay the efficiency of CD46 down-regulation is sufficient to observe functional consequences for respective ligand interactions.

Gonococci bind to CD46-down-regulated epithelial cells. For analyses of pilus-mediated bacterial binding to CD46-down-regulated cells, siRNA-treated HeLa and Hec1B cells were infected with the piliated gonococcal strain N138 (P^+ , Opa⁻). The nonpiliated but adherent Opa₅₀-expressing strain N303 and the nonadherent, nonpiliated Opa-negative strain N302 were included as controls. Semiconfluent monolayers were infected for 90 min, and the amount of bound bacteria was quantified. As shown in Fig. 6, the adherence of piliated gonococci to CD46-down-regulated cells is not reduced compared to control cells. Similar results were obtained from binding studies using isogenic PilC1 (N558)- or PilC2 (N557)-expressing strains (data not shown). To confirm this observation, we performed immunofluorescence staining and analyzed gonococcal binding to CD46-down-regulated cells on the single-cell level. Piliated bacteria (N138) bound to CD46-positive as well as CD46-negative cells (Fig. 7A and B). On HeLa cells we observed slight differences in the distribution of cell-associated bacteria. Whereas gonococci bound locally by forming microcolonies on the cell surface of nontransfected cells, bacteria exhibited a more scattered binding on CD46-down-regulated cells (Fig. 7B). Such differences were not detected in Hec1B cells (Fig. 7A).

PilC2 protein binds to down-regulated epithelial cells. Finally, we wanted to determine the impact of CD46 down-regulation on PilC2 binding to epithelial cells. Therefore, CD46 expression on Hec1B and HeLa cells was suppressed by

FIG. 5. Down-regulation of CD46 expression by RNA interference. (A and B) Hec1B (A) and HeLa (B) cells were transfected with siRNA duplexes targeting CD46 and lamin A/C. After 72 h, the CD46 expression was analyzed. For flow cytometric analyses, cells were stained with anti-CD46 antibody J4.48. In both cell lines the CD46 expression was specifically down-regulated by RNA interference. The assay was performed in duplicate and repeated two times. Shown is the mean fluorescence $\pm$ standard deviation from one representative experiment (upper panel). The bottom panels show Western blotting of siRNA-transfected cell lysates. CD46 expression (N19 antibody) and lamin A/C expression (MAb 3211) were specifically down-regulated in Hec1B and HeLa cells (arrows). Arrowheads indicate unspecific binding of the secondary antibody. (C) siRNA-treated HeLa cells were infected with eGFP-expressing measles virus Edmonston strain for 3 days at an MOI of 0.5 or 5. GFP expression was measured by flow cytometry. Shown is the percentage of eGFP-positive cells $\pm$ standard deviation of triplicates. (D) C3b cleavage assay. siRNA-treated HeLa cells were incubated with factor I, C3b, or both for 2 h at 37°C, and the supernatant was analyzed by Western blotting. The C3b α' chain and the 40-kDa cleavage fragment are recognized by MAb 755. MAb G-3E detects the 67-kDa cleavage fragment. Cell lysates were probed with α -tubulin antibody.

RNA interference, and PilC2 binding was monitored by flow cytometry. Cells transfected with lamin A/C-specific siRNA were included as controls. CD46 was down-regulated in 73.18% of the transfected Hec1B cells and in 80.04% of the transfected HeLa cells (Fig. 8; Table 1). Both cell lines, Hec1B and HeLa, bound PilC2 protein effectively. Alteration of CD46 expression had no effect on binding, since the CD46-expressing and the CD46-down-regulated cell population showed similar amounts of cell-bound PilC2 protein. These data clearly show that PilC2 protein does not bind to epithelial cells in a CD46-dependent manner.

DISCUSSION

Pilus-mediated adherence of pathogenic neisseriae has critical functions both in the initial phase and during the subse-

quent course of an infection. Studies with human volunteers using low-dose inocula indicated that successful infection requires the application of piliated *Neisseria gonorrhoeae* variants, while nonpiliated ones appeared to be noninfectious. These studies emphasize the pivotal function of pili in establishing infection (18, 45). The highly flexible surface appendages may facilitate the anchoring of the bacteria in the human mucosa, trigger initial host response mechanisms, and thus prepare the pathogens for subsequent steps of the infection process. Type IV pilus retraction, which seems to occur subsequent to the binding of pili (25, 34), may facilitate proximal contact of molecules associated with the pathogen and host cell surfaces (5). Later in the infection, invasive *N. meningitidis* strains make use of their pili to interact with endothelial cells of the blood-brain barrier, while other surface-associated fac-

FIG. 6. Gonococci adhere to CD46-down-regulated human epithelial cells. Hec1B (A) and HeLa (B) cell monolayers, transfected with the CD46 or lamin A/C siRNA duplex for 3 days, were infected with the piliated strain N138, the nonpiliated Opa₅₀-expressing strain N303, and the nonpiliated Opa-negative strain N302 for 90 min. Cell-associated CFU were quantified by saponin lysis. The bars show the mean number of cell-associated bacteria $\pm$ the standard deviation from one representative experiment.

tors appear to be buried by the extracellular polysaccharide capsule (29). Both epithelial and endothelial cell interactions with the neisserial type IV pilus seem to follow similar principles and involve identical receptor molecules (27, 42). Also, piliated meningococci and gonococci exhibit similar binding specificities, indicating the existence of identical or closely related PilC receptors for both species (41, 42). Identifying the neisserial pilus receptor on human cells is therefore of vital importance to our understanding of the basic events taking place during the initial phases of pathogen-host cell contact. Furthermore, since pili constitute a major determinant of the neisserial species tropism, receptor identification would provide a rationale for generating more suitable animal models, e.g., via the construction of receptor-proficient transgenic mice.

Previous studies had shown that the abundant transmem-

brane glycoprotein CD46 may act as receptor for gonococcal type IV pili (17), yet no direct interaction between CD46 and any pilus component, such as the PilC adhesin, has been demonstrated. In our present work we could not confirm the role of CD46 as an essential receptor for neisserial pili. Neither piliated bacteria nor the purified adhesin molecule PilC2 bound to CHO or MDCK cells expressing certain isoforms of human CD46. The reverse approach, down-regulation of CD46 in a human epithelial cell system by RNA interference, offered an ideal opportunity to assess the role of this receptor in a functionally positive background. Here we could clearly demonstrate that the interaction of piliated gonococci as well as of the purified pilus adhesin PilC2 with human epithelial cells is unaffected by CD46 expression, since the binding efficiency is not reduced on CD46-down-regulated cells. Additionally, preincubation of Me180 cells with a polyclonal CD46-specific antibody

FIG. 7. Confocal analyses of pilus-mediated gonococcal binding to CD46-down-regulated cells. Hec1B (A) and HeLa (B) cell monolayers, transfected with siRNA targeting lamin A/C or CD46, were infected with the piliated strain N138 for 90 min. Cells were fixed and stained for CD46 (green) and gonococci (red). Piliated bacteria bound to both CD46-expressing and nonexpressing cells.

did not result in reduced pilus-mediated gonococcal adherence (data not shown). Moreover, by comparing different human cell lines, we could not observe any direct correlation between the level of CD46 surface expression on human cell lines and pilus-mediated bacterial or PilC2 protein binding efficiency. Our results are consistent with previously published data (47), which showed an inverse relationship between pilus-mediated adherence and CD46 surface expression. The authors of that

work concluded that CD46 does not act as a classical pilus receptor, questioning the precise role of that molecule in neisserial infection. The fact that CD46 is typically expressed on the basolateral site of human epithelial tissues (46) makes the possibility that CD46 acts as a prime receptor for neisserial pili in mucosal colonization unlikely (23).

Together our results show that piliated gonococci adhere to human epithelial cells in a CD46-independent manner, thus

FIG. 8. PilC2 binding to CD46-down-regulated cells. Hec1B and HeLa cells, nontransfected and transfected with siRNA duplexes targeting lamin A/C or CD46, were incubated or not with purified PilC2 protein and double stained for CD46 (mouse) and PilC2 (rabbit). CD46 is specifically down-regulated in siRNA CD46-transfected cells (lower populations). siRNA targeting lamin A/C did not affect CD46 expression. PilC2 protein bound to both CD46-expressing and CD46-down-regulated cells (right populations). Shown is the percentage of CD46-positive (y axis) and PilC2-positive (x axis) cells.

questioning the role of CD46 as the receptor for gonococcal pili. However, our study does not rule out the possibility of a different function of CD46 in neisserial infection. The alteration in microcolony formation on CD46-down-regulated HeLa cells described here could lead to speculation about a role of CD46 in this particular process. In previous studies it

was shown that pilus-induced cytosolic Ca^{2+} flux involves CD46 interaction (16). Furthermore, CD46 is phosphorylated upon neisserial adherence and also is down-regulated later in infection, although this down-regulation could not be inhibited by preincubation with various antibodies against CD46 before infection (10, 20). CD46 transgenic mice develop disease after

TABLE 1. CD46 expression and PilC2 binding after 3 days of treatment with siRNA duplexes targeting lamin A/C or CD46^a

Cell line	siRNA	CD46-positive cells (%)	PilC2-positive cells (%)
Hec1B	Lamin A/C	93.35	76.58
	CD46	26.82	76.93
HeLa	Lamin A/C	74.78	56.25
	CD46	19.96	56.10

^a Data were obtained from flow cytometry.

challenge with *Neisseria meningitidis* (13); however, bacteremia and mortality were not restricted to pilus-producing strains. Indeed, CD46 transgenic mice were more sensitive to infection with nonpilated strains when injected intraperitoneally. The mice used in that study appeared to harbor an additional alpha/beta interferon defect (26). This intrinsic defect rather than the transgenic expression of CD46 might explain the increased susceptibility of mice to neisserial infection (13).

Given that pilated gonococci bind to epithelial cells in a CD46-independent manner, our current investigations are focused on the characterization and identification of host cell factors with receptor function for gonococcal and meningococcal type IV pili. Preliminary data indicate that the putative pilus/PilC receptor of *Neisseria gonorrhoeae* is proteinous and located in cholesterol-rich microdomains (M. Kirchner et al., unpublished data). Signaling through such a receptor could lead to infection-induced CD46 phosphorylation. Further research appears to be necessary to understand the complex nature of the cellular microdomains as well as the function of the underlying cortical plaques (24).

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