Molecular Microbiology (2005) **56**(4), 945–957 doi:10.1111/j.1365-2958.2005.04600.x

The PilC adhesin of the *Neisseria* type IV pilus – binding specificities and new insights into the nature of the host cell receptor

Marieluise Kirchner and Thomas F. Meyer*
Department of Molecular Biology, Max Planck Institute for Infection Biology, Schumannstr. 21/22, 10117 Berlin, Germany.

Summary

Type IV pili of *Neisseria gonorrhoeae* and *Neisseria meningitidis* mediate the first contact to human mucosal epithelial cells, an interaction which is also critical for the interaction with vascular endothelial cells. The PilC proteins have been characterized as the principal pilus-associated adhesin. Here we show that PilC2 exhibits a defined cell and tissue tropism, as it binds to human epithelial and endothelial cell lines, but not to human T cells or fibroblasts. Piliated gonococci and PilC2 exhibit similar patterns of binding to human epithelial and endothelial cells, supporting the function of PilC as the key pilus adhesin. Although CD46 has previously been suggested to be a pilus receptor, several observations indicate that neisserial type IV pili and the pilus adhesin PilC2 interact with epithelial cells in a CD46 independent manner. Biochemical approaches were used to characterize the nature of host cell factors mediating binding of piliated gonococci and PilC2 protein. Our data indicate that the putative host cell receptor for gonococcal pili and the PilC2 pilus adhesin is a surface protein. Glycostructures were found to not be involved in binding. Moreover, we observed the uptake of purified PilC2 protein together with its receptor via receptor-mediated endocytosis and subsequent receptor re-exposure on the cell surface. Our data support the existence of a specific pilus receptor and provide intriguing information on the nature of the receptor.

Introduction

Specific adherence of microbes to the host tissue represents the initial step in pathogenesis. Host specificity and tissue tropism are determined by the molecular interaction between bacterial adhesins and their complementary receptors on the host cell surface. Many bacterial adhesins are organized as thin thread-like organelles called fimbriae or pili. In most Gram-negative pathogens these organelles mediate the bacterial attachment to mucosal epithelial surfaces (Soto and Hultgren, 1999). Pili are complex structures with a long pilus tail composed of a structural protein, which often serves as a scaffold for the display of a few copies of the actual adhesive protein, typically located at the tip of the organelle (Jones *et al*., 1995; Kuehn *et al*., 1994).

Neisseria gonorrhoeae, the causative agent of the sexually transmitted disease gonorrhoea, is an extremely host-restricted pathogen with infections limited to humans (Meyer *et al*., 1994; Nassif and So, 1995). The pathogen colonizes the epithelium of the urogenital tract and, less frequently, of the rectum, oropharynx and conjunctiva (Handsfield, 1990). *Neisseria meningitidis*, a closely related pathogen, colonizes the human oro- and nasopharynx, and can cause septicaemia and/or meningitis. For both pathogens the target specificity can be attributed to surface-associated fimbrial organelles, called type IV pili, which mediate the initial attachment to the host cell and are essential for establishing infection *in vivo* (Kellogg Jr *et al*., 1963; Swanson, 1973; Johnson *et al*., 1977; Heckels, 1989; Virji *et al*., 1992; Nassif *et al*., 1993; Virji *et al*., 1993; Seifert *et al*., 1994). Neisserial type IV Pili are composed of a major structural subunit, the pilin or PilE protein, which assembles into a helical pilus fibre (Parge *et al*., 1995). The pilin subunit undergoes antigenic variation (Meyer *et al*., 1994; Seifert, 1996) and can be post-translationally modified (Stimson *et al*., 1996; Weiser *et al*., 1998; Forest *et al*., 1999; Hegge *et al*., 2004). The protein PilC was localized to the pilus tip and revealed as the major pilus adhesion molecule (Rudel *et al*., 1995b), as piliated *pilC* mutants failed to adhere to epithelial cells (Rudel *et al*., 1992; Nassif *et al*., 1994). In addition, purified PilC protein binds to host cells and thereby blocks the pilus-mediated adherence of both *N. gonorrhoeae* and *N. meningitidis* (Scheuerpflug *et al*., 1999). The adhesive property of the PilC protein relies on its amino acid sequence, and the adhesion promoting regions are suggested to be in the N-terminal part of the molecule (Morand *et al*., 2001).

Accepted 31 January, 2005. *For correspondence. E-mail meyer@mpiib-berlin.mpg.de; Tel. (+49) 302 846 0400; (+49) 302 846 0402; Fax (+49) 302 846 0401.

PilC protein, which also resides in the bacterial outer membrane (Rahman *et al*., 1997), has additional functions in pilus biogenesis and natural transformation competence (Jonsson *et al*., 1991; Rudel *et al*., 1995a). The proposed dual location is consistent with a model put forward for type IV pilus assembly (Fussenegger *et al*., 1997; Wolfgang *et al*., 2000). Two high-molecular weight (110 kDa) PilC proteins, designated PilC1 and PilC2, which are functionally similar, are usually found in *N. gonorrhoeae*.

Several studies have suggested a role for PilE in receptor recognition, as different antigenic forms of PilE influence the adherence of *Neisseria* to epithelial cells and tissue tropism (Virji and Heckels, 1984; Rudel *et al*., 1992; Nassif *et al*., 1993; Jonsson *et al*., 1994). Moreover, it has been reported that PilE itself is responsible for pilus-mediated agglutination of human erythrocytes (Schoolnik *et al*., 1984; Rudel *et al*., 1992; Scheuerpflug *et al*., 1999), however, the biological significance of this *in vitro* activity for pilus-mediated gonococcal adherence remains unclear. Besides pilin and PilC, several other accessory proteins, the minor pilins, are required for type IV pili biogenesis and function (Parge *et al*., 1990; Drake and Koomey, 1995; Freitag *et al*., 1995; Wolfgang *et al*., 2000; Park *et al*., 2002). One of them, the PilV protein, was suggested to promote the functional display of PilC within the pilus fibre (Winther-Larsen *et al*., 2001).

Neisserial type IV pili mediate adhesion to several human cell types including epithelial and endothelial cells. It is likely that the pilus adhesin PilC contributes to this interaction because it has been described as the adhesin for both epithelial and endothelial cells (Rudel *et al*., 1992; Nassif *et al*., 1994). Previous studies suggested the cell surface protein CD46 as a receptor for neisserial pili on human epithelial cells (Kallstrom *et al*., 1997). Transfected Chinese hamster ovary cells (CHO) expressing CD46-bound piliated but not non-piliated gonococci. This adherence could be blocked with recombinant CD46 protein and anti-CD46 antibodies. Moreover, neisserial type IV pili triggered a transient increase in internal calcium levels in target cells, which can be blocked by anti-CD46 antibodies (Kallstrom *et al*., 1998). Recently, Johansson and colleagues described a CD46 transgenic mouse model, which is susceptible to fatal infection with *N. meningitidis* (Johansson *et al*., 2003). However, no studies have demonstrated a direct interaction between the pilus adhesin PilC and CD46. Tobiason and colleagues found that the level of pilus-mediated gonococcal adherence does not correlate with the amount of cell surface expressed CD46 (Tobiason and Seifert, 2001). Furthermore, CD46 is typically expressed on the basolateral cell surface (Maisner *et al*., 1996; Teuchert *et al*., 1999), questioning its role as a target molecule for piliated *Neisseria* in initial steps of mucosal adherence. Work from our laboratory also does not support the pilus receptor function of CD46. Using a variety of approaches we observed a CD46 interaction with neither piliated *Neisseria* nor the isolated pilus adhesin PilC on human host cells, thus questioning the function of CD46 as a receptor for neisserial pili (Kirchner *et al*., 2005).

To determine the nature of the potential eukaryotic receptor for gonococcal type IV pili, we have taken various approaches to examine the pilus-mediated host cell interaction of gonococci. In this study we confirmed the function of PilC as the pilus adhesin of *N. gonorrhoeae*. Binding specificities of piliated gonococci and purified PilC2 protein were compared, and the potential epithelial receptor was characterized biochemically. Furthermore, we could show, that purified PilC2 protein is internalized by epithelial cells. The underlying mechanisms of this uptake, as well as the role of the potential receptor in this process were investigated.

Results

Purified PilC2 protein inhibits pilus-mediated adherence of gonococci to epithelial and endothelial cells in a dose-dependent manner

Previously, Scheuerpflug and colleagues showed, that purified gonococcal PilC protein binds to human epithelial and endothelial cells and prevents the attachment of piliated *Neisseria* to these cells (Scheuerpflug *et al*., 1999). However, dose-dependent inhibition of the gonococcal adherence and saturable binding of the PilC protein remains to be demonstrated. In this study, we used the human cervix carcinoma epithelial cell line ME180 and the human endothelial cell line HBMEC to confirm the specific function of PilC as the pilus adhesin and to determine the host cell binding capacity for PilC protein. Cell monolayers were incubated with different concentrations of purified PilC2 protein followed by infection with the piliated strain N138 or the non-piliated Opa_{57} expressing strain N313. As shown in Fig. 1, preincubation of cells with purified PilC2 protein inhibited the adherence of piliated gonococci (N138) to ME180 as well as HBMEC cells in a dose-dependent manner. Fifty per cent of inhibition was reached with 500 ng (ME180) or rather 300 ng (HBMEC) protein and approximately 700 ng of purified PilC2 protein was sufficient to saturate cell surface exposed pilus receptor molecules of 2×10^5 cells (3.5 pg $cell^{-1}$), resulting in a 91.7% reduction of bacterial binding. Preincubation of cells with heat inactivated PilC2 protein did not inhibit the adherence of piliated gonococci (data not shown). Opa_{57}-mediated binding (N313) was not affected by preincubation with PilC2 protein. Thus, the inhibition of gonococcal adherence by PilC2 protein is pilus specific.

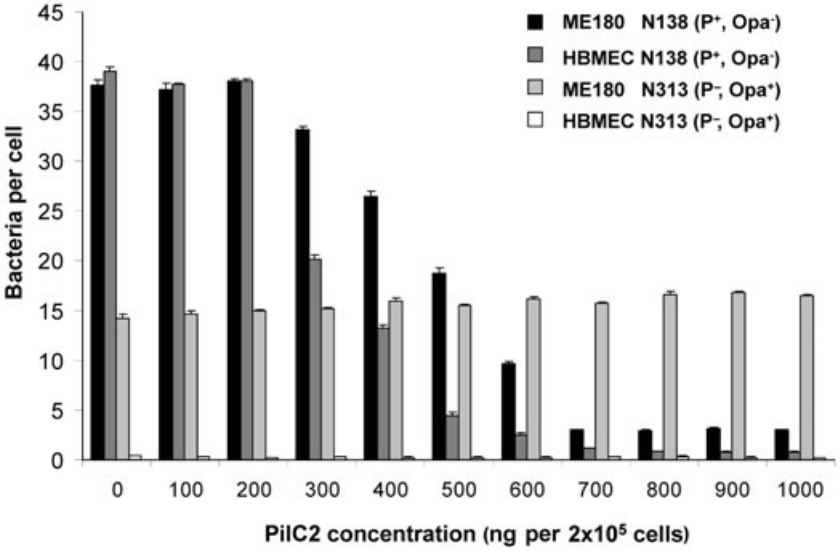

Fig. 1. Purified PilC2 protein inhibits the pilus-mediated binding of *Neisseria gonorrhoeae* to epithelial and endothelial cells in dose-dependent manner. ME180 and HBMEC cells were incubated with indicated concentrations of purified PilC2 protein for 30 min, washed and infected with the piliated gonococcal strain N138 or the non-piliated Opa_{57}-expressing strain N313. Cell-associated colony forming units were quantified by saponin lyses. The assay was performed in duplicates and repeated three times. N138 binding to ME180 and HBMEC cells is inhibited by PilC2 protein in a dose-dependent manner. N313 binding is not affected by PilC2 preincubation. The bars show the mean number of bacteria associated per cell ± standard deviation.

PilC2 protein and piliated gonococci exhibit similar binding specificities

In order to confirm the function of PilC as the major pilus adhesin, we compared the binding specificity of purified PilC2 protein with piliated gonococci. Human cell lines of different origin and one non-human epithelial cell line, the hamster cell line CHO, were used for experiments. Cell monolayers were infected with the piliated strain N138 and cell-associated bacteria were quantified. Piliated bacteria (N138) adhered to the human cell lines ME180, HeLa, Chang and HBMEC, but not to the non-human cell line CHO (Fig. 2A). On Chang cells a two-time less efficient binding was observed compared with the other human epithelial (ME180, HeLa) and endothelial (HBMEC) cells.

In parallel, the capacity of these cells to bind PilC protein was determined by PilC specific staining and flow cytometric analyses. The quantification of PilC2 binding in Fig. 2B shows, that purified PilC2 protein bound to ME180, HeLa and HBMEC cells approximately two times more efficiently than to Chang cells. No binding was observed with the non-human cell line CHO, as well as with human lung fibroblasts (HL) and T cells (Jurkat). Taken together, these results clearly show, that piliated gonococci and purified PilC protein exhibit similar binding specificities to human epithelial and endothelial cells, probably by interacting with the same host cell receptor.

PilC2 binds exclusively to cells of human epithelial origin

As seen in Fig. 2B, PilC2 protein efficiently bound to ME180, HeLa and HBMEC cells, and twofold less to Chang cells. No cell-bound PilC could be detected on HL (human lung fibroblasts), Jurkat (human T-cell line) and CHO (non-human) cells. These data suggest that the potential receptor for PilC is exclusively expressed on the

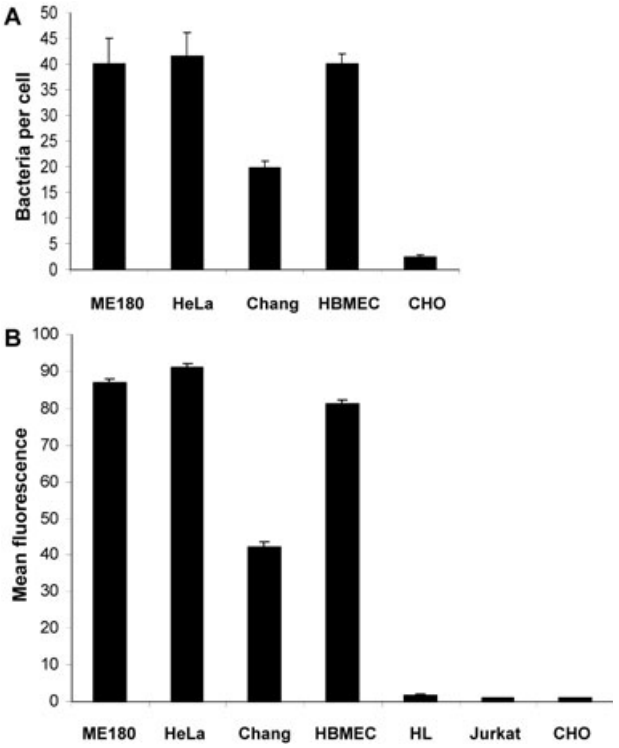

Fig. 2. PilC2 protein and piliated gonococci exhibit similar binding specificities.
A. cells were incubated with piliated gonococci (N138) for 60 min and cell-associated colony forming units were quantified by saponin lyses. Bacteria adhere to ME180, HeLa, Chang and HBMEC. No significant binding is observed on CHO cells. The bars show the mean number of bacteria associated per cell ± standard deviation.
B. cells were incubated with purified PilC2 protein and protein binding was quantified by flow cytometry. The bars show the mean fluorescence ± standard deviation. PilC2 protein binds to ME180, HeLa, Chang and HBMEC, but not to HL, Jurkat or CHO cells. The experiments were performed in duplicates and repeated three times.

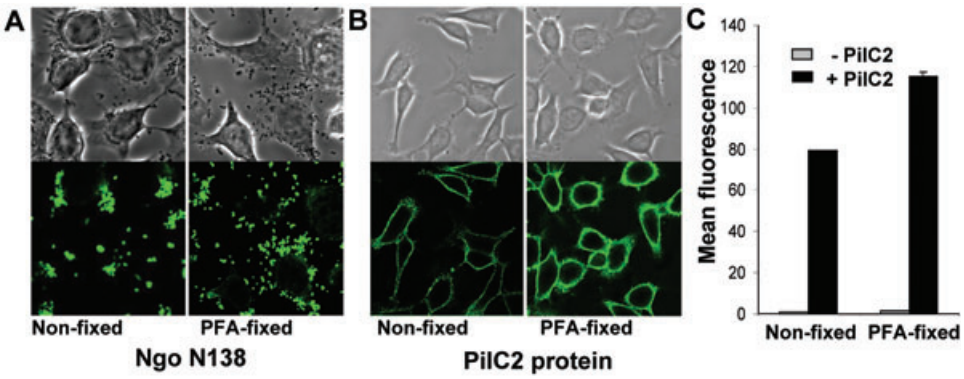

Fig. 3. Piliated gonococci and purified PilC2 protein bind to viable and PFA-fixed ME180 cells.
A. non-fixed and PFA-fixed ME180 cells were infected with the piliated strain N138. Cell-associated bacteria were visualized by immunofluorescence staining. Bacteria (green) bind to non-fixed and PFA-fixed cells. The bacterial aggregation on live cells is absent on fixed cells.
B and C. non-fixed and PFA-fixed ME180 cells were incubated with purified PilC2 protein for 1 h at 4°C.
B. Cell surface-bound PilC2 protein (green) was visualized by immunofluorescence staining.
C. Quantification of cell surface-bound PilC2 protein by flow cytometry. The bars show the mean fluorescence ± standard deviation. PilC2 protein binds to non-fixed and PFA-fixed cells.

cell surface of human epithelial and endothelial cells. In terms of epithelial origin, the cervix-derived cells (ME180, HeLa) seem to exhibit the highest expression level.

PilC2 protein and piliated gonococci bind to fixed ME180 cells

In order to determine whether cellular responses are necessary for efficient binding of piliated gonococci and PilC protein, cells were fixed with 3.7% PFA before incubation with bacteria or PilC2 protein. Microscopical analyses showed that piliated gonococci (N138) as well as purified PilC protein bound to fixed cells (Fig. 3A and B) in contrast to non-piliated Opa_{57}-expressing gonococci, which adhered to living, but not to fixed ME180 cells (data not shown). However, distribution of N138 strain was different on living compared with fixed cells. While on living cells piliated gonococci started to form microcolonies, these aggregates were not present on fixed cells (Fig. 3A). Additionally, more surface-bound PilC protein was detected on fixed cells compared with living cells (Fig. 3B), what could be confirmed by quantitative flow cytometric analyses (Fig. 3C). Thus, these data demonstrate that cellular host responses are not needed for efficient binding of piliated gonococci or purified PilC protein to the host cell.

The pilus receptor on epithelial cells is a protein and sugar components are not involved in this interaction

To assess the biochemical nature of the epithelial receptor for neisserial type IV pili, ME180 cells were pretreated with various chemical reagents and enzymes before adding piliated gonococci (N138) or purified PilC2 protein. FITC-conjugated cholera toxin B (CTB), concanavalin A (ConA) and *Arachis hypogaea* lectin (PNA) were included as controls, because of their known binding specificity (Goldstein *et al.*, 1965; Whitehouse *et al.*, 1997). Cell-associated bacteria and PilC2 protein were visualized by immunofluorescence staining and binding was determined semi-quantitatively by microscopical analyses. The results are summarized in Table 1. In addition, quantitative data obtained from flow cytometric analyses are represented in Fig. 4A. In order to evaluate, whether the pilus receptor is a protein, PFA-fixed ME180 cells were pretreated with trypsin and proteinase K to eliminate cell surface exposed protein structures. The binding of piliated gonococci and PilC2 protein was completely abolished. To determine the role of carbohydrate structures, cells were treated with specific inhibitors of glycosylation before infection, Tunicamycin, which blocks an early step in the N-glycosylation pathway (Elbein , 1983) and benzylGalNAc, a competitive inhibitor of the transferase (N-acetyl-alpha-D-galactosamyltransferase) involved in the first step of the biosyn-

Table 1. Biochemical characterization of the pilus receptor

	Binding	
Treatment	N138	PilC2
untreated	++	++
Trypsin	–	–
Proteinase K	–	–
Sodium periodate	++	++
Tunicamycin	++	+++
Benzyl Gal NAc	++	++
PNGase F	++	+++
Endo-O-Glycosidase	++	++
Sialidase	++	++

Binding of piliated gonococci (N138) and purified PilC2 protein was analysed semi-quantitatively by fluorescence microscopy.
–, no binding; ++, binding like control; +++, increased binding.

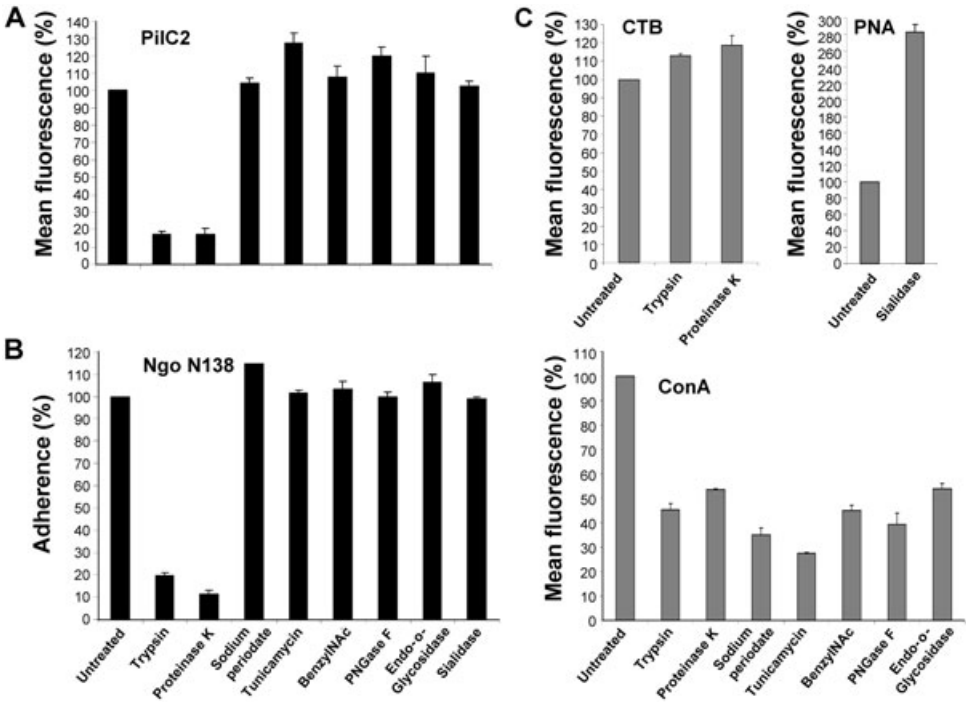

Fig. 4. The pilus receptor is a protein and sugar components are not involved in binding. ME180 cells were treated biochemically (see *Experimental procedures*) and used for binding studies.
A. cells were infected with piliated gonococci (N138) for 1 h and cell-associated colony forming units were quantified by saponin lyses. Shown is the percentage of adherent bacteria in relation to untreated control cells.
B. Quantification of cell surface-bound PilC2 protein on ME180 cells by flow cytometry. Shown is the percentage of mean fluorescence in relation to untreated control cells.
C. FITC conjugated cholera toxin B subunit (CTB), *Arachis hypogaea* lectin (PNA) and concanavalin A (ConA) were included as controls for biochemical treatment. Binding was quantified by flow cytometry and is shown as the percentage of mean fluorescence in relation control cells ± standard deviation.

thesis of most types of O-linked carbohydrates (Brockhausen *et al*., 1992). Furthermore, surface exposed glycostructures were cleaved with PNGase F, Endo-o-Glycosidase or Sialidase, or modified with sodium periodate. None of the sugar structure disrupting or modifying treatments had an effect on pilus-mediated gonococcal or PilC2 binding. Successful drug treatment was confirmed by the impaired binding of CTB, ConA and PNA. Taken together, these data argue that the putative epithelial host cell receptor for gonococcal pili and the pilus adhesin PilC2 is a surface protein.

PilC2 protein is endocytosed via receptor-mediated endocytosis

PilC2 protein was shown to bind to epithelial cell surface. Binding studies were generally performed at 4°C. In order to investigate host cell binding of PilC2 in more detail, assays was performed at different temperatures. ME180 cells were incubated with purified PilC2 protein at 4°C followed by shifting the temperature to 37°C for various time points. Figure 5A and C show, that PilC2 bound to the cells at 4°C. Interestingly, after 30 min at 37°C PilC2 protein was not detectable on the cell surface. This observation was not made with cells incubated at 4°C (Fig. 5A and C) or 16°C (data not shown). To determine whether adherent PilC2 protein is internalized by the host cell, intracellular staining of PilC2 was included. After extracellular staining, cells were permeabelized and staining was repeated using a differently labelled secondary antibody. The time course experiment (Fig. 5B) shows that PilC2 was detectable inside the cell after 15 min of incubation at 37°C. PilC2 was almost exclusively localized within the cell after 30 min. However, when cells were preincubated with monodansylcadaverine (MDC), an inhibitor of the transglutaminase enzyme essential for receptor-mediated endocytosis (Van Leuven *et al*., 1980), the internalization of PilC2 protein was blocked (Fig. 5C). Altogether, these observations indicate that cell-bound PilC2 protein is internalized, most likely via receptor-mediated endocytosis.

Internalization of PilC depletes the PilC receptor from the host cell surface

The observed internalization of PilC2 raised the question, whether the protein is endocytosed together with its recep-

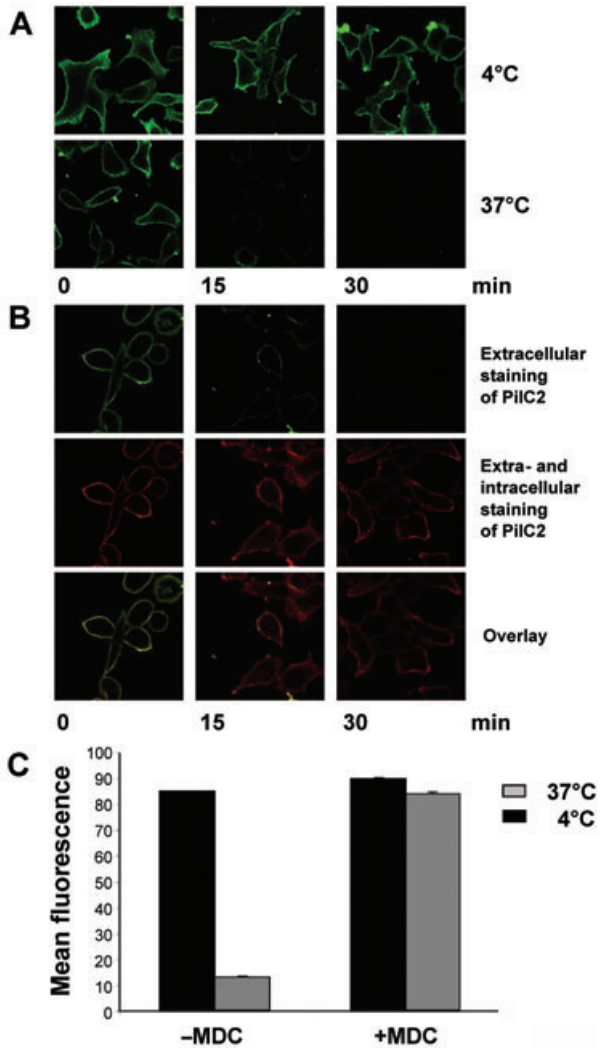

Fig. 5. PilC2 protein is internalized by ME180 cells via receptor-mediated endocytosis. Cell-associated PilC2 protein was visualized by immunofluorescence staining (A and B) of non-permeabilized cells (green) and permeabilized cells (red).
A. ME180 cells were incubated with PilC2 protein for 1 h at 4°C followed by incubation at 4°C or 37°C for indicated time points. PilC2 protein (green) binds to the cell surface at 4°C. PilC2 protein is not detectable on the cell surface after 30 min at 37°C.
B. PilC2 protein was added to ME180 cells and incubated for 1 h at 4°C followed by 37°C for indicated time points. PilC2 protein is internalized within 30 min at 37°C. In the overlay picture surface-bound PilC2 protein appears in yellow and intracellular PilC2 protein appears in red.
C. ME180 cells were treated or not with monodansylcadaverine (MDC), incubated with PilC2 protein for 1 h at 4°C followed by 30 min at 4°C or 37°C. Cell surface-bound PilC2 protein was quantified by flow cytometry. The bars show the mean fluorescence ± standard deviation.

tor. In order to answer that question, we analysed the capacity of ME180, which were already challenged with PilC2, to bind PilC2 protein anew. ME180 cells were incubated with or without PilC2 protein for 1 h at 4°C. The cells temperature was shifted to 37°C for 30 min to permit the internalization of PilC2. Both samples were than incubated again with PilC2 protein for 1 h at 4°C. Cell-bound PilC2 was visualized by immunofluorescence staining. Cells that were not challenged with PilC2 during the first incubation period, bound PilC2. In contrast, no cell-bound PilC2 protein was detected on cells, which bound and internalized PilC2 protein before (Fig. 6A). From this experiment we assume that the PilC2 receptor disappears from the cell surface upon PilC2 binding and internalization. It is likely that PilC2 protein is endocytosed together with its receptor.

PilC competitively inhibits binding of piliated gonococci

PilC2 protein was shown to inhibit the pilus-mediated gonococcal adherence to epithelial host cells (Scheuerp-

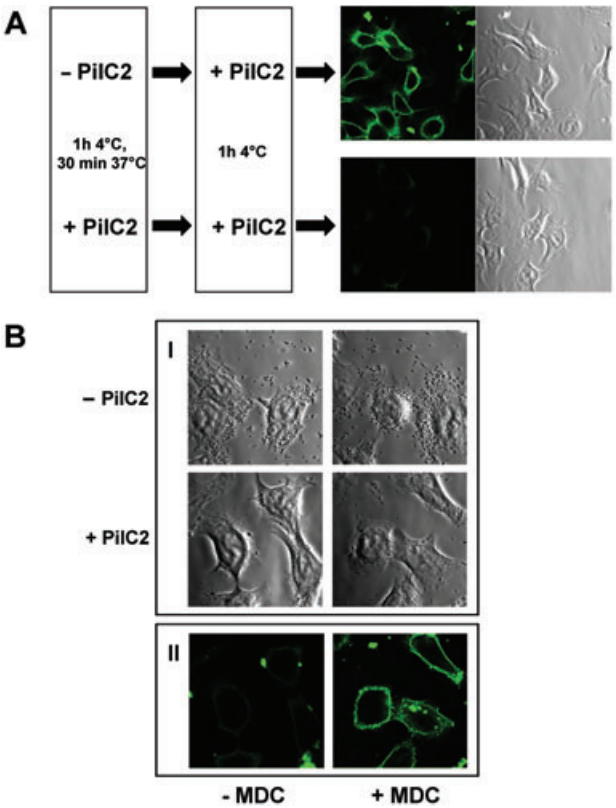

Fig. 6. The pilus receptor is internalized upon endocytosis of PilC2.
A. ME180 cells were treated with purified PilC2 protein as indicated. PilC2 protein (green) binds to cells not pulsed with PilC2 protein before (upper panels). No PilC2 protein binding is seen on cells previously treated with PilC2 protein (bottom panels).
B. gonococcal adherence is inhibited by surface-associated PilC2 protein or upon PilC2 protein internalization. ME180 cells were treated or not with Monodansylcadaverine (MDC) and incubated with or without PilC2 protein for 1 h at 4°C followed by 30 min at 37°C. After fixation cells were infected with the piliated gonococcal strain N138. Piliated gonococci adhere to cells, which were not preincubated with PilC2 protein (phase contrast, upper panels). Bacterial binding is inhibited, if cells were pulsed with PilC2 protein before. Inhibition is observed either with or without MDC treatment (I). The surface distribution of PilC2 protein on MDC treated or untreated cells is shown in II.

flug *et al*., 1999). In the context of the above results, we analysed by which mechanism the inhibition may occur, by competing for receptor binding sites or just by removing the receptor from the cell surface. ME180 cells were incubated with or without PilC2 protein at 37°C, followed by infection with piliated gonococci (N138). In order to prevent internalization of PilC2 protein, cells were treated with MDC before PilC2 loading. Cell-associated bacteria and PilC protein were visualized by immunofluorescence staining. Bottom panels in Fig. 6B show that PilC2 protein inhibited gonococcal adherence irrespective of whether PilC remained on the cell surface (MDC treatment) or was internalized (no MDC treatment). Because piliated gonococci bound to fixed ME180 cells that were not pretreated with PilC2 protein before infection MDC treatment itself did not affect bacterial binding (upper panels). Altogether we conclude that PilC2 protein inhibits pilus-mediated adherence of gonococci competitively by occupying the receptor-binding site.

The putative pilus receptor is recycled to the cell surface

Because we could show that the PilC receptor is endocytosed together with the adhesin PilC2, we investigated the time course of PilC receptor recycling. ME180 cells were incubated with or without PilC2 protein at 37°C for various time periods prior the infection with piliated gonococci. In order to prevent PilC2 induced receptor internalization, cells were treated with MDC before PilC2 loading. Subsequent infection with piliated gonococci (N138) was stopped after one hour and cell-associated bacteria were quantified. Figure 7 shows that PilC2 preincubation for up to 90 min inhibited gonococcal binding. When PilC2 was incubated for 120 min and longer prior the infection, gonococci were found to adhere to cells, indicating that the pilus receptor was re-exposed on the cell surface. Gonococcal binding to MDC treated cells was inhibited over the whole time period tested (180 min). MDC by itself did not affect binding of bacteria (Fig. 7).

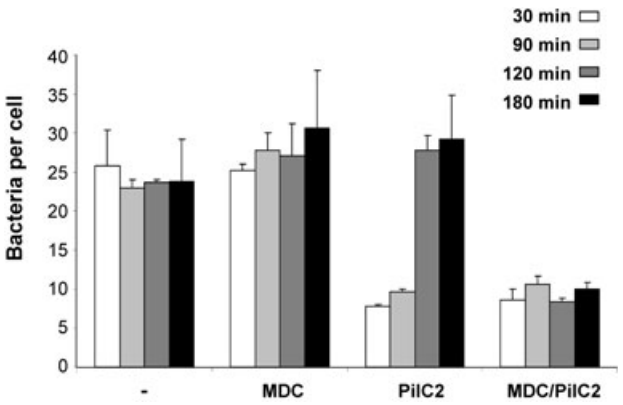

Fig. 7. Upon internalization the pilus receptor is recycled within 2 h. ME180 cells, grown in 24-well plates, were treated with or without MDC and incubated with or without 1 μg PilC2 protein for 30, 90, 120 or 180 min at 37°C. The piliated gonococcal strain N138 was added for 1 h and cell-associated colony forming units were quantified by saponin lyses. The assay was performed in triplicates and repeated three times. The bars show the mean number of bacteria associated per cell ± standard deviation. Ninety minutes after initial PilC2 protein pulsing, gonococcal adherence is still inhibited. One hundred and twenty minutes after PilC2 protein challenge, gonococcal binding is observed again. On MDC treated cells bacterial binding is inhibited by PilC2 protein over the whole period tested.

Discussion

The type IV pilus-mediated adherence of pathogenic *Neisseria* species to epithelial cell surfaces is considered a critical first event during the course of an infection. The PilC proteins, initially identified in the gonococcus as pilus assembly factors (Jonsson *et al*., 1991), were shown to function as tip located adhesins and to establish the initial host cell contact (Rudel *et al*., 1995b; Scheuerpflug *et al*., 1999). Experiments with neisserial strains expressing distinct mutant forms of PilC, confirmed its adhesive function and suggested that the adhesive properties of the protein are defined by particular amino acid sequences in the N-terminal part (Morand *et al*., 2001).

Thus far, only a few investigations have been reported with the purified adhesin. We have previously succeeded in isolating a full length His-tagged pilus adhesin from pilin depleted gonococci via induced expression and affinity purification (Scheuerpflug *et al*., 1999). The purified protein maintained its native properties, thus allowing precise studies on its adhesive function independent of other pilus or bacterial components.

In this study, we analysed the adhesive function and host specificity of the purified PilC2 protein in relation to the binding properties of piliated gonococci. We could show that PilC2 protein specifically inhibited the pilus-mediated gonococcal adherence to epithelial cells in a dose-dependent and saturable manner, confirming its function as the type IV pilus adhesin of *Neisseria*. PilC2 protein, like piliated gonococci, selectively binds to different cell lines of human origin *in vitro*, with both PilC2 and piliated gonococci showing similar binding patterns. This observation correlates with the strict host specificity of neisserial infections and characteristic cell and tissue tropisms, as it binds efficiently to human epithelial and endothelial cells (Scheuerpflug *et al*., 1999), whereas no binding was detected on human T cells or fibroblasts. This selective binding of PilC2 protein emphasizes its particular role in the interaction of *Neisseria* with epithelial and endothelial cells.

Pilus-mediated attachment of *Neisseria* to epithelial cells is affected by PilE subunit variation. The mechanism by which PilE variation can influence the binding properties of neisserial pili is presently not understood. PilE

variants, because of their amino acid sequence and distinct forms of glycosylation, may influence the pilus-mediated adherence of gonococci to epithelial cells indirectly, i.e. by affecting the topology and display of PilC proteins at the pilus tip. The existence of additional pilus components like the minor pilins makes a full understanding of the complex interactions even more complicated. In contrast, the assay system we used here is independent of PilE and its variation. Purified PilC2 protein was shown to be a representative tool for studying host cell interactions as it maintained the similar binding properties to epithelial cells as the gonococcal type IV pili themselves.

The ultimate function of bacterial adhesins is to target specific host cell receptors. The nature of host cell factors involved in pilus-mediated neisserial adhesion has been studied intensively. Kallstrom and colleagues have report CD46 as the receptor for neisserial type IV pili (Kallstrom *et al*., 1997). However, studies from our laboratory indicate that piliated gonococci and purified PilC2 protein both exhibit a pronounced lack of interaction with the suggested pilus receptor CD46 (manuscript in preparation). Therefore, we used biochemical approaches to characterize the nature of epithelial cell factors that interact with neisserial type IV pili and PilC2.

According to our results obtained with purified PilC2 protein or piliated gonococci, the potential host cell receptor for pili/PilC is a protein and sugar components do not seem to be involved in the interaction. These data are consistent with observations from other groups who found that a eukaryotic protein or glycoprotein could be a potential pilus receptor, whereas sugar structures are not involved in binding (Watt and Ward, 1977; Trust *et al*., 1983; Jonsson *et al*., 1994). In contrast, Buchanan and colleagues could inhibit the adherence of piliated gonococci by host cell treatment with various gangliosides or exoglycosidases (Buchanan *et al*., 1978). As type IV pili represent complex structures, capable of expressing additional distinguishable binding specificities (Rudel *et al*., 1992; Scheuerpflug *et al*., 1999; Park *et al*., 2002), contradictory observations may result from differences in the respective pilus compositions and in the use of target cells. The above-mentioned homogeneity of purified PilC2 protein, however, reveals the binding properties of the adhesin itself and reflects the binding of piliated *Neisseria* to human epithelial and endothelial cells.

Intriguingly, we observed the internalization of surface-bound PilC2 protein by epithelial cells. The rapid internalization of PilC2 within a time range of 15–30 min and prevention of PilC2 uptake by MDC, an inhibitor of receptor-mediated endocytosis, and low temperatures suggests that PilC2 is internalized by receptor-mediated endocytosis (Folk and Chung, 1973; Davies *et al*., 1980; Mazella *et al*., 1991; Vanisberg *et al*., 1991; Chabry *et al*., 1993). Receptor-mediated endocytosis is characterized by the formation of specific ligand-receptor complexes within clathrin-coated pits, which are subsequently internalized leading to depletion of the receptor from the plasma membrane. After pH-induced ligand release from the early endosomes, the receptor may be recycled to the plasma membrane surface (Schwartz, 1995; Mukherjee *et al*., 1997). We could show that PilC2 protein internalization resulted in a complete depletion of its receptor from the cell surface. After some time the cells regained the capacity to bind PilC2 protein. These observations suggested that the PilC receptor is endocytosed upon ligand binding (PilC2 protein) and involved in recycling pathways. Endocytosed receptors can be recycled by different pathways, using the endocytic recycling compartments or the Golgi complex (Schwartz, 1991; Man *et al*., 2000). Future studies using specific vesicle markers should help to determine the exact intracellular route of the PilC receptor.

Receptor-mediated endocytosis is a common feature in bacterial pathogenesis. A number of bacteria, like *Chlamydia spp.* (Soderlund and Kihlstrom, 1983; Wyrick *et al*., 1989), *Campylobacter jejuni*, *Citrobacter freudii* (Oelschlaeger *et al*., 1993) and *Staphylococcus aureus* (Ellington *et al*., 1999) are considered to enter their host cell via this uptake mechanism. Several bacterial toxins were also shown to reach their intracellular target through receptor-mediated endocytosis (FitzGerald, 1996; Lahiri, 2000). Harvey and colleagues could show that *N. gonorrhoeae* uses receptor-mediated endocytosis for the internalization into host cells, whereby the gonococcal lipooligosaccharide (LOS) and the human asialoglycoprotein receptor (ASGP-R) seemed to be the factors responsible for the uptake (Harvey *et al*., 2001). ASGP-R was shown to not be constitutively present on the cell surface, but rather after gonococcal infection. In contrast, the putative pilus receptor, in our hands, appeared to be constitutively expressed on the cell surface, because efficient binding of gonococci was also observed to fixed cells. This renders an involvement of ASGP-R in the receptor-mediated endocytosis of PilC2 protein unlikely.

A potential role of receptor-mediated endocytosis of PilC2 in gonococcal pathogenesis is not yet evident. One might speculate that, upon retraction of the pili, PilC might mediate internalization that could guide adherent bacteria into the cell. Also, PilC2 has not only been found in association with pili but also with the outer membrane. A function of PilC2 as an invasin is possible, as previously described for the type 1 pilus adhesin FimH from *E. coli* (Martinez *et al*., 2000). Yet, the role of type IV pili in neisserial invasion is controversial. Pili were shown to inhibit Opa protein-dependent invasion in some cell types but enhance the invasion in others (Makino *et al*., 1991; Virji *et al*., 1995; Merz *et al*., 1996; Pujol *et al*., 1997). In our model system gonococcal pili do not support invasion (data not shown). In other cell models a role for PilC2 in

gonococcal invasion cannot be excluded. The future identification of the PilC receptor, as well as an in-depth characterization of the cellular response mechanisms induced by the binding of piliated bacteria, may help to shed light on the early events of the neisserial infection.

Taken together, our results show that the potential pilus receptor is of proteinous nature and highly expressed on cervix carcinoma-derived epithelial cells. It is likely that pili bind directly to protein structures, because sugar-disrupting treatments did not affect binding. Moreover, the potential receptor is constitutively present on the host cell surface. Differences in gonococcal distribution upon infection of living or fixed cells suggest an involvement of cellular responses in latter steps of infection. Indeed, several findings demonstrate that the initial pilus binding causes host cell responses such as Ca^{2+} fluxes and cortical plaque formation (Kallstrom *et al*., 1998; Merz *et al*., 1999; Ayala *et al*., 2001). The observed receptor-mediated endocytosis further supports the existence of a specific pilus receptor on human epithelial cells and provides additional information on its nature. The purified PilC2 protein should be a valuable tool for revealing the nature of the type IV pilus neisserial receptor.

Experimental procedures

Bacterial strains

The gonococcal strains used in the present work are derived from N. gonorrhoeae MS11. N138, a piliated strain exhibiting the transparent phenotype (P+, Opa–), as well as the non-piliated Opa_{57}-expressing strain N313 (P–, Opa+) have been previously described (Kupsch *et al*., 1993; Scheuerpflug *et al*., 1999). Strain N560 was used for PilC2 protein overexpression and purification (Scheuerpflug *et al*., 1999). Strains were grown on GC agar base with vitamin supplement and proteose peptone at 37°C and 5% CO_2. Piliation and Opa phenotypes of gonococci were monitored by colony morphology and Western blot.

Cell lines and growth conditions

The cell lines used in the experiments were ME180 human cervix carcinoma (ATCC HTB33), Chang human conjunctiva (ATCC CCL20.2), HeLa human cervix carcinoma (ATCC CCL2), HL human lung fibroblasts (ECACC 96121720), Jurkat human T-cell lymphoma (ATCC TIB-152), CHO-K1 hamster ovary cell line (CHO, ATCC CCL-61). The ME180 cells were maintained in McCoy's 5A medium supplemented with L-glutamine and 10% FCS. Chang, HeLa, HL and Jurkat cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% FCS. CHO-K1 were maintained in Ham's 12 medium with L-glutamine and 10% FCS. Human brain microvascular endothelial cells (HBMEC) were cultured in DMEM medium with 4500 mg l^{-1} glucose, L-glutamine and supplement (MVGS, Cascade Biologics) on gelatine-coated plates.

PilC2 purification

The PilC2 protein was purified as previously described (Scheuerpflug *et al*., 1999). Briefly, the gonococcal strain N560 ($PilC2_{His6}$) was grown overnight on GC agar plates containing tetracycline (10 μg ml^{-1}), IPTG (100 μg ml^{-1}) and 10 mM $MgCl_2$ at 37°C in 5% CO_2. Bacteria were collected in buffer containing 50 mM Tris-HCl (pH 8.0) and 150 mM NaCl, and lysed by sonification. After centrifugation at 4000 r.p.m. for 20 min at 4°C, the supernatant (membrane fraction) was separated and centrifuged for 1 h at 20 000 r.p.m. at 4°C. The PilC2 protein containing membrane pellet was dissolved by incubation in 2% lauryldimethylamine N-oxide (LDAO) in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 500 mM NaCl for 1 h at 37°C. After centrifugation at 20 000 r.p.m. for 1 h at 4°C, the supernatant was loaded onto a nickel-nitrilotriacetic acid-agarose (Ni-NTA) column equilibrated with 2% LDAO in 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 500 mM NaCl. After washing with 50 mM imidazole in PBS and 10% glycerine in PBS, the PilC2 protein was eluted by a shift of the pH from 8.0 to 4.0 with 10 mM sodium citrate buffer containing 150 mM NaCl. The pH was neutralized by adding sodium phosphate buffer to the eluate.

Adherence and adherence inhibition experiments

Cells were grown in 24-well cell culture plates to 70–80% confluency. Monolayers were washed three times with medium and bacteria were added to the cells with a moi of 100. Infection was performed in medium without FCS. Infected monolayers were centrifuged for 3 min at 120 *g* to synchronize infection and incubated for 1 h at 37°C in 5% CO_2. For inhibition experiments, cells were preincubated with various concentrations of purified PilC2 protein at 37°C in FCS free medium and washed twice before infection. To stop the infection and to remove non-adherent bacteria, cells were washed three times with medium. Cell-associated bacteria were quantified by lysing the monolayer with 1% saponin in medium for 7–10 min and suspending gonococci by vigorous pipetting. Colony forming units were determined by plating of serial dilutions. For microscopic analyses, cells were grown on glass cover slips. PilC2 protein was added as indicated, samples were washed and incubated with bacteria for indicated time points. After removing non-adherent bacteria, cells were fixed with 3.7% PFA and proceeded for immunofluorescence staining.

PilC2 protein binding

For flow cytometric analyses, suspended cells were washed with cold medium. PilC2 protein was added (3 μg protein/ 1×10^6 cells) and incubated for 1 h at 4°C. Cells were washed three times with PBS, and suspended in PBS containing 3% FCS.

For immunofluorescence studies, semi-confluent cell monolayers, grown on glass coverslips in a 24-well plate, were incubated with 1 μg purified PilC2 protein for 1 h at 4°C, washed three times, proceeded as indicated and fixed with 3.7% PFA. For inhibition studies cell monolayers were pretreated with 150 μM MDC (Sigma) for 3 h at 37°C before incubation with PilC2 protein.

Experiments with fixed cells

Pre-confluent ME180 cells, grown on glass cover slips, were washed with PBS and treated with 3.7% PFA for 15 min at RT. Cells were washed with PBS and infected with bacteria or incubated with PilC protein as described above.

Biochemical treatment of cells

ME180 cells were grown to 70–80% confluency and treated as described below. For microscopical analyses, samples were grown on glass coverslips before treatment.

Protease treatment. ME180 cell were fixed with 3.7% PFA for 10 min, washed with PBS and incubated with 40 μg ml^{-1} trypsin or 6 μg ml^{-1} proteinase K for 30 min. Cells were washed three times with PBS and blocked in medium with 7% FCS.

Inhibition of n- and o-glycosylation. Cell monolayers were incubated with 2 μg ml^{-1} tunicamycin (Boehringer) for 24 h or with 2 mM benzyl-N-acetyl-α-D-galactosamide (benzyl-GalNAc, Sigma) for three days in growth medium with 10% FCS. After treatment with the respective drug, cells were washed with PBS and used in binding experiments.

Cleavage of carbohydrate residues. PNGase F (peptide-N-glycosidase F), Endo-O-glycosidase (O-glycopeptide-endo-D-galactosyl-N-acetyl-α-galactosaminohydrolase) and Sialidase A were obtained from Prozyme, USA (GLYCOPRO™ Deglycosylation Kit). 0.5 μl of each enzyme was added to the cells and incubated for 24 h. Before infection and protein binding studies, cells were washed three times with medium.

Sodium periodate treatment. Cells were fixed with 3.7% PFA and incubated with 30 mM sodium periodate in 50 mM sodium acetate buffer (pH 4.5) for 1 h at room temperature in the dark. To convert the aldehyde groups generated by periodate oxidation to alcohols, the samples were incubated with 50 mM sodium borohydride for 30 min. After washing with PBS and blocking with 7% FCS in medium, cells were used for experiments.

Immunofluorescence microscopy

Cell-associated gonococci were detected by using the rabbit antiserum AK213 (raised against gonococcal lysate) and goat anti-rabbit-Cy2. For PilC2 detection a mouse anti-PilC serum (raised against purified native PilC2 protein) was used. PilC2 protein was visualized with goat anti-mouse-Cy2 (extracellular staining) and goat anti-mouse-Cy3 (intracellular staining). All secondary antibodies were purchased from Jackson Immunoresearch (Baltimore, MD). Cells were grown on glass coverslips and fixed with 3.7% PFA for 15 min at room temperature, washed with PBS and blocked for 30 min with 7% FCS in PBS. Coverslips were incubated with the first antibodies for 1 h, washed in PBS and then incubated with secondary antibody for an additional hour. For intracellular staining, cells were permeabelized with 0.2% Triton in PBS/3% FCS for 30 min before repeating antibody incubations. After washing, slides were mounted with Mowiol (Merck, Darmstadt, Germany), dried and analysed by confocal laser scanning microscopy (Leica TCS NT).

Flow cytometry

For quantitative binding studies cells were incubated with purified PilC2 protein (1 μg ml^{-1}), FITC conjugated CTB subunit (8 μg ml^{-1}, Sigma), FITC conjugated ConA (10 μg ml^{-1}, Sigma) or FITC conjugated *A. hypogaea* lectin (PNA; 8 μg ml^{-1}, Sigma) for 1 h at 4°C and washed three times with PBS. Cell surface-bound PilC2 protein was stained using a PilC mouse antiserum (raised against purified PilC protein). A total of 1×10^6 cells suspended in 100 μl of PBS containing 3% FCS were incubated with 2 μl of PilC antiserum for 1 h at 4°C. Cells were washed three times with PBS/3% FCS and then incubated for an additional hour at 4°C in 100 μl of a 1:100 dilution of Cy2-conjugated goat anti-mouse antibody (Jackson Immunoresearch, Baltimore, MD). Cells were washed three times and suspended in 300 μl PBS. One microlitre of propidium iodide was added to stain dead cells.

The samples were analysed on a FACScalibur (Becton Dickinson) with Cell Quest™ software.

Acknowledgements

We gratefully acknowledge our stimulating discussions with Drs Thomas Rudel and Yuri Churin. This work was supported by the DFG priority program SPP1130 Grant No. Me705/7–1.

References

Ayala, B.P., Vasquez, B., Clary, S., Tainer, J.A., Rodland, K., and So, M. (2001) The pilus-induced Ca2+ flux triggers lysosome exocytosis and increases the amount of Lamp1 accessible to Neisseria IgA1 protease. *Cell Microbiol* **3:** 265–275.

Brockhausen, I., Moller, G., Pollex-Kruger, A., Rutz, V., Paulsen, H., Matta, K.L. (1992) Control of O-glycan synthesis: specificity and inhibition of O-glycan core 1 UDP-galactose: N-acetylgalactosamine-alpha-R-beta-3-galactosyltransferase from rat liver. *Biochem Cell* **70:** 99–108.

Buchanan, T.M., Pearce, W.A., Chen, K.C.S. (1978) Attachment of *Neisseria gonorrhoeae* pili to human cells and investigations of the chemical nature of the receptor for gonococcal pili. In: *Immunbiology of Neisseria gonorrhoeae.* Brooks, G.F., Gotschlich, E.C., Holmes, K.K., Sawyer, W.D., and Young, F.E. (eds). Washington, DC: American Society for Microbiology, pp. 242–249.

Chabry, J., Gaudriault, G., Vincent, J.P., and Mazella, J. (1993) Implication of various forms of neurotensin receptors in the mechanism of internalization of neurotensin in cerebral neurons. *J Biol Chem* **268:** 17138–17144.

Davies, P.J., Davies, D.R., Levitzki, A., Maxfield, F.R., Milhaud, P., Willingham, M.C., and Pastan, I.H. (1980) Transglutaminase is essential in receptor-mediated endocytosis of alpha 2-macroglobulin and polypeptide hormones. *Nature* **283:** 162–167.

Drake, S.L., and Koomey, M. (1995) The product of the pilQ gene is essential for the biogenesis of type IV pili in *Neisseria gonorrhoeae*. *Mol Microbiol* **18:** 975–986.

Elbein, A. (1983) Inhibitors of glycoprotien synthesis. *Methods Enzymol* **98:** 135–155.

Ellington, J.K., Reilly, S.S., Ramp, W.K., Smeltzer, M.S., Kellam, J.F., and Hudson, M.C. (1999) Mechanisms of *Staphylococcus aureus* invasion of cultured osteoblasts. *Microb Pathog* **26:** 317–323.

FitzGerald, D. (1996) Why toxins!. *Semin Cancer Biol* **7:** 87–95.

Folk, J.E., and Chung, S.I. (1973) Molecular and catalytic properties of transglutaminases. *Adv Enzymol Relat Areas Mol Biol* **38:** 109–191.

Forest, K.T., Dunham, S.A., Koomey, M., and Tainer, J.A. (1999) Crystallographic structure reveals phosphorylated pilin from *Neisseria*: phosphoserine sites modify type IV pilus surface chemistry and fibre morphology. *Mol Microbiol* **31:** 743–752.

Freitag, N.E., Seifert, H.S., and Koomey, M. (1995) Characterization of the pilF-pilD pilus-assembly locus of *Neisseria gonorrhoeae*. *Mol Microbiol* **16:** 575–586.

Fussenegger, M., Rudel, T., Barten, R., Ryll, R., and Meyer, T.F. (1997) Transformation competence and type-4 pilus biogenesis in *Neisseria gonorrhoeae* – a review. *Gene* **192:** 125–134.

Goldstein, I.J., Hollerman, C.E., and Smith, E.E. (1965) Protein–carbohydrate interaction. II. Inhibition studies on the interaction of concanavalin A with polysaccharides. *Biochemistry* **41:** 876–883.

Handsfield, H.H. (1990) Old enemies. Combating syphilis and gonorrhea in the 1990s. *JAMA* **264:** 1451–1452.

Harvey, H.A., Jennings, M.P., Campbell, C.A., Williams, R., and Apicella, M.A. (2001) Receptor-mediated endocytosis of *Neisseria gonorrhoeae* into primary human urethral epithelial cells: the role of the asialoglycoprotein receptor. *Mol Microbiol* **42:** 659–672.

Heckels, J.E. (1989) Structure and function of pili of pathogenic *Neisseria* species. *Clin Microbiol Rev* **2**(Suppl.): S66–S73.

Hegge, F.T., Hitchen, P.G., Aas, F.E., Kristiansen, H., Lovold, C., Egge-Jacobsen, W., *et al.* (2004) Unique modifications with phosphocholine and phosphoethanolamine define alternate antigenic forms of *Neisseria gonorrhoeae* type IV pili. *Proc Natl Acad Sci USA* **101:** 10798–10803.

Johansson, L., Rytkonen, A., Bergman, P., Albiger, B., Kallstrom, H., Hokfelt, T., *et al.* (2003) CD46 in meningococcal disease. *Science* **301:** 373–375.

Johnson, A.P., Taylor-Robinson, D., and McGee, Z.A. (1977) Species specificity of attachment and damage to oviduct mucosa by *Neisseria gonorrhoeae*. *Infect Immun* **18:** 833–839.

Jones, C.H., Pinkner, J.S., Roth, R., Heuser, J., Nicholes, A.V., Abraham, S.N., and Hultgren, S.J. (1995) FimH adhesin of type 1 pili is assembled into a fibrillar tip structure in the Enterobacteriaceae. *Proc Natl Acad Sci USA* **92:** 2081–2085.

Jonsson, A.B., Nyberg, G., and Normark, S. (1991) Phase variation of gonococcal pili by frameshift mutation in pilC, a novel gene for pilus assembly. *EMBO J* **10:** 477–488.

Jonsson, A.B., Ilver, D., Falk, P., Pepose, J., and Normark, S. (1994) Sequence changes in the pilus subunit lead to tropism variation of *Neisseria gonorrhoeae* to human tissue. *Mol Microbiol* **13:** 403–416.

Kallstrom, H., Liszewski, M.K., Atkinson, J.P., and Jonsson, A.B. (1997) Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic *Neisseria*. *Mol Microbiol* **25:** 639–647.

Kallstrom, H., Islam, M.S., Berggren, P.O., and Jonsson, A.B. (1998) Cell signaling by the type IV pili of pathogenic *Neisseria*. *J Biol Chem* **273:** 21777–21782.

Kellogg, D.S., Jr., Peacock, W.L., Jr., Deacon, W.E., Brown, L., and Pirkle, D.I. (1963) *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J Bacteriol* **85:** 1274–1279.

Kirchner, M., Hener, D., and Meyer, T.F. (2005) CD46 independent binding of neisserial type IV pili and the major pilus adhesin PilC to human epithelial cells. *Infect Immun* **73,** in press.

Kuehn, M.J., Jacob-Dubuisson, F., Dodson, K., Slonim, L., Striker, R., and Hultgren, S.J. (1994) Genetic, biochemical, and structural studies of biogenesis of adhesive pili in bacteria. *Methods Enzymol* **236:** 282–306.

Kupsch, E.M., Knepper, B., Kuroki, T., Heuer, I., and Meyer, T.F. (1993) Variable opacity (Opa) outer membrane proteins account for the cell tropisms displayed by *Neisseria gonorrhoeae* for human leukocytes and epithelial cells. *EMBO J* **12:** 641–650.

Lahiri, S.S. (2000) Bacterial toxins – an overview. *J Nat Toxins* **9:** 381–408.

Maisner, A., Liszewski, M.K., Atkinson, J.P., Schwartz-Albiez, R., and Herrler, G. (1996) Two different cytoplasmic tails direct isoforms of the membrane cofactor protein (CD46) to the basolateral surface of Madin-Darby canine kidney cells. *J Biol Chem* **271:** 18853–18858.

Makino, S., van Putten, J.P., and Meyer, T.F. (1991) Phase variation of the opacity outer membrane protein controls invasion by *Neisseria gonorrhoeae* into human epithelial cells. *EMBO J* **10:** 1307–1315.

Man, H.Y., Ju, W., Ahmadian, G., and Wang, Y.T. (2000) Intracellular trafficking of AMPA receptors in synaptic plasticity. *Cell Mol Life Sci* **57:** 1526–1534.

Martinez, J.J., Mulvey, M.A., Schilling, J.D., Pinkner, J.S., and Hultgren, S.J. (2000) Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. *EMBO J* **19:** 2803–2812.

Mazella, J., Leonard, K., Chabry, J., Kitabgi, P., Vincent, J.P., and Beaudet, A. (1991) Binding and internalization of iodinated neurotensin in neuronal cultures from embryonic mouse brain. *Brain Res* **564:** 249–255.

Merz, A.J., Enns, C.A., and So, M. (1999) Type IV pili of pathogenic Neisseriae elicit cortical plaque formation in epithelial cells. *Mol Microbiol* **32:** 1316–1332.

Merz, A.J., Rifenbery, D.B., Arvidson, C.G., and So, M. (1996) Traversal of a polarized epithelium by pathogenic Neisseriae: facilitation by type IV pili and maintenance of epithelial barrier function. *Mol Med* **2:** 745–754.

Meyer, T.F., Pohlner, J., and van Putten, J.P. (1994) Biology of the pathogenic Neisseriae. *Curr Top Microbiol Immunol* **192:** 283–317.

Morand, P.C., Tattevin, P., Eugene, E., Beretti, J.L., and

Nassif, X. (2001) The adhesive property of the type IV pilus-associated component PilC1 of pathogenic *Neisseria* is supported by the conformational structure of the N-terminal part of the molecule. *Mol Microbiol* **40:** 846–856.

Mukherjee, S., Ghosh, R.N., and Maxfield, F.R. (1997) Endocytosis. *Physiol Rev* **77:** 759–803.

Nassif, X., and So, M. (1995) Interaction of pathogenic neisseriae with nonphagocytic cells. *Clin Microbiol Rev* **8:** 376–388.

Nassif, X., Lowy, J., Stenberg, P., O'Gaora, P., Ganji, A., and So, M. (1993) Antigenic variation of pilin regulates adhesion of *Neisseria meningitidis* to human epithelial cells. *Mol Microbiol* **8:** 719–725.

Nassif, X., Beretti, J.L., Lowy, J., Stenberg, P., O'Gaora, P., Pfeifer, J., *et al.* (1994) Roles of pilin and PilC in adhesion of *Neisseria meningitidis* to human epithelial and endothelial cells. *Proc Natl Acad Sci USA* **91:** 3769–3773.

Oelschlaeger, T.A., Guerry, P., and Kopecko, D.J. (1993) Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. *Proc Natl Acad Sci USA* **90:** 6884–6888.

Parge, H.E., Bernstein, S.L., Deal, C.D., McRee, D.E., Christensen, D., Capozza, M.A., *et al.* (1990) Biochemical purification and crystallographic characterization of the fiber-forming protein pilin from *Neisseria gonorrhoeae*. *J Biol Chem* **265:** 2278–2285.

Parge, H.E., Forest, K.T., Hickey, M.J., Christensen, D.A., Getzoff, E.D., and Tainer, J.A. (1995) Structure of the fibre-forming protein pilin at 2.6 A resolution. *Nature* **378:** 32–38.

Park, H.S., Wolfgang, M., and Koomey, M. (2002) Modification of type IV pilus-associated epithelial cell adherence and multicellular behavior by the PilU protein of *Neisseria gonorrhoeae*. *Infect Immun* **70:** 3891–3903.

Pujol, C., Eugene, E., de Saint, Martin, L., and Nassif, X. (1997) Interaction of *Neisseria meningitidis* with a polarized monolayer of epithelial cells. *Infect Immun* **65:** 4836–4842.

Rahman, M., Kallstrom, H., Normark, S., and Jonsson, A.B. (1997) PilC of pathogenic *Neisseria* is associated with the bacterial cell surface. *Mol Microbiol* **25:** 11–25.

Rudel, T., van Putten, J.P., Gibbs, C.P., Haas, R., and Meyer, T.F. (1992) Interaction of two variable proteins (PilE and PilC) required for pilus-mediated adherence of *Neisseria gonorrhoeae* to human epithelial cells. *Mol Microbiol* **6:** 3439–3450.

Rudel, T., Facius, D., Barten, R., Scheuerpflug, I., Nonnenmacher, E., and Meyer, T.F. (1995a) Role of pili and the phase-variable PilC protein in natural competence for transformation of *Neisseria gonorrhoeae*. *Proc Natl Acad Sci USA* **92:** 7986–7990.

Rudel, T., Scheurerpflug, I., and Meyer, T.F. (1995b) Neisseria PilC protein identified as type-4 pilus tip-located adhesin. *Nature* **373:** 357–359.

Scheuerpflug, I., Rudel, T., Ryll, R., Pandit, J., and Meyer, T.F. (1999) Roles of PilC and PilE proteins in pilus-mediated adherence of *Neisseria gonorrhoeae* and *Neisseria meningitidis* to human erythrocytes and endothelial and epithelial cells. *Infect Immun* **67:** 834–843.

Schoolnik, G.K., Fernandez, R., Tai, J.Y., Rothbard, J., and Gotschlich, E.C. (1984) Gonococcal pili. Primary structure and receptor binding domain. *J Exp Med* **159:** 1351–1370.

Schwartz, A.L. (1991) Trafficking of asialoglycoproteins and the asialoglycoprotein receptor. *Targeted Diagn Ther* **4:** 3–39.

Schwartz, A.L. (1995) Receptor cell biology: receptor-mediated endocytosis. *Pediatr Res* **38:** 835–843.

Seifert, H.S. (1996) Questions about gonococcal pilus phase- and antigenic variation. *Mol Microbiol* **21:** 433–440.

Seifert, H.S., Wright, C.J., Jerse, A.E., Cohen, M.S., and Cannon, J.G. (1994) Multiple gonococcal pilin antigenic variants are produced during experimental human infections. *J Clin Invest* **93:** 2744–2749.

Soderlund, G., and Kihlstrom, E. (1983) Effect of methylamine and monodansylcadaverine on the susceptibility of McCoy cells to *Chlamydia trachomatis* infection. *Infect Immun* **40:** 534–541.

Soto, G.E., and Hultgren, S.J. (1999) Bacterial adhesins: common themes and variations in architecture and assembly. *J Bacteriol* **181:** 1059–1071.

Stimson, E., Virji, M., Barker, S., Panico, M., Blench, I., Saunders, J., *et al.* (1996) Discovery of a novel protein modification: alpha-glycerophosphate is a substituent of meningococcal pilin. *Biochem J* **316:** 29–33.

Swanson, J. (1973) Studies on gonococcus infection. IV. Pili: their role in attachment of gonococci to tissue culture cells. *J Exp Med* **137:** 571–589.

Teuchert, M., Maisner, A., and Herrler, G. (1999) Importance of the carboxyl-terminal FTSL motif of membrane cofactor protein for basolateral sorting and endocytosis. Positive and negative modulation by signals inside and outside the cytoplasmic tail. *J Biol Chem* **274:** 19979–19984.

Tobiason, D.M., and Seifert, H.S. (2001) Inverse relationship between pilus-mediated gonococcal adherence and surface expression of the pilus receptor, CD46. *Microbiology* **147:** 2333–2340.

Trust, T.J., Gillespie, R.M., Bhatti, A.R., and White, L.A. (1983) Differences in the adhesive properties of *Neisseria meningitidis* for human buccal epithelial cells and erythrocytes. *Infect Immun* **41:** 106–113.

Van Leuven, F., Cassiman, J.J., and Van Den, Berghe, H. (1980) Primary amines inhibit recycling of alpha 2M receptors in fibroblasts. *Cell* **20:** 37–43.

Vanisberg, M.A., Maloteaux, J.M., Octave, J.N., and Laduron, P.M. (1991) Rapid agonist-induced decrease of neurotensin receptors from the cell surface in rat cultured neurons. *Biochem Pharmacol* **42:** 2265–2274.

Virji, M., and Heckels, J.E. (1984) The role of common and type-specific pilus antigenic domains in adhesion and virulence of gonococci for human epithelial cells. *J Gen Microbiol* **130:** 1089–1095.

Virji, M., Makepeace, K., Ferguson, D.J., Achtman, M., Sarkari, J., and Moxon, E.R. (1992) Expression of the Opc protein correlates with invasion of epithelial and endothelial cells by *Neisseria meningitidis*. *Mol Microbiol* **6:** 2785–2795.

Virji, M., Makepeace, K., Ferguson, D.J., Achtman, M., and Moxon, E.R. (1993) Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells. *Mol Microbiol* **10:** 499–510.

Virji, M., Makepeace, K., Peak, I.R., Ferguson, D.J., Jennings, M.P., and Moxon, E.R. (1995) Opc- and pilus–

dependent interactions of meningococci with human endothelial cells: molecular mechanisms and modulation by surface polysaccharides. *Mol Microbiol* **18:** 741–754.

Watt, P.J., Ward, M.E. (1977) The interaction of gonococci with human epithelial cells. In: *The Gonococcus*. Roberts, R.B. (ed.). New York, NY: John Wiley and Sons, pp. 355–368.

Weiser, J.N., Goldberg, J.B., Pan, N., Wilson, L., and Virji, M. (1998) The phosphorylcholine epitope undergoes phase variation on a 43-kilodalton protein in Pseudomonas aeruginosa and on pili of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *Infect Immun* **66:** 4263–4267.

Whitehouse, C., Burchell, J., Gschmeissner, S., Brockhausen, I., Lloyd, K.O., and Taylor-Papadimitriou, J. (1997) A transfected sialyltransferase that is elevated in breast cancer and localizes to the medial/trans-Golgi apparatus inhibits the development of core-2-based O-glycans. *J Cell Biol* **137:** 1229–1241.

Winther-Larsen, H.C., Hegge, F.T., Wolfgang, M., Hayes, S.F., van Putten, J.P., and Koomey, M. (2001) *Neisseria gonorrhoeae* PilV, a type IV pilus-associated protein essential to human epithelial cell adherence. *Proc Natl Acad Sci USA* **98:** 15276–15281.

Wolfgang, M., van Putten, J.P., Hayes, S.F., Dorward, D., and Koomey, M. (2000) Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. *EMBO J* **19:** 6408–6418.

Wyrick, P.B., Choong, J., Davis, C.H., Knight, S.T., Royal, M.O., Maslow, A.S., and Bagnell, C.R. (1989) Entry of genital *Chlamydia trachomatis* into polarized human epithelial cells. *Infect Immun* **57:** 2378–2389.