Release of host-derived membrane vesicles following pilus-mediated adhesion of *Neisseria gonorrhoeae*

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**Summary**

Following attachment of *Neisseria gonorrhoeae* to human epithelial cell lines, the cellular pilus receptor CD46 is shed from the cell and accumulates in the media. In this report, we assess *Neisseria*-induced alterations in CD46 surface distribution and characterize this complement regulatory protein following its release from the infected cell. Within 3 h of attachment of gonococci to human epithelial cell lines, CD46 is enriched beneath sites of microcolony adhesion. By 6 h post infection, differential ultracentrifugation of culture media from ME-180 monolayers resulted in sedimentation of structurally and functionally intact CD46. Electron microscopy of these 100 000 g pellets revealed 30–200 nm vesicles. These vesicles likely originated from the host cell as they contained additional host cell surface proteins including CD55 and the epidermal growth factor receptor. Further, these vesicles were visualized by quick-freeze, deep-etch electron microscopy in association with the surface of infected ME-180 cells and with pili of adherent gonococci. Like CD46 shedding, CD46 redistribution and vesicle release were insensitive to colchicine and cytochalasin-D but dependent on expression of the pilus retraction protein PilT. This vesiculation may represent a host cell defence response in which surface proteins that are commonly exploited by pathogens, such as CD46, are removed from the cell.

**Introduction**

The type IV pilus is a multifunctional, filamentous appendage expressed by many Gram-negative bacteria including members of the family Neisseriaceae (reviewed in Craig *et al*., 2004). Type IV pili of the two pathogenic members of this family, *Neisseria gonorrhoeae* and *Neisseria meningitidis*, mediate bacterial motility (Merz and So, 2000), DNA transformation competency (Wolfgang *et al*., 1998) and adhesion to host cells (reviewed in Merz and So, 2000).

The human complement regulator CD46, or membrane cofactor protein (MCP), is a cellular pilus receptor for *N. gonorrhoeae* (Kallstrom *et al*., 1997; reviewed in Gill and Atkinson, 2004). CD46 protects host cells against autologous complement attack by serving as a cofactor for the plasma serine protease factor I to cleave and thereby inactivate C3b and C4b deposited on a host cell (reviewed in Riley-Vargas *et al*., 2004). In addition to *Neisseria*, CD46 is a cellular receptor for a growing list of pathogens (reviewed in Cattaneo, 2004) including some strains of the measles virus (reviewed in Manchester *et al*., 2000), Streptococcus pyogenes (Okada *et al*., 1995), human herpesvirus 6 (Santoro *et al*., 1999; Greenstone *et al*., 2002; Mori *et al*., 2003), certain group B and D adenoviruses (Gaggar *et al*., 2003; Segerman *et al*., 2003; Wu *et al*., 2004) and bovine diarrhoea virus (BDV) (Maurer *et al*., 2004). CD46 is also incorporated in the envelope of human immunodeficiency virus (HIV) (Montefiori *et al*., 1994; Saifuddin *et al*., 1997).

Pilus-mediated attachment of *N. gonorrhoeae* to human epithelial cell lines results in adhesion-promoting signalling responses including the induction of a CD46-dependent calcium response (Kallstrom *et al*., 1998) and tyrosine phosphorylation of CD46 by the src kinase c-yes (Lee *et al*., 2002). Additionally, CD46 clusters beneath gonococcal microcolonies adhering to primary cervical epithelial cells (Edwards *et al*., 2000) and cellular CD46 decreases following incubation with piliated gonococci (Gill *et al*., 2003). *Neisseria*-induced loss (shedding) of CD46 is accompanied by accumulation of CD46 in the cell culture media (Gill *et al*., 2003). The extracellular CD46 has a relative electrophoretic mobility (M) equivalent to that of membrane CD46, suggesting that the mechanism by which CD46 is released does not involve proteolysis (Gill *et al*., 2003). CD46 shedding and
certain other cellular responses to *Neisseria* attachment, such as the formation of a cortical actin plaque at the site of adhesion (Merz *et al.*, 1999), are dependent at least in part on expression of the *Neisseria* protein PilT. PilT is an inner membrane ATPase that mediates pilus retraction (Wolfgang *et al.*, 1998) and is necessary for many bacterial processes including transformation competency and twitching motility (reviewed in Merz and So, 2000).

In this study, we further explore the effects on CD46 of gonococcal adhesion to human epithelial cells. We characterize the PilT-dependent enrichment of CD46 at the site of bacterial adhesion and describe the *N. gonorrhoeae*-induced release of host-derived vesicles containing CD46.

**Results**

*CD46 is enriched at the site of microcolony adhesion*

Attachment of *N. gonorrhoeae* results in accumulation of host cell surface proteins at the site of microcolony adhesion in the form of a cortical actin plaque that is enriched in CD44, epidermal growth factor receptor (EGFR) and tyrosine-phosphorylated proteins (Merz *et al.*, 1999). Also, immunofluorescence microscopy of *N. gonorrhoeae*-infected primary tissue demonstrated aggregation of CD46 in response to gonococcal adhesion (Edwards *et al.*, 2000). To further explore the effects of *N. gonorrhoeae* adherence on surface distribution of CD46, green fluorescent protein (GFP) chimeras of two of the most abundantly expressed isoforms of CD46, BC1 and BC2 were constructed. These isoforms differ only in their cytoplasmic tails. GFP was fused to the N-terminus (GFP-BC1, GFP-BC2) and in other constructs, the C-terminus (BC1-GFP, BC2-GFP). ME-180 cells transiently expressing one GFP-CD46 chimera were incubated with gonococci and then visualized by confocal microscopy (Fig. 1). Adhesion of piliated gonococci resulted in enrichment of each of the GFP-CD46 chimerae at the site of microcolony attachment (Fig. 1B and C). In contrast, uninfected cells exhibited relatively uniform expression of GFP-CD46 over the ME-180 cell surface (Fig. 1C). This enrichment was observed as early as 90 min post infection and increased in intensity over 6 h as the size of adherent microcolonies increased (Fig. 2). Similar results were observed with all four GFP-CD46 chimerae, indicating that the location of the GFP moiety or the cytoplasmic tail did not influence enrichment of CD46 at the site of adhesion (not shown). Enrichment of endogenous CD46 was also observed in non-transfected cells using immunofluorescence microscopy employing anti-CD46 monoclonal antibodies (mAbs) (not shown). Additionally, cells were visually monitored at each time point for morphological changes indicative of apoptosis (nuclear condensation, rounding up, blebbing, etc.). None of these morphological changes were observed. Similarly, no evidence of toxicity was revealed when membrane integrity of monolayers incubated with gonococci for up to 24 h was assessed by staining with trypan blue or propidium iodide or by assessing the release of lactate dehydrogenase using the Cytotoxicity Detection Kit (Roche Applied Sciences) (not shown).

Due to the PilT-dependent nature of CD46 shedding and certain other *Neisseria*-induced host cell responses (Merz *et al.*, 1999), the ability of PilT-deficient gonococci to enrich for CD46 at the site of adhesion was assessed. ME-180 cells expressing GFP-CD46 were incubated with PilT-deficient gonococci for 6 h as described above. The PilT mutant was hyperadherent and associated with the cell in microcolonies as previously described (Wolfgang *et al.*, 1998; Gill *et al.*, 2003). However, enrichment of CD46 was less pronounced than that associated with wild-type gonococci, indicating that PilT contributes to the localization of CD46 at the site of adhesion (Fig. 1C).

CD46 shedding was observed in ME-180 and the colorectal epithelial cell line T84 but not in the pharyngeal epithelial cell line Hep-2, likely due to low levels of bacterial adhesion to the latter cells (Gill *et al.*, 2003). Similarly, enrichment of GFP-CD46 at the site of microcolony adhesion was observed in transfected T84 cells but not in transfected Hep-2 cells (not shown).

*CD46 localization and shedding are not inhibited by colchicine or cytochalasin-D*

BC2-GFP-expressing ME-180 cells were incubated with the microtubule disrupting agent colchicine or the F-actin polymerization inhibitor cytochalasin-D (Fig. 3). Colchicine had little effect on CD46 distribution in the presence or absence of adherent gonococci (Fig. 3B). Cytochalasin-D disrupted the actin cytoskeleton, as evidenced by the non-uniform, aggregated pattern of phalloidin staining (Fig. 3C and D). In uninfected cells, the uniform distribution of BC2-GFP (Fig. 3A) changed to a pattern of small, punctate, diffusely distributed patches following treatment with cytochalasin-D (Fig. 3C). Unexpectedly, cytochalasin-D pre-treatment followed by incubation with gonococci resulted in enhanced aggregation of CD46 at the site of bacterial adhesion (Fig. 3D). Similarly, colchicine and cytochalasin-D did not inhibit the *N. gonorrhoeae*-induced loss of CD46 from ME-180 cells or its accumulation in the media (Gill *et al.*, 2003) (Fig. 3E). Surprisingly, treatment of ME-180 cells with colchicine or cytochalasin-D enhanced these effects, particularly in the case of cytochalasin-D treatment (Fig. 3E).
Neisseria gonorrhoeae attachment results in shedding of CD46-containing vesicles

Adherence of *N. gonorrhoeae* to ME-180 cells results in loss of CD46 from the cell, accompanied by accumulation of CD46 in the medium (Gill *et al.*, 2003). This extracellular CD46 exhibits a normal Mr, suggesting that it is released from the cell intact. Further, following its release into the supernatant, CD46 retains cofactor activity (not shown). In order to further characterize the CD46 following its release from the cell, the culture medium from *N. gonorrhoeae*-infected ME-180 cells was fractionated by differential ultracentrifugation. Following a 1 h centrifugation at 30 000 *g* to remove unbound bacteria and cell debris, culture media was subjected to sequential 1 h centrifugations at 50 000 *g* and 100 000 *g*. The CD46 content of each pellet was assessed by Western immunoblot. Little to no CD46 was detected in the 30 000 *g* or 50 000 *g* pellets but CD46 accumulated in the 100 000 *g* pellet (Fig. 4A). Characterization of the 100 000 *g* pellet by electron microscopy revealed predominantly round, membranous structures of 30–200 nm with mean size of approximately 85 nm (*n* = 200, standard deviation = 43) (Fig. 4B). Such structures were not observed following centrifugation of the media from uninfected ME-180 cells or cells infected with non-piliated bacteria.

In accord with the dependence of CD46 shedding and localization on PilT expression, incubation of ME-180 cells...
with PilT-deficient gonocci did not induce accumulation of CD46 or vesicles in the 100 000 g pellet. However, when PilT expression was derepressed by addition of isopropyl-beta-D-thiogalactopyranoside (IPTG) to the culture medium, CD46 and vesicles were observed in the 100 000 g pellet (Fig. 4B). Additionally, CD46 and vesicles were observed in the medium from infected T84 cells (which also exhibit CD46 shedding) (Gill et al., 2003) but not in the medium from infected Hep-2 cells (which do not exhibit CD46 shedding) (not shown). Similar to CD46 shedding, accumulation of CD46 and vesicles in the medium was not inhibited by pre-treatment with colchicine or cytochalasin-D (Fig. 3F).

Neisseria gonorrhoeae-induced vesicles are associated with the host cell surface and with pili

Quick-freeze, deep-etch electron microscopy (Heuser, 1981) was used to visualize the interaction of piliated gonococci with the surface of ME-180 cells (Fig. 5A–C). Pili appear as long filaments radiating from the bacterial surface and networking with the host cell membrane, other bacteria and other pili. Both wild-type and PilT-deficient gonococci were associated with membrane ruffling and microvillus elongation as has been previously described (Edwards et al., 2000; and reviewed in Merz and So, 2000).

Vesicles of the same approximate shape and size as those isolated from the culture media of N. gonorrhoeae-infected cells were observed in association with the ME-180 cells infected with wild-type but not PilT-deficient bacteria. Vesicles were also observed trapped in the network of pili formed by the adherent microcolony but not in tight association with the bacterial surface. Vesicles were not observed on the surface of ME-180 cells infected with PilT-deficient gonococci (Fig. 5D–F).

Following incubation of ME-180 cells with wild-type gonococci, culture media were harvested and centrifuged as above. The 100 000 g pellet was passed through a 2.6–0.25 M sucrose gradient. Gradient fractions were assessed for CD46 by Western immunoblot and for vesicles by electron microscopy. CD46 was detected in fractions from the centre of the gradient corresponding with a concentration of approximately 1.0–1.5 M sucrose (Fig. 6A). Electron microscopy revealed the presence of vesicles in CD46-containing fractions but not in non-CD46-containing fractions (Fig. 6B). Such co-fractionation of CD46 with vesicles suggests that the CD46 in the media of N. gonorrhoeae-infected cells is associated with these membranous, vesicular structures. These vesicles likely originated from the host cell surface as additional membrane-bound host cell proteins (CD55 and EGFR) were present in the CD46- and vesicle-containing fractions of a sucrose gradient of these pellets (Fig. 6A). Inter-
Interestingly, previous reports have linked both proteins to *N. gonorrhoeae* adhesion as CD55 is also lost from the host cell in response to *N. gonorrhoeae* infection (Gill et al., 2003) and EGFR accumulates at the site of *Neisseria* attachment (Merz et al., 1999).

**Discussion**

Herein we describe a phenomenon in which attachment of *N. gonorrhoeae* to cultured human epithelial cells results in enrichment of CD46 at the site of the adherent microcolony. This interaction leads to a reduction in surface expression of CD46 and its appearance in the culture media. By differential ultracentrifugation and sucrose gradient separation, functionally intact CD46 co-fractionates with ~80 nm vesicles derived from the host cell. Quick-freeze, deep-etch electron microscopy demonstrated similar vesicles on the surface of infected cells, both near adherent gonococci and interacting with the pilus network. CD46 enrichment at the site of adhesion, shedding and vesicle release appear to be related based on evidence reported here and in our prior study (Gill et al., 2003). First, these three processes require expression of the pilus retraction protein PilT. Second, these effects are...
observed in the human cell lines ME-180 and T84 but not Hep-2. Third, they are not inhibited by cytochalasin-D and colchicine. We propose that these three processes are part of a pathway whereby: (i) gonococci adhere to the host cell via a pilus-mediated attachment event, (ii) this attachment induces responses within the host cell resulting in recruitment of CD46 to adherent microcolonies and (iii) CD46-containing vesicles are released from the epithelial cell surface near the site of the adherent bacteria.

Adhesion of *N. gonorrhoeae* to human epithelial cells induces membrane ruffling and microvillus extension (reviewed in Merz and So, 2000), as well as rearrangements of the cortical actin cytoskeleton and clustering of tyrosine-phosphorylated proteins such as ICAM-1, CD44 and EGF-R and actin-associated proteins such as ezrin and vinculin (Merz et al., 1999; Edwards et al., 2000). This clustering phenomenon is thought to promote bacterial infectivity by increasing the local concentration of adhesion molecules and by facilitating the activation of host signalling pathways (Merz et al., 1999). Aggregation of CD46 beneath the site of gonococcal microcolonies (Merz et al., 1999) were insensitive to cytochalasin-D and were enhanced by expression of PilT.

While the role of PilT in this process remains unclear, its influences are likely related to retraction of the pili of adherent gonococci. PilT is an inner membrane ATPase that is essential to retraction of the pilus. Mutations in *pilT* produce a number of phenotypes including reduced transformation competency, a partial defect in cortical actin plaque formation and loss of twitching motility (reviewed in Merz and So, 2000). It has been proposed that retraction of the pili of adherent gonococci exerts tensile forces upon the membrane that, in turn, initiate signalling cascades within the host cell (reviewed in Merz and So, 2000). The PilT dependence of the formation of CD46 aggregates at the site of microcolony adhesion and the release of CD46-containing vesicles may be similarly explained.

In a recent paper further exploring the PilT-dependent mechanical signal transduction of *N. gonorrhoeae* in infected cells, Howie et al. (2005) describe a role for mechanotransduction in the activation of survival (e.g. antiapoptotic) signals within the host cell. In this work, the
authors suggest a model in which bacterial contact initiates apoptotic cascades that are counteracted by anti-apoptotic signals. These pro-viability signals arise from the exertion of tensile forces upon the membrane by the co-ordinate retraction of the pili of multiple gonococci in a bound microcolony (Howie et al., 2005). These findings support our observations that the prolonged incubations with live gonococci performed in the current study are not accompanied by a decrease in host cell viability or visual evidence of apoptosis.

Potential function(s) of vesicles released in response to gonococcal attachment can be divided into two broad, non-exclusive categories: those that benefit the bacteria (infection promoting) and those that benefit the host (infection limiting). Localization and release of CD46 may represent a complement evasion strategy wherein gonococci orchestrate release of CD46 on vesicles and then decorate their surface with these vesicles as a means to limit complement activation. Alternatively, vesicle release may represent an innate cell defence mechanism by...
Neisseria gonorrhoeae-induced host cell vesiculation

which membrane components are selectively removed from the host cell membrane. In the scenario of *N. gonorrhoeae* infection, CD46 cross-linking by microbial ligands may result in expulsion of host cell proteins that are commonly exploited by the pathogen. Such a process would serve the dual purpose of decreasing the concentration of available receptors on the cell surface while increasing receptor density in the extracellular milieu. These receptors could then act as a decoy to neutralize microbial adhesins before cellular contact.

Regarding the origin of these vesicles and the mechanism of their formation, ongoing investigations are assessing several possibilities. First, *N. gonorrhoeae*-induced vesiculation of the host cell membrane could result from budding directly from the plasma membrane. Interestingly, such vesiculation has been described following insertion of pore-forming agents such as the membrane attack complex of the complement system and streptolysin O (SLO), presumably to release pore formers and circumvent their lytic properties (Iida *et al.*., 1991; Xie and Low, 1995). A second potential mode of host cell-derived vesicle formation and release is intraluminal budding of the limiting membrane of a late endosome (forming a multivesicular body) followed by fusion of the endosome with the plasma membrane. This process results in the release of an intensely studied class of vesicles termed exosomes (reviewed in Fevrier and Raposo, 2004). While the function of exosomes remains elusive, these structures have been related to a number of cellular and immunomodulatory functions including facilitating intercellular communication (reviewed in Denzer *et al.*, 2000; Fevrier and Raposo, 2004). While CD46 has not been demonstrated on mature (released) exosomes, it has been demonstrated in association with the limiting membrane and within the contents of multivesicular bodies following internalization via clathrin-coated vesicles (Crimeen-Irwin *et al.*., 2003). Also, HIV budding employs the same pathway as exosome biogenesis (Pornillos *et al.*, 2002), and CD46 is incorporated into the envelope of HIV where it protects against complement-mediated virolysis (Marischang *et al.*, 1993; Montefiori *et al.*, 1994; Saifuddin *et al.*, 1995).

A third possible origin of CD46-containing vesicles is the ‘pinching off’ of microvilli. In the colonic epithelium, vesiculation of microvilli is a normal response to conditions such as fasting, thermal injury, treatment with antibodies or lectins, and bacterial or viral infection (Baranov *et al.*, 1994; Fahlgren *et al.*, 2003). Carcinoembryonic antigen (CEA) and related carcinoembryonic antigen-related cell adhesion molecules (CEACAMs), a family of proteins that are commonly exploited by microorganisms including *Neisseria*, are constitutively shed into the glyco-

Fig. 6. Sucrose gradient fractionation of the 100 000 g pellets. Following infection with piliated gonococci, cell culture media from ME-180 cells were centrifuged for 1 h at 50 000 g and then 2 h at 100 000 g. The 100 000 g pellets were suspended in PBS (0.5 ml) and 30 μl removed for Western blot analysis (‘P’ in the left-most lane). Remaining material was floated through a gradient of 2.6–0.25 M sucrose. Fractions (0.5 ml) of the gradient were analysed by Western immunoblot for CD46, CD55 and EGF-R (A) and by negative staining electron microscopy for vesicles (B) (magnification = 40 000×; scale bars = 100 nm).
calyx of colonic epithelial cells (Fahlgren et al., 2003). This likely occurs via a calcium-dependent vesiculation of microvilli. The blebbing of microvilli may result in a high concentration of CEA, CEACAMs and other proteins in the glyocalyx. In this location, they can bind and trap microorganisms and thereby inhibit invasion of the colonic epithelium (Fahlgren et al., 2003). This vesiculation could also result in the release of microorganisms already adherent to receptors on the microvilli (Fahlgren et al., 2003). The early stages of gonococcal adhesion are accompanied by elongation of microvilli followed by localized disappearance of microvilli as the infection progresses (reviewed in Merz and So, 2000). N. gonorrhoeae-induced release of CD46 may occur through a similar mechanism.

Distinction among these modes of vesicle formation outlined above is being pursued along several lines including proteomic and biochemical characterizations of vesicle constituents. Preliminary proteomic analyses have revealed no evidence of bacterial proteins in the CD46- and vesicle-containing fractions of a sucrose gradient, supporting our hypothesis that these vesicles originate from the host cell. Additionally, these preliminary analyses have not demonstrated common exosomal markers associated with the vesicles yet have suggested the presence of one or more members of the CEACAM family (D.B. Gill and P. Atkinson, unpublished). Future studies will focus on characterization of the vesicles induced by _N. gonorrhoeae_ adhesion and their mode of biogenesis.

**Experimental procedures**

**Cell lines and bacterial strains**

The cell lines ME-180, Hep-2 and T84 (HTB-33, CCL-23 and CCL-248 respectively) were obtained from American Type Culture Collection. ME-180 cervical epithelial cells were maintained in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum. Hep-2 (pharyngeal epithelium) and T84 (colorectal epithelium) were maintained in Dulbecco's modified eagle medium (Gibco) with 10% heat-inactivated fetal bovine serum. Hep-2 (pharyngeal epithelium) and T84 (colorectal epithelium) were maintained in McCoy's 5A medium supplement with 10% heat-inactivated fetal bovine serum. ME-180 cervical epithelial cells were maintained in Dulbecco's modified eagle medium (Gibco) as per the recommendations of the manufacturer.

_Neisseria gonorrhoeae_ strain N401 and its derivative GT104 were previously described (Wolfgang et al., 1998). Strain N401 expresses wild-type pili while strain GT104 expresses the pilus. Strain N401 (0.01 mg ml⁻¹) was grown on Isovitalex (Gibco). Strain N401 and its derivative GT104 Neisseria gonorrhoeae were previously described (Wolfgang et al., 1998). Strain N401 (∞, 2003) and strain N401 (∞, 2003) were obtained from American Type Culture Collection. ME-180 cervical epithelial cells were maintained in McCoy's 5A medium supplemented with 10% heat-inactivated fetal bovine serum.

**Construction and transfection of GFP-CD46 chimeras**

CD46 is predominantly expressed as four major isoforms (BC1, BC2, C1 and C2) that arise from alternative splicing of a single gene. These isoforms differ in their quantity of O-glycosylation due to presence or absence of the ‘B’ module and in expression of one of two possible cytoplasmic tails (Cyt-1 or Cyt-2) (reviewed in Gill and Atkinson, 2004). In most individuals, CD46 bearing the ‘B’ module is predominant and may contain either the 23 Cyt-1 cytoplasmic tail or the 16-amino-acid Cyt-2. These two tails are structurally unrelated and contain distinct signalling motifs (reviewed in Riley-Vargas et al., 2004).

For the direct visualization of human CD46, we tagged the BC1 and BC2 with the enhanced GFP at the N-terminus (GFP-BC1 and GFP-BC2) or C-terminus (BC1-GFP and BC2-GFP).

To insert the GFP-encoding DNA sequence at the N-terminus of CD46 (BC1 isoform), we first engineered a basic construct, designated pNILM-BC1, by combining a 1415 bp Asel/BsiWI fragment of sT-DAF (Spitzer et al., 2004) [including the SV40 promoter region of pSBC-1 (Dirks et al., 1993) and the signal peptide of CD59], a 382 bp BsiWI/BsGI flanked polymerase chain reaction (PCR) fragment (5' primer: MCPdSP5P5, 3' primer: MCP3-PCR) of pMCP-BC1 (Post et al., 1991) and a 2249 bp BsrGI/Asel fragment of pMCP-BC1 in a three-frame ligation. The BC2 isoform was obtained by replacing the 1802 bp Asel/BsrGI fragment from pNILM-BC1 with the 2379 bp BsrGI/BsrGI fragment of pMCP-BC2 (Post et al., 1991). Both N-terminally tagged isoforms were generated by insertion of a 740 bp PstI/BsiWI/BsGI flanked PCR-amplified GFP sequence (5' primer: EGFPSpSt, 3' primer: EGFPS3BsI) from pEGFP-1 (Clontech) into the PstI/BsiWI cut pNILM-BC1 and pNILM-BC2, resulting in GFP-BC1 and GFP-BC2 respectively.

For the addition of GFP to the C-terminus of CD46 isoforms BC1 and BC2, unique PstI restriction sites were introduced by PCR immediately upstream of the stop codon of the respective MCP-encoding plasmids (BC1, 5' primer: MCPdSP5P5, 3' primer: MBC13P; BC2, 5' primer: MCPdSP5P5, 3' primer: MBC23P).

Three-fragment ligation of the 4596 bp BsrGl/BglII fragment of pMCP-BC1, a 740 bp PstI/BglII GFP PCR fragment (5' primer: EGFPSpSt, 3' primer: EGFPS3BsI) and either a 652 bp BsrGl/Pstl PCR fragment of pMCP-BC2 or a 674 bp BsrGl/Pstl PCR fragment of pMCP-BC2 resulted in the generation of BC1-GFP and BC2-GFP respectively. All PCR-generated amplification products were verified by DNA sequencing.

Vectors were transfected into ME-180 cells using the FuGene (Roche Applied Sciences) transfection reagent as per the instructions of the manufacturer.

**Assessment of membrane integrity.** Following incubation with gonococci or medium alone, ME-180 cells were washed with PBS, treated with 37°C trypsin-EDTA for 2 min, scraped into PBS containing 0.8 mM trypan blue and then counted employing a haemacytometer. Additionally, cells were washed and trypsinized as above then resuspended in 1% fetal calf serum in PBS containing 0.01 mg ml⁻¹ propidium iodide for Fluorescence Activated Cell Sorting (FACS) analysis. Lactate dehydrogenase release was assessed using the Cytotoxicity Detection Kit (Roche Applied Sciences) as per the recommendations of the manufacturer.

**Confocal microscopy.** ME-180 cells (1 × 10⁶) were transfected with GFP-CD46 and cultured overnight in 35 mm glass-bottom dishes (MatTek). Following two washes of the monolayer, 2 ml of serum-free medium containing suspended gonococci were added to the dish to achieve a multiplicity of infection (moi) of 100 (assuming OD₅₅₀ of 1 corresponds to 1 × 10⁹ bacteria ml⁻¹). Following incubation for 0, 1.5, 3 or 6 h at 37°C in a 5% CO₂ atmosphere, dishes were washed three times with serum-free culture media and then fixed for 10 min at room temperature in PBS containing 2% paraformaldehyde and 0.5% glutaraldehyde.

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Following two washes in PBS, PBS containing 0.01 mg ml⁻¹ propidium iodide was added. Samples were examined using a Zeiss LSM 510 laser scanning confocal microscope and images were processed using Image Examiner Software (Zeiss). Z-stacks were collected and used to generate three-dimensional (3D) projections of the cell. Regions of increased fluorescence were identified using Velocity image analysis software version 3.0.2 (Improvision). These regions were identified by applying a ‘classifier’ which specified regions of greater than 10 cubic pixels with fluorescence intensities of 200 or greater on a scale of 1–255.

**Differential ultracentrifugation and sucrose gradient separation.** Medium from ME-180 cells was collected following a 6 or 12 h incubation with piliated gonococci (moi 100) and subjected to sequential 1 h centrifugations at 30 000 g, 50 000 g and 100 000 g at 4°C. Pellets were resuspended in PBS. For sucrose density gradient separation, pellets were washed once in PBS and then resuspended in 2 ml of 2.6 M sucrose. The 100 000 g pellets were floated through a gradient consisting of 2 ml each of 2.0, 1.5, 1.0, 0.5 and 0.25 M sucrose by centrifugation at 270 000 g for 16 h at 4°C. Fractions (0.5 ml) were collected and analysed by Western immunoblot and electron microscopy (see below).

**Western immunoblot.** Samples were separated by SDS-PAGE then transferred to a nitrocellulose membrane using an XCell II Blot Module (Novex) as per the manufacturer’s recommendations. Membranes were incubated for 1 h at 37°C in blocking buffer (Tris-buffered saline with 0.05% Tween-20 containing 2% non-fat dry milk) for 1 h, washed, and immunoblotted using rabbit polyclonal antiserum (1:7000) followed by horseradish peroxidase (HRP)-conjugated donkey anti-rabbit immunoglobulin G (1:7000). They were developed using Supersignal West Pico chemiluminescent substrate (Pierce). Additional antibodies used were rabbit anti-CD55 (1:100) and rabbit anti-EGF-R (1:100) (both from Santa Cruz Biologicals).

**Transmission electron microscopy of vesicles.** Samples were allowed to adsorb onto formvar-carbon-coated grids for 10 min. Grids were washed in dH₂O and stained with 1% aqueous uranyl acetate (Ted Pella) for 1 min. Excess liquid was gently wicked off and grids were allowed to air-dry. Samples were viewed on a JEOL 1200EX transmission electron microscope (JEOL USA).

**Quick-freeze deep-etch electron microscopy of infected ME-180 cells.** Methods used were a modification of those described in Heuser (1980; 1989). ME-180 cells were grown on 3 mm² pieces of #1 glass coverslips. 5.5 h post-infection, the coverslips were immersed in fixative (30 mM Hepes, 100 mM NaCl, 2 mM CaCl₂, pH 7.4 with 2% glutaraldehyde) for 2–4 h. Coverslips were then washed by brief immersion in several successive dishes of distilled water.

Each coverslip was then mounted on a 3 mm² slab of aldehyde-fixed and water-washed rabbit lung (0.8 mm thick). This served as a ‘cushion’ for quick-freezing by abrupt impact against an ultrapure copper block cooled with liquid helium to 4°C above absolute zero. Following storage in liquid nitrogen, coverslips were mounted in a Balzers’ Model 301 vacuum evaporator. In this device, the sample was freeze-dried by warming to ~80°C for 15 min followed by rotary replication with a thin (~2 nm) film of Pt (evaporated for 5–10 s from an electron beam gun mounted 15–20° above the horizontal during rotation of the coverslip at 5 Hz). This Pt ‘replica’ of the coverslip and the cells remaining attached to it were then immediately supported or ‘backed’ by evaporating ~10 nm of pure carbon onto it, using a standard carbon-arc supply mounted 10° off of the vertical.

Next, the coverslip was removed from the Balzers device, allowed to thaw, and the replica was floated off by immersing it at an ~45° angle into full strength (47%) hydrofluoric acid (HF). Immediately thereafter, the replica was picked up off the surface of the HF with a glass rod and transferred through several washes of distilled water. The replica was then floated on standard household bleach followed by more washing in distilled water and then adsorbed to a 75 mesh formvar-coated EM grid. For electron microscopy, the grid was mounted in a eucentric side-entry goniometer stage of a JEOl 200CX electron microscope, imaged at 30–70 K magnification, and photographed in stereo at ±10° of tilt off the vertical axis.

‘Anaglyph’ stereo images were generated as described (Heuser, 2000a,b). Briefly, the two stereo micrographs were digitized using a Canon EOS1 Ds Mark II digital camera. Using Adobe Photoshop, the right view was converted to a pure red-channel RGB image and the left view to a blue-green-channel RGB image. Next, one image was copied directly onto the other, and the two were imaged simultaneously using the ‘screen’ command in the ‘layers’ menu of Photoshop. The anaglyph was finally brought into alignment by using the ‘free translate’ command in Photoshop on one of the two layers.

**Cofactor assay.** Cofactor assays were performed as previously described (Liszewski et al., 2000). Briefly, CD46-containing vesicles were isolated by sucrose density gradient separation, washed once in PBS and resuspended in low-salt buffer (LSB) 10 mM Tris, pH 7.2, 25 mM NaCl. The vesicle suspension (10 µg ml⁻¹) was incubated for 1.5 h at 37°C with 5 µl of a 1:50 dilution of factor I (Quidel) and 5 µl of a 1:100 dilution of biotinylated human C3b (Liszewski et al., 1998). Samples were combined with 3× SDS-PAGE sample buffer containing β-mercaptoethanol and then electrophoresed on a 10% SDS-PAGE followed by transfer to a nitrocellulose membrane (as described above for Western immunobLOTS). Following incubation in blocking buffer, the membrane was probed with extravidin-HRP (Sigma) and developed with Supernisignal West Pico Luminol reagents (Pierce).

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Supplementary Material

The following supplementary material is available for this article online:

**Fig. S1A–E.** Membrane vesicles associate with the host cell surface and with pilus networks. Quick freeze-deep etch electron microscopy was performed on ME-180 cells following incubation with wild-type (A–C) or PilT-deficient (D–F) gonococci for 5.5 h. Following incubation with wild-type gonococci, membrane vesicles were associated with the host cell surface and trapped in the pilus networks (A, B). Regions of adherent gonococci were also observed that were devoid of vesicles but exhibited elongated microvilli, often interacting with the bacterial surface or entwined with pili (C). Vesicles were not found on the host cell surface or in the pilus networks of adherent, PilT-deficient gonococci (D) but attachment of PilT deficient gonococci did result in elongation of microvilli similar to that of the wild-type (E). Short microvilli were observed in regions of infected cells with no adherent gonococci. Scale bar = 300 nm. Images are stereo “anaglyph” images of Fig. 5A–E, respectively, as they are presented in the print article. Anaglyph images are best viewed with red/green 3D glasses.