Binding of vitronectin and Factor H to Hic contributes to immune evasion of Streptococcus pneumoniae serotype 3.

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Published in:
Thrombosis and Haemostasis

DOI:
10.1160/TH14-06-0561

2015

What is known about this topic?

- *Streptococcus pneumoniae* (pneumococcus) has evolved various strategies to encounter humans and establish itself in different host niches. For instance, pneumococci interact with vitronectin and Factor H. Both human glycoproteins are fluid-phase complement inhibitors, bind to integrins or to the extracellular matrix of various human tissues, and vitronectin is additionally implicated in haemostasis and angiogenesis.
- Serotype 3 *Streptococcus pneumoniae* strains express Hic, a surface-exposed PspC-like protein, which was shown to recruit the human complement inhibitor protein Factor H to evade complement-mediated phagocytosis.

What does this paper add?

- Hic was identified as vitronectin-binding adhesin of serotype 3 pneumococci.
- Factor H and vitronectin simultaneously bind to Hic and the binding sites for Factor H and vitronectin within the Hic molecule were characterised. In addition, the Hic-binding site was localised to the C-terminal heparin-binding domain within human vitronectin. Importantly, vitronectin bound to Hic efficiently inhibited the terminal complement pathway.
- By binding Factor H and vitronectin, Hic may contribute to both immune evasion at different levels of the complement cascade and pneumococcal adhesion.

Binding of vitronectin and Factor H to Hic contributes to immune evasion of *Streptococcus pneumoniae* serotype 3

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Running title: Pneumococcal Hic binds vitronectin

Financial support:
This work was supported by Grants from the Deutsche Forschungsgemeinschaft DFG HA 3125/4-2 and the DFG-GRK840 (to S. H.) as well as the Anna and Edwin Berger foundation and the Swedish Medical Research Council (grant number 521-2010-4221) (to K. R.).

Summary

Streptococcus pneumoniae serotype 3 strains are highly resistant to opsonophagocytosis due to recruitment of the complement inhibitor Factor H via Hic, a member of the pneumococcal surface protein C (PspC) family. In this study, we demonstrated that Hic also interacts with vitronectin, a fluid-phase regulator involved in haemostasis, angiogenesis, and the terminal complement cascade as well as a component of the extracellular matrix. Blocking of Hic by specific antiserum or genetic deletion significantly reduced pneumococcal binding to soluble and immobilised vitronectin and to Factor H, respectively. In parallel, ectopic expression of Hic on the surface of *Lactococcus lactis* conferred binding to soluble and immobilised vitronectin as well as Factor H. Molecular analyses with truncated Hic fragments narrowed down the vitronectin-binding site to the central core of Hic (aa 151-201). This vitronectin-binding region is separate from that of Factor H, which binds to the N-terminus of Hic (aa 38-92). Binding of pneumococcal Hic was localised to the C-terminal heparin-binding domain (HBD3) of vitronectin. However, an N-terminal region to HBD3 was further involved in Hic-binding to immobilised vitronectin. Finally, vitronectin bound to Hic was functionally active and inhibited formation of the terminal complement complex. In conclusion, Hic interacts with vitronectin and simultaneously with Factor H, and both human proteins may contribute to colonization and invasive disease caused by serotype 3 pneumococci.

Keywords: complement, Hic, pathogenesis, PspC11, serotype 3, *Streptococcus pneumoniae*, vitronectin

Introduction

Gram-positive *Streptococcus pneumoniae* (*S. pneumoniae*, pneumococci) are often carried as harmless commensals in the human nasopharynx. However, pneumococci have evolved several sophisticated strategies to evade the immune system in order to cause mucosal and respiratory diseases such as sinusitis, otitis media and pneumonia. They are also responsible for severe life-threatening systemic diseases such as pneumococcal sepsis and meningitis associated with vascular injury due to pneumococcal pneumolysin, coagulation and complement activation, production of reactive oxygen and nitrogen species, and secretion of pro-inflammatory cytokines (1-3). The polysaccharide capsule, which occurs in at least 94 varieties, is one main pneumococcal virulence determinant (4). However, surface-exposed adhesive proteins are also considered being important virulence factors (5, 6). In spite of the ability to treat and prevent pneumococcal infections with antibiotics and conjugate vaccines, the incidence of invasive pneumococcal disease remains high. Risk groups are especially children <5 years of age, the elderly, and immunocompromised individuals (7). Serotype 3 pneumococci belong to the most frequent strains in clinical isolates and are associated with invasive diseases in young children and high case-fatality rates (8-10). The resistance of pneumococci, and especially serotype 3, to complement-mediated opsonization and phagocytosis is closely related to the recruitment of complement inhibitor Factor H to the bacterial surface (11). Factor H (FH), a 150-kDa single chain plasma glycoprotein, is composed of 20 modules referred to as short consensus repeat (SCR) and complement control protein (CCP), respectively. Factor H regulates the alternative pathway (AP) of complement at the C3 convertase level (12). In addition, Factor H binds to integrins via its arginine-glycine-aspartate (Arg-Gly-Asp, RGD) motif and to surface-associated molecules, such as glycosaminoglycans (12, 13). Pneumococci exploit host-cell bound Factor H for adherence and dissemination, and they recruit soluble Factor H to inhibit complement activation during infections via the pneumococcal surface protein C (PspC) (13-16). Based on their anchorage

to the bacterial surface, the 11 distinct PspC subtypes are classified into two subgroups. Classical PspC proteins (subtypes 1-6, members of subgroup I) possess a conserved C-terminal choline-binding domain, which enables non-covalent attachment to phosphorylcholine moieties of the surface-displayed teichoic acids. In contrast, non-classical or PspC-like proteins (subtypes 7-11, members of subgroup II) harbor a conserved C-terminal pentapeptide sequence (LPxTG-motif), which is cleaved by the transpeptidase sortase A and covalently linked to the bacterial peptidoglycan (17). Only little is known on the allelic distribution of *pspC* proteins among serotype 3 pneumococci but it is suggested that either group 8 or group 11 PspC proteins are expressed (17) (F. Iannelli, personal communication). PspC protein group 11 so far consists of four members (PspC11.1-4), which show 100% nucleotide and amino acid identity in their N-terminal region and C-terminal LPSTG-containing part but vary in their proline-rich region (17). In serotype 3 strain A66 a *pspC* locus-encoded surface-exposed protein was identified as major Factor H-binding adhesin and termed Hic (factor H-binding inhibitor of complement, class II PspC, PspC11.4) (11, 18, 19). Hic is a ~70-kDa protein, composed of a 37-aa leader peptide, a long stretch with regions of predicted α-helical conformation, followed by 25 proline-rich repeats and a sortase signal motif (LPSTG) for covalent anchorage to the pneumococcal cell wall (17). The N-terminal part (aa 38-155) of Hic shows considerable sequence homology to the Factor H-binding region (residues 38-158) of classical PspC proteins, suggesting that Hic and PspC share a sequence motif with Factor H-binding activity (17, 18).

Similar to Factor H, human vitronectin (Vn) plays a role in pneumococcal pathogenesis. Monomeric vitronectin is an abundant plasma glycoprotein of ~75 kDa promoting microvascular angiogenesis, regulating the fibrinolytic, coagulation and plasminogen activation system, and inhibiting the terminal complement pathway both in fluid phase and on cell surfaces. In contrast, multimeric vitronectin constitutes the extracellular matrix of

different tissues promoting cell adhesion, spreading and differentiation (20). Vitronectin consists of an N-terminal somatomedin-B domain, an RGD motif as recognition site for integrins, four hemopexin-like and three heparin-binding domains (HBD1: aa 82-137; HBD2: aa 175-219; HBD3: aa 348-361) (20, 21). *S. pneumoniae* expressing class I PspC proteins were shown to recruit vitronectin efficiently to their surface possibly preventing formation of the terminal complement complex (TCC) during the intimate contact of pneumococci with the human host (22-24). In addition, pneumococci exploit host-cell-bound multimeric vitronectin to adhere to epithelial cells of the respiratory tract, thereby activating $\alpha_v\beta_3$-integrin signaling pathways, triggering cytoskeleton reorganization, and subsequently promoting pneumococcal internalization (22).

The purpose of this study was to analyse vitronectin-binding to PspC11-expressing serotype 3 pneumococci and to characterise the interaction of Hic (PspC11.4) with the human plasma glycoproteins vitronectin and Factor H.

Material and methods

Reagents and antibodies

DNA and protein markers were from Fermentas (Thermo Fisher Scientific, MA, USA) or SERVA (Germany). Coomassie brilliant blue R250 and bovine serum albumin (BSA) were purchased from Roth (Germany). Heparin (potassium salt) was purchased from ICN Biomedicals (OH, USA). Human Factor H was provided by Calbiochem (Merck Millipore, Germany) or Complement Technology (TX, USA). Multimeric vitronectin was obtained from Millipore (Merck Millipore, Germany). Biotin-labeled multimeric vitronectin was provided by Loxo (Germany). Recombinant C-terminally His_6-tagged multimeric vitronectin fragments were heterologously expressed in HEK293T cells and purified as described previously (25).

Paraformaldehyde (PFA), glutaraldehyde, Nisin, IPTG, fluorescein isothiocyanate (FITC), heparan sulfate, chondroitin sulfate A, hyaluronic acid, and Bradford Reagent were purchased from Sigma-Aldrich (Germany). Dulbecco's modified Eagle medium (DMEM, 1 g/l glucose) and fetal calf serum (FCS) were provided by PAA Laboratories (Germany). Rabbit anti-human vitronectin antiserum, goat anti-human Factor H antiserum, and the complement proteins C5b-6, C7, C8, and C9 were obtained from Complement Technology. Alexa Fluor 488-conjugated goat anti-rabbit IgG as well as Alexa Fluor 647-conjugated anti-rabbit, anti-goat, and anti-mouse IgG were provided by Molecular Probes (Life Technologies, NY, USA). HRP-conjugated swine anti-rabbit antibodies, HRP-conjugated rabbit anti-goat antibodies, mouse anti-human C5b-9 monoclonal antibodies, and HRP-conjugated swine anti-mouse antibodies were purchased from Dakopatts (Denmark). Cy5-conjugated goat anti-mouse antibodies and Alexa Fluor 488-conjugated streptavidin were provided by Dianova (Germany). The ELISA substrate 1,2-phenylenediamine dihydrochloride (OPD) was obtained from Biozol (Germany). Anti-Hic2 antiserum was generated by immunization of mice with Hic2 protein derivative according to standard methods. Mouse control serum was generated by similar immunization procedure without protein. Human serum was derived from healthy volunteers (normal human serum, NHS) or a patient with glycosylation defects suffering from atypical haemolytic uremic syndrome (aHUS; patient human serum, PHS; obtained upon informed consent; unpublished data).

Bacterial strains, culture conditions, and transformation

S. pneumoniae were cultivated on solid blood agar plates (Oxoid, Germany) at 37°C and 5% CO_2 or in liquid Todd-Hewitt-broth (Roth, Germany) supplemented with 0.5% yeast extract (Roth, Germany) and appropriate antibiotics to mid-log phase (3.5-4 x 10^8 cfu/ml). Pneumococcal wild type strains, as well as isogenic mutants of *S. pneumoniae* A66 deficient

for capsule expression and Hic are listed in Table 1. Low encapsulated *S. pneumoniae* A66 (serotype 3) were isolated from infected type II pneumocytes (A549; ATCC CCL-185) (24), herein referred to as $A66^{ccv}$ (Table 1). Pneumococcal serotype 3 isolates from the National Reference Center for Streptococci, Germany, are listed in Table 2. *Escherichia coli* strains were cultivated at 30°C on Luria-Bertani agar or broth (Roth, Germany) supplemented with appropriate antibiotics. Transformation of *E. coli* strains with plasmid DNA (Table 1) was carried out with $CaCl_2$-treated competent cells according to standard procedures.

DNA sequencing and analysis

Chromosomal DNA of *S. pneumoniae* was isolated and purified using a standard Phenol/Chloroform extraction method as described previously (26). DNA amplification was performed by PCR using the primers NNHic and CCHic (Table 3) in 50 µl volumes either using the Pfu Ultra II Fusion HS DNA polymerase (Agilent Technologies, CA, USA) or the Finnzymes' Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, MA, USA) as recommended by the distributors. PCR products were directly purified using the Zymoclean DNA Clean & Concentrator Kit or purified from agarose gels using the Zymoclean Gel DNA Recovery Kit according to the manufacturer´s instructions (Zymo Research, CA, USA). DNA sequencing was carried out by Eurofins MWG Operon (Germany) using the primers listed in Table 3. BLAST programs were used to compare nucleotide and amino acid sequences with database entries available at NCBI. The program ClustalOmega available at EMBL-EBI was used to align nucleotide and amino acid sequences of annotated alleles for PspC11 (17, 18) with sequences obtained for the clinical isolates. Sequence data for the identified PspC11 alleles have been submitted to the EMBL-EBI database (Table 4).

Culture conditions, cloning and recombinant procedures in *Lactococcus lactis*

L. lactis MG1363 was grown statically at 30°C in M17 agar or broth (Oxoid) supplemented with 0.5% glucose and 5 µg/ml erythromycin. The shuttle vector pMSP3545 was used for the inducible extracellular expression of Hic (PspC11.4) in *L. lactis* (Table 1). Briefly, the complete coding sequence of *hic* was amplified by PCR using chromosomal DNA of *S. pneumoniae* A66 with the primers NNHic and CCHic containing incorporated *Nco*I and *Xba*I restriction sites, respectively. The PCR product was digested with *Nco*I and *Xba*I (NEB, MA, USA) and cloned into the similarly digested pMSP3545 vector to generate the pHic plasmid (Table 1). The coding sequence of *hic* was verified by DNA sequencing (Eurofins MWG Operon, Germany). Sequence comparisons were performed with the blast programs from the NCBI database. The recombinant plasmid was first selected after transformation into *E. coli* DH5α and then transformed into electrocompetent *L. lactis* as described recently (23, 27). Expression of Hic was induced by adding Nisin (0.1 µg/ml) to mid-log grown recombinant lactococci and verified by flow cytometry using mouse anti-Hic2 antiserum.

Cloning, expression and purification of N-terminally His_6-tagged Hic protein derivatives

The *S. pneumoniae* A66 DNA sequences coding for the four Hic protein derivatives (Fig. 3B) were PCR amplified using the primers listed in Table 3 and chromosomal DNA as template. The PCR products were cloned after *Nhe*I/*Hin*dIII (NEB, MA, USA) digestion into the similarly digested pTP1 vector (28) resulting in the plasmids pHic2, pHic5, pHic6, and pHic7, respectively (Table 1). The integrity of the inserted DNA was confirmed by sequencing (Eurofins MWG Operon, Germany) and sequence comparison with the NCBI blast programs. For protein production, the plasmids were transformed into *E. coli* BL21(DE3) and expression of the recombinant N-terminally His_6-tagged protein derivatives Hic2, Hic5, Hic6, and Hic7, respectively, was induced with 1 mM IPTG. The His_6-tagged protein derivatives were

purified by Ni^{2+} affinity chromatography with the Protino Ni-TED prepacked column kit according to the manufacturer's instructions (Macherey-Nagel, Germany). Proteins were dialysed against appropriate buffers before experiments and concentrations were measured by using a Bradford protein assay. The purity of expressed proteins was controlled on SDS-PAGE stained with Coomassie brilliant blue R250 or silver nitrate.

Deglycosylation of vitronectin and Factor H

Vitronectin or Factor H (each 5 µg) was incubated with N-glycosidase F (5 U) (Roche, Germany) for 3h at 37°C. Deglycosylated vitronectin or Factor H (each 1 µg) was separated by SDS-PAGE the proteins were transferred to a nitrocellulose membrane and analysed by using rabbit anti-human vitronectin antibodies or goat anti-human Factor H antibodies, in combination with HRP-conjugated secondary antibodies. Development was performed with ECL Western blotting detection reagents (Applichem).

Flow cytometric analysis

To detect binding of vitronectin and Factor H, 1 x 10^8 bacteria were incubated with vitronectin, recombinant vitronectin fragments, and Factor H, respectively, in 100 µl DMEM for 45 min at 37°C in 96-well U-shaped plates (Greiner Bio-One, Germany). For the detection of protein-binding to the surface of pneumococcal clinical isolates, bacteria were incubated with 3% PFA and 2.5% glutaraldehyde in cacodylate buffer (0.1 M cacodylate, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, 0.09 M sucrose, pH 6.9) for 1 h on ice, washed twice with cacodylate buffer as well as DMEM prior to incubation with vitronectin and Factor H, respectively (24). To inhibit protein-binding to the pneumococcal surface, pneumococci were incubated with anti-Hic2 mouse antiserum or mouse control serum (1:50 dilution) for 15 min prior to the addition of vitronectin or Factor H. After washing with 0.5% FCS/PBS (pH 7.4), bacteria

were incubated with rabbit anti-human vitronectin antibodies or goat anti-human Factor H antibodies, washed, and incubated with Alexa Fluor 488-conjugated anti-rabbit IgG or FITC-conjugated anti-goat IgG. In competitive inhibition experiments with Factor H, pneumococci were incubated in DMEM for 15 min with Factor H, and thereafter biotin-labeled vitronectin (1 µg/ml) was added to the pneumococci. After extensive washing, bacteria were incubated with Alexa Fluor 488-conjugated streptavidin (2 µg/ml). Finally, bacteria were washed and fixed using 1% PFA/0.5% FCS/PBS overnight at 4°C. The samples were analysed using a FACS Calibur™ flow cytometer and data acquisition was performed using CellQuestPro 6.0 (Becton Dickinson, NJ, USA). To assess whether glycosylation of vitronectin and Factor H plays a role for interaction with bacteria, both host proteins were treated with N-glycosidase F. Pneumococci were incubated with treated or untreated vitronectin or Factor H (each 10 µg/ml), NHS (1%) or PHS (1%) for 30 min at 37°C. Thereafter, bacteria were washed with 1% BSA/PBS and bound vitronectin or Factor H was detected with rabbit anti-human vitronectin antiserum or goat anti-human Factor H antiserum followed by incubation with Alexa Fluor 647-conjugated secondary antibodies. After washing, bound human proteins were identified by flow cytometry (FACScan LRII™) and data acquisition was performed using FlowJo (Becton Dickinson, NJ, USA). Data analysis was conducted using WinMDI 2.9 (The Scripps Research Institute, CA, USA) or Flowing Software 2.5.0 (Turku Centre for Biotechnology, Finland). The bacteria were detected and gated as described previously (22, 29) and protein binding is shown as the total fluorescence (geometric mean fluorescence intensity (GMFI) multiplied with the percentage of fluorescently labeled and gated events).

Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (F96, Polysorb^{TM}, Nunc, Thermo Fisher Scientific, MA, USA) were coated with *S. pneumoniae* Hic constructs Hic2, Hic5, Hic6, Hic7, and BSA, as control, over night at

4°C. The plates were washed four times with 0.05% Tween20/PBS (pH 7.4) and blocked for 1 h at RT with 2% BSA/0.1% Tween20/PBS (blocking buffer). The plates were then incubated for 1 h at RT with vitronectin, the various recombinant vitronectin constructs or Factor H diluted in blocking buffer. Thereafter, wells were washed and incubated with a rabbit anti-human vitronectin antiserum (1:1000 in blocking buffer) or goat anti-human Factor H antiserum (1:500 in blocking buffer), followed by HRP-conjugated anti-rabbit antibodies or HRP-conjugated anti-goat antibodies (1:1000 in blocking buffer). The assay was also performed vice versa. Vitronectin, Factor H, recombinant vitronectin fragments or BSA, as control, were immobilised on microtiter plates (F96, Maxisorb^{TM}, Nunc, Thermo Fisher Scientific, MA, USA), blocked, and incubated with recombinant Hic peptides diluted in blocking buffer. Thereafter, wells were incubated with mouse anti-Hic2 antiserum (in blocking buffer) followed by HRP-conjugated anti-mouse IgG (1:1000 in blocking buffer). In inhibition assays, the binding of a constant concentration of vitronectin (5 µg/ml) was measured to immobilised Hic2 in the presence of increasing concentrations of NaCl (100 µl of 0-1.0 M), heparin (100 µl of 0-5000 µg/ml), heparan sulfate (100 µl of 0-100 µg/ml), chondroitin sulfate A (100 µl of 0-1000 µg/ml), and hyaluronic acid (100 µl of 0-1000 µg/ml), respectively. In competition assays, vitronectin, used at different concentrations (100 µl of 0-1 µg/ml), was incubated with a constant amount of Factor H (10 µg/ml) and vitronectin-binding to immobilised Hic2 was measured. Similarly, Factor H was used at different concentrations (0-1 µg/ml), combined with a constant amount of vitronectin (5 µg/ml), and Factor H-binding to immobilised Hic2 was assayed. The reactions were developed with OPD and the absorbance was measured at 492 nm.

Surface plasmon resonance (SPR)

Direct protein–protein interactions were analysed by surface plasmon resonance (SPR) using a Biacore T100 optical biosensor (GE Healthcare, Germany). Covalent immobilization of vitronectin (5 µg/ml), Vn(80-396), Vn(80-339), and Vn(80-229) (each 25 µg/ml), or Hic2 (80 µg/ml) onto an NHS/EDC-activated carboxymethyl-dextran (CM5) sensor chip was performed as described previously (22, 23). The control flow cell was similarly prepared but in the absence of added protein. Binding of the analytes was performed in 0.05% Tween20/PBS (pH 7.4) or HNET buffer (50 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% Tween20, pH 7.5) at 25°C using a flow rate of 10 µl/min. Bound protein was removed between subsequent sample injections using 12.5 mM sodium hydroxide or 2 M sodium chloride. Each measurement was repeated at least three times. The representative sensorgrams show the response unit (RU) values after subtraction of the blank run (control flow cell) and values without analyte(s) from the corresponding sensorgrams. Binding was analysed using the BiacoreT100 Evaluation Software (Version 2.0.1.1).

Microscale Thermophoresis (MST)

Molecular protein-protein interactions in solution were analysed by microscale thermophoresis. Human vitronectin at a concentration of 13.3 µM was labeled with NT-647 using Nano Temper´s protein labeling kit RED-NHS (NanoTemper Technologies, Germany). A series of 1:1 dilutions (0.001-40 µM) of unlabeled Hic2 or BSA, as control, was added to 12.5 nM of NT-647-labeled vitronectin. Samples were diluted in MST buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM $MgCl_2$ and 0.05% Tween20, pH 7.6). Thermophoresis was measured at 60% LED power and 40% MST power for 30 s in a Monilith NT.115 instrument (NanoTemper Technologies, Germany). All measurements were performed at room temperature using hydrophilic capillaries.

Binding of bacteria to immobilised human proteins

Microtiter plates (F96, MaxiSorp™, Nunc, Thermo Fisher Scientific, MA, USA) were coated with human vitronectin, recombinant vitronectin fragments, Factor H, or BSA in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.2) at 4°C overnight. The surfaces of the wells were washed and subsequently blocked with 1% BSA/PBS (pH 7.4) for at least 3 h at RT. Labeling of bacteria with FITC was performed as described previously (29). Extensively washed FITC-labeled bacteria (100 µl of 2 x 10^8 bacteria) were added to the washed wells and incubated for 1 h at 37°C. Fluorescence was measured at 485 nm/520 nm (excitation/emission) using a microplate reader (Fluostar Omega, BMG Labtech, Germany) prior to the first washing step (total fluorescence of inoculated bacteria) and after each of the four washing steps with 100 µl PBS (fluorescence of bound bacteria).

TCC formation assays

Microtiter plates (F96, Medisorb™, Nunc, Thermo Fisher Scientific, MA, USA) were coated with Hic2 (100 µl of 5 µg/ml) or Factor H (100 µl of 50 µg/ml) over night at 4°C in PBS (pH 7.4). After washing with 0.05% Tween20/PBS (pH 7.4), the wells were blocked for 1 h at RT with 2% BSA/0.05% Tween20/PBS. Thereafter, vitronectin (100 µl of 0-50 µg/ml) was added to immobilised Hic2 and incubated for 1 h at RT. After washing, the terminal complement proteins C5b-6 (1.5 µg/ml) and C7 (1 µg/ml) were added. Following incubation for 10 min at RT, C8 (0.2 µg/ml) and C9 (1 µg/ml) were added to a total volume of 100 µl and the mixture was further incubated for 30 min at 37°C. TCC assembly was detected using mouse anti-human C5b-9 mAb and HRP-conjugated swine anti-mouse antibodies. The reaction was developed with OPD and the absorbance was measured at 492 nm.

To investigate whether vitronectin inhibits TCC formation on the pneumococcal surface, pneumococci were incubated with vitronectin (0-25 µg/ml) for 30 min at RT. Bacteria were

washed with 1% BSA/PBS, C5b-6 (1 µg/ml) was added for 5 min at RT and thereafter C7 (1 µg/ml), C8 (0.4 µg/ml) and C9 (1 µg/ml) were added. Following incubation for 30 min at 37°C, deposited C5b-9 was detected with mouse anti-human C5b-9 mAb and Alexa Fluor 647-conjugated swine anti-mouse antibodies. After washings the binding was analysed using a FACScan LRII™ flow cytometer and data acquisition was performed using FlowJo (Becton Dickinson, NJ, USA). Data analysis and gating of bacteria was conducted as described above.

Statistical analysis

All data are reported as mean ± SD. Results were statistically analysed using the unpaired two-tailed Student's test. A *P*-value ≤0.05 was considered statistically significant.

Results

Vitronectin binds to pspC11-expressing bacteria

Given the functional diversity of various pneumococcal surface-exposed proteins (e.g., class I PspC interacting with Factor H, C4b-binding protein (C4BP), secretory component of the human pIgR, vitronectin, and 67kDa-laminin receptor) (6, 15, 23, 30-32), we were interested whether the Factor H-binding protein Hic and related PspC11 proteins also bind vitronectin. Therefore, we first screened for *pspC*11 in genomic DNA samples of serotype 3 clinical isolates received from the NRZ, Germany (Table 2). By using the primers Hic4F and Hic5R (Table 3), a PCR fragment specific for *pspC11* (17) was amplified from chromosomal DNA of *S. pneumoniae* A66 and the serotype 3 clinical isolates, except for N025, whereas no PCR product was obtained from D39 genomic DNA (data not shown). The PCR products of the clinical isolates correspond to nucleotides 112-796 or amino acids 38-266 of the annotated sequences for Hic in *S. pneumoniae* A66 and related PspC11 proteins (Table 4), suggesting a

high conservation of this protein domain among PspC11 proteins. To define the *pspC*11 allele in the serotype 3 clinical isolates, the complete *pspC*11 gene was amplified with the primer pair NNHic and CCHic, sequenced and aligned using the primers listed in Table 3. Strikingly, when aligned against the amino acid sequence of Hic (PspC11.4), the N-terminal domain and the C-terminal domain, including the LPSTG motif, showed 100% identity, whereas the proline-rich domains varied in length and sequence.

In order to explore vitronectin-binding activity, strain A66 and *pspC*11-positive serotype 3 clinical isolates were pretreated with paraformaldehyde and glutaraldehyde to reduce the amount of capsule hindering protein-binding (15, 22, 24, 26, 29), incubated with increasing amounts of soluble vitronectin, and bound vitronectin was measured by flow cytometry. In addition, A66Δ*cps* and A66^{ccv}, a low-encapsulated variant of A66 recovered from the intracellular compartment of epithelial cells (24), were examined for binding of vitronectin. Vitronectin bound dose-dependently to the surface of all tested pneumococcal strains (Fig. 1, A and C). In addition, Factor H-binding to A66 and the clinical isolates was assayed. Factor H, similar to vitronectin, was recruited in a dose-dependent fashion (Fig. 1B). To prove that treatment of pneumococci with aldehydes does not interfere with the recruitment of human plasma proteins to the pneumococcal surface, A66Δ*cps* was treated with paraformaldehyde and glutaraldehyde and thereafter incubated with vitronectin and Factor H. Both human glycoproteins bound with comparable intensity to A66Δ*cps* when compared with untreated pneumococci (data not shown).

To verify if Hic recruits vitronectin to the pneumococcal surface, vitronectin-binding to A66Δ*cps* and its isogenic *hic* deletion mutant was analysed. Recruitment of vitronectin to the surface of Hic-deficient A66Δ*cps* was substantially reduced, but not completely diminished, compared with its isogenic A66Δ*cps* strain. In contrast, binding of Factor H was abolished in the Hic-deficient mutant (Fig. 1, D and E). In a complementary approach, pretreatment of pneumococci with specific anti-Hic serum diminished binding of vitronectin and Factor H,

whereas the control serum showed no effect (Fig. 1F). To demonstrate the direct role of Hic in vitronectin-binding to intact bacteria in the absence of other potential vitronectin-binding proteins, Hic was heterologously expressed on the surface of non-pathogenic, Gram-positive *Lactococcus lactis* and binding of vitronectin and Factor H, respectively, was examined by flow cytometry. The results revealed a significant binding of vitronectin and Factor H to Hic-expressing lactococci compared to control lactococci harboring only the vector pMSP3545 (Fig. 1G).

N-linked glycosylation is not required for binding of vitronectin and Factor H to pneumococcal Hic

To determine whether oligo-saccharide residues of vitronectin and Factor H are involved in the interaction with Hic, pneumococci were incubated with N-glycosidase F-treated or untreated vitronectin and Factor H, respectively, or with serum obtained from patients with glycosylation defects. Binding of deglycosylated vitronectin and Factor H to the pneumococcal surface was similar and did not differ significantly from the unmodified protein. Likewise, binding of Factor H or vitronectin derived from serum of patients with glycosylation defects was not significantly altered compared to normal human serum (Fig. 2).

Vitronectin and Factor H simultaneously bind to Hic

The PspC-like proteins of *S. pneumoniae* serotype 3 exhibit both vitronectin- and Factor H-binding activity. It has been suggested that the Factor H-binding region is localised to the N-terminal part of Hic, which displays high sequence homology to classical PspC proteins (11, 18). To determine whether both human glycoproteins bind simultaneously or compete for binding to Hic, binding of biotin-labeled vitronectin to A66Δ*cps* in presence of Factor H was assayed by flow cytometry. In this approach, Factor H did not inhibit the recruitment of

biotinylated vitronectin to the pneumococcal surface (Fig. 3A). Furthermore, the N-terminal part of Hic, comprising amino acid residues 38 to 245, was produced as His_6-tagged fragment Hic2 in *E. coli* (Fig. 3B), and employed in competitive ELISA. Vitronectin and Factor H simultaneously bound to immobilised Hic2, even when both proteins were applied at excess concentrations compared with the molar ratio that corresponds to their plasma levels, i.e., vitronectin:Factor H of 1:2.5 (Fig. 3, C and D). These results demonstrate that vitronectin and Factor H recognise different regions within the Hic protein.

Localization of the vitronectin and Factor H binding regions within Hic

To further characterise the Hic-vitronectin interaction and to localise the vitronectin- and Factor H-binding region in the Hic protein, amino- and carboxy-terminal truncated fragments of the mature Hic protein were produced in *E. coli* (Fig. 3B) and employed in binding studies. In a first attempt, binding of soluble Factor H and vitronectin, respectively, to immobilised Hic fragments was assayed by ELISA. Factor H bound dose-dependently to the Hic peptides Hic2, Hic5, and Hic6, whereas no binding to immobilised Hic7 was detected (Fig. 4A). In contrast, vitronectin bound dose-dependently to the Hic fragments Hic2, Hic5, and Hic7, whereas only minor binding was observed to Hic6 (Fig. 4B). When performed vice versa, binding of soluble Hic peptides to immobilised vitronectin and Factor H was examined. While Hic5 and Hic7 bound equally well to immobilised vitronectin, a remarkably reduced binding was detected for Hic6. In contrast to Hic5 and Hic6 showing similar binding activities, Hic7 bound with lower intensity to immobilised Factor H (Fig. 4C). Surface plasmon resonance (SPR) experiments were performed to verify and compare the binding of Hic fragments to immobilised vitronectin. In contrast to the other Hic peptides, Hic2 displayed a high binding activity as indicated by the high response values (R_{max} = 221.6 RU at 1 μM) and the slow dissociation of the Hic2-vitronectin complex upon removal of the Hic2 analyte (Fig. 4D).

Although similar association and dissociation profiles were obtained for the Hic peptides Hic5, Hic6, and Hic7, the maximal RU values at identical molarity (1 μM) markedly differed: for Hic5 and Hic7 similar R_{max} values were measured (107.1 RU and 105.4 RU, respectively), whereas Hic6 exhibited the lowest R_{max} value (64.9 RU) (Fig. 4E). These results strongly suggest that the N-terminal part of the Hic molecule comprising amino acid residues 38 to 92 is involved in Factor H-binding, whereas the central part of Hic comprising amino acid residues 151 to 201 is essential for the binding of vitronectin. The affinity of the interaction between Hic and vitronectin was defined by using SPR and microscale thermophoresis (MST). The equilibrium dissociation constant (K_D) of Hic2 for immobilised vitronectin was determined by SPR and calculated to be (1.51±0.56) $x10^{-6}$ M, assuming a simple 1:1 Langmuir binding mode (Fig. 4F). In a complementary approach, MST was performed to measure the affinity of Hic2 to soluble vitronectin. Here, the K_D was found to be (1.39±0.19) $x10^{-7}$ M (Fig. 4G).

Binding of Hic to vitronectin is mediated by ionic interactions and inhibited by glycosaminoglycans

To determine if ionic forces play a role in the interaction of Hic with vitronectin, the effect of sodium chloride on vitronectin-binding to Hic was assayed. Sodium chloride significantly affected binding of vitronectin to immobilised Hic2, e.g., at a concentration of 125 mM, NaCl reduced vitronectin-binding to Hic2 by almost 30% (Fig. 5A). To verify whether Hic competes with glycosaminoglycans for binding to vitronectin (20), the effect of heparin, heparan sulfate, chondroitin sulfate A, and hyaluronic acid on vitronectin-binding to immobilised Hic2 was investigated. In contrast to non-sulfated hyaluronic acid (Fig. 5E), the sulfated glycosaminoglycans heparin, heparan sulfate and chondroitin sulfate A dose-dependently inhibited the Hic2-vitronectin interaction (Fig. 5, B-D). For instance, heparin

used at a concentration of 1 µg/ml decreased vitronectin-binding by ~40% (Fig. 5B). These data suggest a charge-dependent interaction of Hic with the heparin-binding domain(s) of vitronectin.

The Hic-binding region is located within the C-terminal HBD of vitronectin

To pinpoint the heparin-binding domain (HBD) of vitronectin recognised by Hic, a series of truncated fragments spanning the amino acid residues 80-396 of vitronectin were heterologously produced in HEK293T cells (Fig. 6A) and used to analyse their binding to Hic. Binding of the recombinant vitronectin fragments to A66Δ*cps* and Hic-expressing lactococci was examined by flow cytometry. The vitronectin fragments Vn(80-396), Vn(80-373) and Vn(80-363) bound to Hic-expressing pneumococci. In contrast, vitronectin fragments lacking the complete C-terminal HBD (HBD3) (i.e., Vn(80-353), Vn(80-339), and Vn(80-229)) bound with low intensities to the pneumococcal surface, i.e., in the range of 2-10% compared to Vn(80-396) (Fig. 6B). A similar binding pattern was observed with Hic-expressing lactococci (Fig. 6C). In the complementary ELISA, immobilised Hic2 was incubated with the various recombinant vitronectin fragments. Vitronectin fragments consisting of amino acid residues 80-363, 80-373, and 80-396 bound to Hic2. In contrast, vitronectin fragments comprising amino acid residues 80-229, 80-339, and 80-353, respectively, did not interact with Hic2 (Fig. 6D). In addition, SPR measurements revealed a high binding intensity of Vn(80-396) to immobilised Hic2 as indicated by the prominent association and slow dissociation of the Hic2-Vn(80-396) complex upon removal of the analyte. In contrast, vitronectin fragments devoid of the complete HBD3 did not bind to Hic2

(Fig. 6E). Taken together, the results indicate that Hic interacts with the C-terminal HBD of vitronectin.

Binding of Hic to immobilised vitronectin requires an N-terminal region to the HBD3

The important role of human serum and extracellular matrix proteins as molecular bridges for *S. pneumoniae* and other human pathogens to integrin receptors on host cells has been studied extensively (6, 22, 33, 34). In addition, group I PspC of *S. pneumoniae* was recently shown to interact with both soluble and immobilised vitronectin by recognizing different conformational states of vitronectin (23). Therefore, binding of Hic to immobilised vitronectin was investigated. The adherence of FITC-labeled *S. pneumoniae* A66Δ*cps* to immobilised vitronectin and the control protein Factor H was analysed. FITC-labeled pneumococci adhered to immobilised vitronectin and Factor H. Importantly, anti-Hic2 antiserum significantly reduced pneumococcal adherence to immobilised vitronectin and also to Factor H (Fig. 7A). Interestingly, the mutant A66Δ*cps*Δ*hic* showed a strong reduction in adherence to immobilised vitronectin. Binding of this mutant to immobilised Factor H was also significantly reduced, however, the reduction was less pronounced when compared to vitronectin (Fig. 7A). In addition, recombinant lactococci exposing Hic on their surface efficiently bound to immobilised vitronectin and Factor H, compared to control lactococci carrying an empty vector (Fig. 7B). In order to verify whether the interaction of Hic with immobilised vitronectin requires additional sequence or structural elements N-terminal to the heparin-binding domain 3, binding of Hic to immobilised truncated vitronectin fragments was tested. FITC-labeled A66Δ*cps* and Hic-expressing recombinant lactococci adhered to the immobilised vitronectin fragments Vn(80–396), Vn(80–379), Vn(80–373), Vn(80–363), as well as to Vn(80–353) and Vn(80–339). In contrast, FITC-labeled bacteria did not bind to the

immobilised vitronectin fragment Vn(80-229) compared with BSA as a negative control (Fig. 7, C and D). Purified soluble Hic2 bound to immobilised vitronectin fragments interacting with Hic-expressing bacteria, while soluble Hic2 did not interact with immobilised Vn(80-229) (Fig. 7E). In SPR experiments, the vitronectin-binding Hic7 derivative shows considerable binding activities to immobilised Vn(80-396) and Vn(80-339), but did not reveal any interaction with immobilised Vn(80-229) (Fig. 7F). These data strongly suggest that the linker region between HBD2 and HBD3 is involved in Hic-binding to immobilised vitronectin.

The vitronectin-Hic complex inhibits TCC formation

The ability of vitronectin bound to Hic to impede TCC formation was assessed by ELISA. Vitronectin bound to immobilised Hic2 significantly reduced the generation of TCC. In contrast, Factor H did not affect TCC formation (Fig. 8A). In a complementary approach, TCC deposition on the surface of A66Δ*cps* and its isogenic *hic* mutant in the presence of increasing concentrations of vitronectin was analysed by flow cytometry. Vitronectin at a concentration of 25 µg/ml significantly inhibited TCC formation on the surface of both pneumococcal strains (Fig. 8B). Taken together, vitronectin bound to the pneumococcal surface - and to Hic - remains active and regulates the terminal complement pathway (33).

Discussion

The efficiency of *S. pneumoniae* to cause life-threatening infections is closely related to their ability to recruit human glycoproteins that interfere with the coagulation and complement system, facilitate attachment to and transmigration through tissue barriers, and modulate

inflammatory responses (5, 6, 10). Serotype 3 strain A66 recruits the complement inhibitor protein Factor H, via surface-exposed Hic, which renders the bacteria resistant to opsonophagocytosis (11, 18). This study demonstrates that Hic, a PspC11 subtype, and related PspC11 proteins specifically and simultaneously interact with vitronectin and Factor H. Both human glycoproteins are fluid-phase complement inhibitors, bind to integrins or to the extracellular matrix of various human tissues, and vitronectin is additionally implicated in haemostasis and angiogenesis (12, 20, 35).

Nucleotide and amino acid sequence analysis of the *pspC*11 gene of serotype 3 clinical isolates revealed four new PspC11 alleles (Table 4), whose N-terminal and C-terminal LPSTG-containing parts were highly conserved when compared with Hic (PspC11.4). In contrast, the proline-rich region showed considerable variability in length and amino acid composition. This has previously been shown for other PspC alleles and PspA, another surface-exposed choline-binding protein and important virulence factor of *S. pneumoniae* (17). Despite the diversity, the proline-rich regions of PspC and PspA are highly conserved compared to the N-terminal parts of both protein families and highly immunogenic, which has raised interest in the development of protein-based vaccines against pneumococcal infections (17, 36).

Binding of vitronectin and Factor H to PspC11-expressing clinical isolates and the A66 reference strain was monitored by flow cytometry. Both human plasma glycoproteins bound in a dose-dependent manner to the pneumococcal surface, after treatment with aldehydes (Fig. 1, A and B). However, the ability to recruit vitronectin and Factor H significantly increased when the capsular polysaccharide was absent or massively reduced as shown for the capsule mutant and the low-encapsulated variant of A66 (Fig. 1C). This effect has also previously been reported for vitronectin-binding to pneumococci expressing choline-bound PspC and for Factor H-binding to A66 (15, 22). Vitronectin-binding to the *hic* mutant was significantly reduced, though the decrease was less pronounced compared to the binding of Factor H and to

the pretreatment of A66Δ*cps* with anti-Hic antiserum (Fig. 1, D-F). The anti-Hic antiserum specifically recognizes surface-exposed Hic. However, the antibodies might also sterically impede binding of vitronectin to other putative binding partners resulting in the dramatic reduction of vitronectin acquisition (Fig. 1F). These data suggest that *S. pneumoniae* A66 expresses another yet unknown protein(s) binding to soluble vitronectin. However, the capacity of Hic to serve as a vitronectin- and Factor H-binding protein of *S. pneumoniae* was verified by using a heterologous *L. lactis* expression system. Lactococci expressing Hic on their surface showed significant vitronectin- and Factor H-binding activity (Fig. 1G). The expression of surface-exposed proteins cooperatively recognising the same host factor is a general strategy among pneumococci and other human pathogens. Acquisition of human plasma proteins and immune inhibitors facilitate colonization and survival, as was demonstrated, for instance, for fibronectin-binding adhesins of *S. pneumoniae* as well as vitronectin-binding proteins of *Haemophilus influenzae* and *Neisseria meningitidis* (37-45).

Deglycosylated vitronectin and Factor H retained their Hic-binding characteristics indicating that oligosaccharide residues within both glycoproteins are not required for recognition of vitronectin and Factor H by Hic-expressing pneumococci (Fig. 2). This was also demonstrated for vitronectin-binding to UspA2 of *M. catarrhalis* (46).

Factor H and vitronectin simultaneously interact with Hic either exposed on the pneumococcal surface or immobilised on a microtiter plate (Fig. 3) (11, 18). To narrow down the binding sites of Factor H and vitronectin in the Hic molecule, truncated Hic peptides were employed in complementary binding assays. The binding data suggest that vitronectin recognises the central part of Hic, most likely the amino acids 151-201, whereas Factor H-binding to pneumococci is likely to be mediated by the N-terminal part of Hic and classical PspC proteins showing >70% sequence identity (Fig. 4) (11, 18). A recent study demonstrated that classical PspC proteins interact with vitronectin via their repeat domains (referred to as R1 and R2). It was reported that the presence of two interconnected repeat domains,

represented by PspC3 proteins, rather than one repeat domain, represented by PspC2 proteins, increases vitronectin-binding efficiency (23). Hic and related PspC11 proteins share only minor sequence and structural homologies to the repeat domain of PspC2 and PspC3 proteins. This suggests that Hic and related PspC11 proteins have adopted a binding mode distinct from classical PspC molecules. The molecular interaction of Hic with vitronectin was analysed in detail and the affinity was in the micromolar range (Fig. 4, C-F). Comparable dissociation constants were reported for other bacterial vitronectin-binding proteins, such as *H. influenzae* Protein E (PE) and F (PF) and *M. catarrhalis* UspA2 (42, 43, 46).

Several human bacterial pathogens bind vitronectin via its heparin-binding domain(s) (HBDs), including PE, PF, and UspA2, as well as Opc of *N. meningitides* (25, 43, 45, 46). The decoration of the bacterial surface with vitronectin or the exploitation of host-cell bound vitronectin facilitates complement evasion and adherence to host cells, respectively (33). Sodium chloride and negatively charged sulfated glycosaminoglycans inhibited the interaction between Hic and vitronectin (Fig. 5), demonstrating the importance of charged amino acids for the interaction of Hic with at least one of the three HBDs of vitronectin. This has also been demonstrated for pneumococci expressing classical PspC and other pathogenic bacteria such as group B streptococci, staphylococci and *H. influenzae* (19, 23, 43, 47). To pinpoint the HBD recognised by Hic, a series of recombinant vitronectin fragments was used. Vitronectin fragments devoid of the complete C-terminal heparin-binding domain (HBD3) failed to interact with Hic (Fig. 6). It is noteworthy that this binding behavior resembles the interaction of classical PspC and other pathogenic bacteria with vitronectin (23, 25, 33, 43). As mentioned, Hic interacts with both the soluble and immobilised form of vitronectin (Fig. 6, Fig. 7, A and B). However, the binding studies suggested that immobilised vitronectin exposes an additional recognition site for Hic because also vitronectin fragment Vn(80-353), which lacks the C-terminal HBD3, bound to immobilised Hic but not to the soluble form (Fig. 6, Fig. 7, C-F). However, whether the HBD3 is dispensable or redundant for the binding of

Hic to immobilised vitronectin needs further investigation. Since a similar binding mechanism has been illustrated for classical PspC, it can be concluded that (i) similar binding profiles can be identified for polymorphic proteins within a family, and that (ii) *S. pneumoniae* discriminates between the soluble and immobilised form of vitronectin (23). Similar observations were reported for *H. influenzae* PE and PF, as well as for *S. pneumoniae* PavA and PavB recognizing both forms of fibronectin (37, 38, 42, 43). Although immobilization of vitronectin on artificial surfaces less likely results in similar multimer formation seen in natural matrices, the interaction of Hic with immobilised, host-cell-bound vitronectin may contribute to colonization and pathogenesis of PspC11-expressing serotype 3 pneumococci, as has been demonstrated for *S. pneumoniae* serotype 35A expressing PspC3 (22). Recruitment of soluble vitronectin to the pneumococcal surface can become highly relevant in terms of inhibition of the terminal complement pathway (20, 48). In general, *S. pneumoniae* and other Gram-positive pathogenic bacteria are naturally protected from complement-mediated lysis via their thick peptidoglycan cell wall and capsular polysaccharide. However, complement activation on the surface of Gram-positive bacteria, including *S. pneumoniae*, resulted in deposition of the terminal complement complex (TCC) at distinct sites on the bacterial cell (49). Furthermore, pneumococci in close contact to human host cells were shown to have a reduced amount of capsule, which could occasionally render them susceptible for complement attack (24). In addition, the TCC also evokes acute pro-inflammatory responses in human endothelial and epithelial cells such as secretion of pro-inflammatory cytokines and chemoattractants, induction of apoptosis, and leukocyte recruitment (50-52). Therefore, inhibition of complement activation and, in particular, TCC-formation might endow pneumococci with an increased capacity to cause invasive infections. Additionally, this might also prevent tissue-damaging effects on the host side. Vitronectin bound to pneumococci and pneumococcal Hic was functionally active and inhibited the TCC formation in a dose-dependent manner (Fig. 8), excluding a conformational change or interruption of the TCC

inhibitory domain of vitronectin upon Hic-binding (53). So far, no data on the surface proteome of *S. pneumoniae* A66 is available, and, consequently, the relative contribution of Hic in inhibition of the terminal complement pathway on the pneumococcal surface remains elusive. However, the results strongly suggest that pneumococci protect themselves from detrimental immune responses by sequestration of vitronectin, either directly to avoid deposition of TCC on their surface or indirectly to suppress pro-inflammatory events.

In conclusion, we show here that (i) the N-terminal part of Hic binds Factor H, (ii) vitronectin interacts with the central part of Hic, and (iii) the C-terminal heparin-binding domain (HBD3) within soluble vitronectin is recognised by Hic. Furthermore, a region N-terminal to the HBD3 is responsible for the interaction of Hic with immobilised vitronectin. Finally, the Hic-vitronectin complex is active in inhibiting the formation of the terminal complement complex. Taken together, the specific interaction of Hic and related PspC11 proteins with vitronectin and Factor H may contribute to the pathogenesis of serotype 3 pneumococci.

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Figure legends

Figure 1: The PspC-like protein Hic recruits vitronectin.

A-G) Dose-dependent recruitment of soluble vitronectin (Vn) and Factor H (FH) to Hic-expressing bacteria. Bound vitronectin was detected using anti-human vitronectin antiserum, followed by an Alexa Fluor 488-conjugated anti-rabbit IgG. Bound Factor H was detected using anti-human Factor H antiserum, followed by incubation with FITC-conjugated anti-goat IgG. Protein binding was analysed by flow cytometry. The results were expressed as GMFI x percentage of fluorescently labeled and gated bacteria. The mean values of at least three independent experiments are shown with error bars corresponding to SD. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

A, B) Encapsulated A66 and clinical isolates expressing Hic or related PspC11 proteins were treated with paraformaldehyde and glutaraldehyde prior to incubation with vitronectin (A) or Factor H (B).

C) *S. pneumoniae* A66Δ*cps* and A66^{ccv} were incubated with increasing concentrations of vitronectin.

D, E) Binding of vitronectin and Factor H to A66Δ*cps* (WT) and its isogenic Δ*hic* mutant. Pneumococci were incubated with increasing amounts of vitronectin and Factor H, respectively, and binding was measured by flow cytometry (D). Recruitment of vitronectin and Factor H to the pneumococcal surface is presented as histograms of a representative flow cytometric analysis. Grey histograms signify staining with primary and secondary antibodies in the absence of protein (Ab ctrl), and white histograms represent staining after incubation with vitronectin or Factor H at indicated concentrations (E).

F) Binding of vitronectin or Factor H was determined in the absence or presence of mouse anti-Hic2 serum and mouse control serum, respectively.

G) Nisin-induced recombinant *L. lactis* either harboring pMSP3545 (ctrl) or pHic (Hic) were incubated with 1 µg/ml vitronectin and Factor H, respectively.

Figure 2: N-deglycosylation of vitronectin and Factor H does not interfere with binding to pneumococcal Hic.

A) Immunoblot analysis of the deglycosylation of vitronectin (upper panel) and Factor H (lower panel) using N-glycosidase F. Equal amounts (1 µg) of untreated and

N-glycosidase F-treated human proteins were used. The control protein was not incubated at 37°C. M, PageRuler^{TM} prestained protein ladder.

B, C) *S. pneumoniae* A66Δ*cps* (1 x 10^8 bacteria) were incubated with glycosylated (untreated) or N-glycosidase F-treated vitronectin and Factor H (each 10 µg/ml), respectively, serum obtained from healthy volunteers (NHS, 1%) or patients with glycosylation defects (PHS, 1%). Bound vitronectin or Factor H was detected with rabbit anti-human vitronectin antiserum or goat anti-human Factor H antiserum, followed by incubation with Alexa Fluor 647-conjugated secondary antibodies. Protein binding was analysed by flow cytometry. The results were expressed as GMFI x percentage of fluorescently labeled and gated bacteria and are the mean values ± SD of three independent experiments (n.s., not significant) (B). Recruitment of native or degylcosylated vitronectin and Factor H to the pneumococcal surface is presented as histograms of a representative flow cytometric analysis. Grey histograms indicate staining with primary and secondary antibodies in the absence of protein (antibody control, Ab ctrl), and white histograms represent staining after incubation with N-glycosidase-treated vitronectin or Factor H (left panel) and normal human serum (NHS) or patient serum (PHS) (right panel), respectively (C).

Figure 3: Vitronectin and Factor H simultaneously bind to pneumococci and pneumococcal Hic.

A) Factor H does not interfere with vitronectin-binding to viable pneumococci. *S. pneumoniae* A66Δ*cps* (1 x 10^8 bacteria) were incubated with 1 µg/ml biotinylated vitronectin (bioVn) in the presence of increasing concentrations of Factor H (0-20 µg/ml). Bound vitronectin was detected using Alexa Fluor 488-conjugated streptavidin and binding was measured by flow cytometry.

B) Schematic model of the PspC subtype 11.4 (Hic) produced as His_6-tagged protein fragments in *E. coli*. Hic protein expression was induced with IPTG. The His_6-tagged proteins were purified by affinity chromatography and their purity was controlled after SDS-PAGE by Coomassie brilliant blue staining (the estimated molecular weight of the individual protein derivative is indicated). The mature domain of Hic is shown in dark grey. LP, leader peptide; LPSTG, sortase-motif; P, proline-rich sequence. M, SERVA unstained protein ladder (6.5-200 kDa).

C, D) Vitronectin does not compete with Factor H for binding to immobilised Hic2. Hic2 was coated on microtiter plates (50 µl of 10 µg/ml). In a total volume of 100 µl,

Factor H (10 µg/ml) was incubated with increasing concentrations of vitronectin (Vn, 0-1 µg/ml) (C), or vitronectin (5 µg/ml) was combined with increasing amounts of Factor H (0-1 µg/ml) (D). Bound vitronectin was detected with anti-human vitronectin antiserum, followed by incubation with HRP-conjugated anti-rabbit antibodies. Bound Factor H was detected using anti-human Factor H antiserum, followed by incubation with HRP-conjugated anti-goat IgG. 1,2-phenylenediamine dihydrochloride was used as a substrate and the absorbance was measured at 492 nm.
In A, C, and D, the mean values of at least three independent experiments are shown with error bars corresponding to SD. n.s., not significant.

Figure 4: Vitronectin and Factor H are recognised by different peptide domains within pneumococcal Hic.

A, B) Dose-dependent binding of soluble vitronectin and Factor H to immobilised Hic protein derivatives. Hic2 (50 µl of 10 µg/ml), Hic5, Hic6, Hic7 (each in equimolar amounts to Hic2), and BSA (50 µl of 10 µg/ml) were coated on microtiter plates and incubated with increasing concentrations (0-1 µg/ml in 50 µl) of Factor H (A) or vitronectin (B). Bound vitronectin and Factor H, respectively, were detected as described in Figure 3. Results are the mean values ± SD of at least three independent experiments.

C) Differential binding of soluble Hic peptides to immobilised vitronectin and Factor H. Vitronectin and Factor H (each 50 µl of 10 µg/ml) were coated on microtiter plates and incubated with Hic2 (50 µl of 10 µg/ml) or Hic5, Hic6, Hic7 (each in equimolar amounts to Hic2). Binding of Hic peptides was detected using anti-Hic2 antiserum, followed by incubation with HRP-conjugated anti-mouse IgG. The reaction was developed with OPD and the absorbance measured at 492 nm.

D-F) Binding of recombinant Hic fragments to immobilised vitronectin was analysed by surface plasmon resonance. Vitronectin was immobilised on a CM5 biosensor chip to a final rate of 300 response units (RU). The recombinant Hic fragments (Hic2, Hic5, Hic6, and Hic7) were used as analytes (at the concentrations of 1, 0.5, 0.25, 0.125, and 0.0625 µM, from top to bottom) in 0.05% Tween20/PBS (pH 7.4) at a flow rate of 10 µl/min. The affinity surface was regenerated between subsequent sample injections of proteins with 2 M NaCl. The amount of Hic-binding to immobilised vitronectin is shown in arbitrary response units. A control flow cell was used to subtract nonspecific signals (D). To compare the binding of Hic fragments to

immobilised vitronectin at equimolar concentrations, the response units (RU) of Hic2, Hic5, Hi6, and Hic7 (each at a concentration of 1 µM) were merged from the individual sensorgrams (E). The equilibrium response units (R_{eq}) obtained for binding of Hic2 to immobilised vitronectin were plotted against the concentration of Hic2, which allowed for estimation of the K_D (F).

G) Binding of Hic2 to NT-647-labeled vitronectin was analysed in fluid-phase using microscale thermophoresis. The normalised fluorescence (F_{norm} [‰] = fluorescence after thermophoresis/initial fluorescence) was plotted against increasing concentrations of Hic2 and, as control, BSA (each 0.001 µM to 40 µM), which permitted calculation of the K_D after non-linear regression. Results are the mean values ± SD of four independent experiments.

Figure 5: Binding of vitronectin to Hic is charge-dependent and inhibited by sulphated glycosaminoglycans.

A-D) Hic2 was coated on microtiter plates (100 µl of 5 µg/ml) and binding of vitronectin (100 µl of 5 µg/ml) was measured in the presence of increasing concentrations of NaCl (0-1 M) (A), heparin (0-100 µg/ml) (B), heparan sulfate (0-100 µg/ml) (C), chondroitin sulfate A (0-1000 µg/ml) (D), and hyaluronic acid (0-1000 µg/ml) (E), respectively. Bound vitronectin was detected as described in Figure 3. Results are shown as the mean values ± SD of at least three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Figure 6: The C-terminal heparin-binding domain HBD3 of vitronectin is recognised by pneumococcal Hic.

A) Schematic models of the recombinant C-terminally truncated vitronectin fragments produced in HEK293T cells. The three heparin-binding domains (HBDs) are depicted in black.

B, C) Binding of recombinant vitronectin fragments (each 5 µg/ml) to Hic-expressing *S. pneumoniae* A66Δ*cps* (B) and recombinant lactococci (C). Detection of bound vitronectin fragments was performed as described in Figure 1. The results were expressed as GMFI multiplied with the percentage of fluorescent and gated bacteria.

D) Binding of soluble recombinant vitronectin peptides (each 100 µl of 5 µg/ml) to immobilised Hic2 or BSA (each 100 µl of 5 µg/ml) was determined by ELISA. Bound vitronectin fragments were detected as described in Figure 3.

E) Hic2 was immobilised on a CM5 biosensor chip to a final rate of 1700 RU. The recombinant vitronectin fragments Vn(80-396), Vn(80-339), and Vn(80-229) were used as analytes in HNET buffer at a concentration of 50 µg/ml and a flow rate of 10 µl/min. The affinity surface was regenerated between subsequent sample injections of proteins with 2 M NaCl. The sensorgram shows the individual binding of each vitronectin fragment to immobilised Hic2 in arbitrary response units (RU).
In B-D, results are shown as the mean values ± SD of at least three independent experiments. **, $p \leq 0.01$; ***, $p \leq 0.001$.

Figure 7: Immobilisation of vitronectin exposes an additional Hic-binding site in a region N-terminal to the HBD3.

A, B) Contribution of Hic to bacterial adherence to immobilised vitronectin or Factor H. Binding of FITC-labeled A66Δ*cps* and A66Δ*cps*Δ*hic* in the absence or presence of anti-Hic2 or mouse control serum (A), and adherence of FITC-labeled recombinant *L. lactis* either expressing Hic (Hic) or carrying an empty vector (ctrl) (B) to immobilised vitronectin and Factor H.
C, D) Adherence of FITC-labeled A66Δ*cps* (C) and recombinant lactococci (D) to immobilised heterologously expressed vitronectin fragments.
E) Binding of Hic2 (100 µl of 5 µg/ml) to immobilised recombinant vitronectin fragments or BSA as negative control (each 50 µl of 10 µg/ml) was determined by ELISA. Bound Hic2 was detected as described in Figure 4.
In A-D binding of 2 x 10^8 FITC-labeled bacteria to immobilised vitronectin, Factor H, or recombinant vitronectin fragments (each 50 µl of 10 µg/ml) was measured at 485 nm/538 nm (excitation/emission) and the number of bound bacteria was calculated. And in A-E, the mean values ± SD of at least three independent experiments are shown. *, $p \leq 0.05$; **, $p \leq 0.01$; and ***, $p \leq 0.001$.
F) The recombinant vitronectin fragments Vn(80-396), Vn(80-339), and Vn(80-229) were immobilised on a CM5 biosensor chip to a final rate of 3400 RU, 1900 RU, and 1600 RU, respectively. Hic7 was used as an analyte at a concentration of 0.25 µM in 0.05% Tween20/PBS (pH 7.4) and a flow rate of 10 µl/min. Sodium chloride (2 M) was used to remove bound protein from the affinity surface. The sensorgram shows the binding of Hic7 to the respective immobilised vitronectin fragment or the control flow cell in arbitrary response units (RU).

Figure 8: Vitronectin bound to Hic inhibits TCC formation

A) Hic2 (100 µl of 5 µg/ml) or Factor H (100 µl of 50 µg/ml) was immobilised over night on a microtiter plate. Thereafter, immobilised Hic2 was pre-incubated with increasing concentrations of vitronectin (Vn; 100 µl of 0-50 µg/ml). Afterwards, the terminal complement proteins C5b-6, C7, C8, and C9 were sequentially added to the indicated wells, and TCC formation was detected using anti-human C5b-9 monoclonal antibodies followed by HRP-conjugated anti-mouse IgG. The reaction was developed using OPD and measured at 492 nm. Results are shown as the mean values ± SD of at least three independent experiments. **, $p \leq 0.01$; ***, $p \leq 0.001$.

B) *S. pneumoniae* A66Δ*cps* or the isogenic *hic* mutant (1 x 10^8 bacteria) was incubated with vitronectin (0-25 µg/ml). After washing, C5b-6, C7, C8 and C9 were sequentially added. Deposited C5b-9 was detected with mouse anti-human C5b-9 mAb and Alexa Fluor 647-conjugated swine anti-mouse antibodies and analysed by flow cytometry. The results were expressed as GMFI x percentage of fluorescently labeled and gated bacteria. Results are the mean values ± SD of three independent experiments with *p* values of 0.036 and 0.049, respectively, for 25 µg/ml versus 0 µg/ml vitronectin and 0.037 for A66Δ*cps*Δ*hic* versus A66Δ*cps*. n.s., not significant; *, $p \leq 0.05$.

Tables

Table 1: Strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference/source
Streptococcus pneumoniae		
P36	A66; serotype 3	NCTC7978
P257	D39; serotype 2	NCTC7466
P217	A66^{ccv}, nonencapsulated variant of A66, derived from epithelial cell line	(24)
PN456	A66Δ*cps* (Δ*cps*::Km^r)	(18)
PN457	A66Δ*cps*Δ*hic* (Δ*hic*::Cm^r)	(18)
Lactococcus lactis		
Lacto 2	strain MG1363, plasmid free and prophage-cured derivative of NCDO 712	(54)
Lacto 16	Lacto 2 pMSP3545	This study
Lacto 17	Lacto 2 pHic	This study
Escherichia coli		
DH5α	*Δ(lac)U169 endA1 gyrA46 hsdR17* Φ80Δ(lacZ)M15 *recA1 relA1 supE44 thi-1*	Novagen
BL21(DE3)	*E. coli* B RP F$^-$ *omp*T *hsdS*(r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* λ(DE3) *endA* Hte [argU proL Cm^r]	Stratagene
Plasmids		
pTP1	pET28a derivative, T7 promotor, IPTG inducible, TEV protease cleavage site, Km^r, Erm^r	(28)
pHic2	pTP1 derivative with nt 112-737 of the *hic* gene for expression, Km^r	This study
pHic5	pTP1 derivative with nt 112-603 of the *hic* gene for expression, Km^r	This study
pHic6	pTP1 derivative with nt 112-454 of the *hic* gene for expression, Km^r	This study
pHic7	pTP1 derivative with nt 274-603 of the *hic* gene for expression, Km^r	This study
pMSP3545	*nis*RK, P*nis*A promotor, ColE1, RepDEG, Erm^r	(55)
pHic	pMSP3545 derivative expressing *hic* from *S. pneumoniae* A66 under P*nis*A promotor control, Erm^r	This study

Km, kanamycin; Erm, erythromycin; Cm, chloramphenicol; r, resistant.

Table 2: Serotype 3 pneumococcal isolates obtained from the NRZ[a]

Isolate[b]	Source	Diagnosis, underlying disease
N025	bronchial secretion	pneumonia, COPD
N039	sputum	pneumonia
I2815	blood	pneumonia
I2830	blood	pneumonia
I2844	blood	pneumonia, prostate cancer, and CRI

a, National Reference Center for Streptococci, Aachen, Germany.
b, Non-invasive ('N') and invasive ('I') strains.
COPD, chronic obstructive pulmonary disease; CRI, chronic renal insufficiency.

Table 3: Primers used in this study

Primer	Sequence[a]	Restriction site	Purpose
NNHic	5'-TCGCCCATGGTTGCATCAAAAAACGAAAG-3'	*Nco*I	cloning, sequencing
CCHic	5'-GCTCTAGACTATTTCATTCTTTTCTTAGC-3'	*Xba*I	cloning, sequencing
PspCR2	5'-TTGCCATCAACTGGATCTATTTCAAATCTA-3'	none	sequencing
PspCF2	5'-CAGGGACTTATTACAACAAGTCG-3'	none	sequencing
PspCF4	5'-CAACAAACGAAAACAAGAAGCAAG-3'	none	sequencing
Hic4F	5'-GCGCGCTAGCACAGAGAAGGAGGTAACTACCC-3'	*Nhe*I	cloning
Hic5F	5'-GCGCGCTAGCCTCACAAAGTTGGGCGCAATTAAAAC-3'	*Nhe*I	cloning, sequencing
Hic5R	5'-GCGCAAGCTTATTTAGTGGAGGAGCCTGAATTCG-3'	*Hind*III	cloning, sequencing
Hic6R	5'-GCGCAAGCTTACTCTTTTTTTAAATTTTTCTCGATGTCATTC-3'	*Hind*III	cloning
Hic8R	5'-GCGCAAGCTTACTTGGCTTTTTTCTGAGCTTCTG-3'	*Hind*III	cloning, sequencing

a, Restriction sites are underlined.

Table 4: PspC alleles in *Streptococcus pneumoniae*

Protein	GenBank Accession Number	Sequence size (bp)[a]	Strain	Capsule type	Reference
Annotated alleles for PspC					
PspC3	ABJ53655	2106	D39	2	(56)
PspC11.1	AF276620	1839	G48	3	(17)
PspC11.2	AF276621	1779	G60	3	(17)
PspC11.3	AF276622	1311	3496	3	(17)
PspC11.4	AF252857	1839	A66	3	(17, 18)
PspC alleles identified in this study					
PspC11.5	HG934980	1641	N039	3	This work
PspC11.6	HG934981	2004	I2815	3	This work
PspC11.7	HG934982	1839	I2830	3	This work
PspC11.8	HG934983	1839	I2844	3	This work

a, Size refers to the length of the coding sequence.

Figures

Figure 1

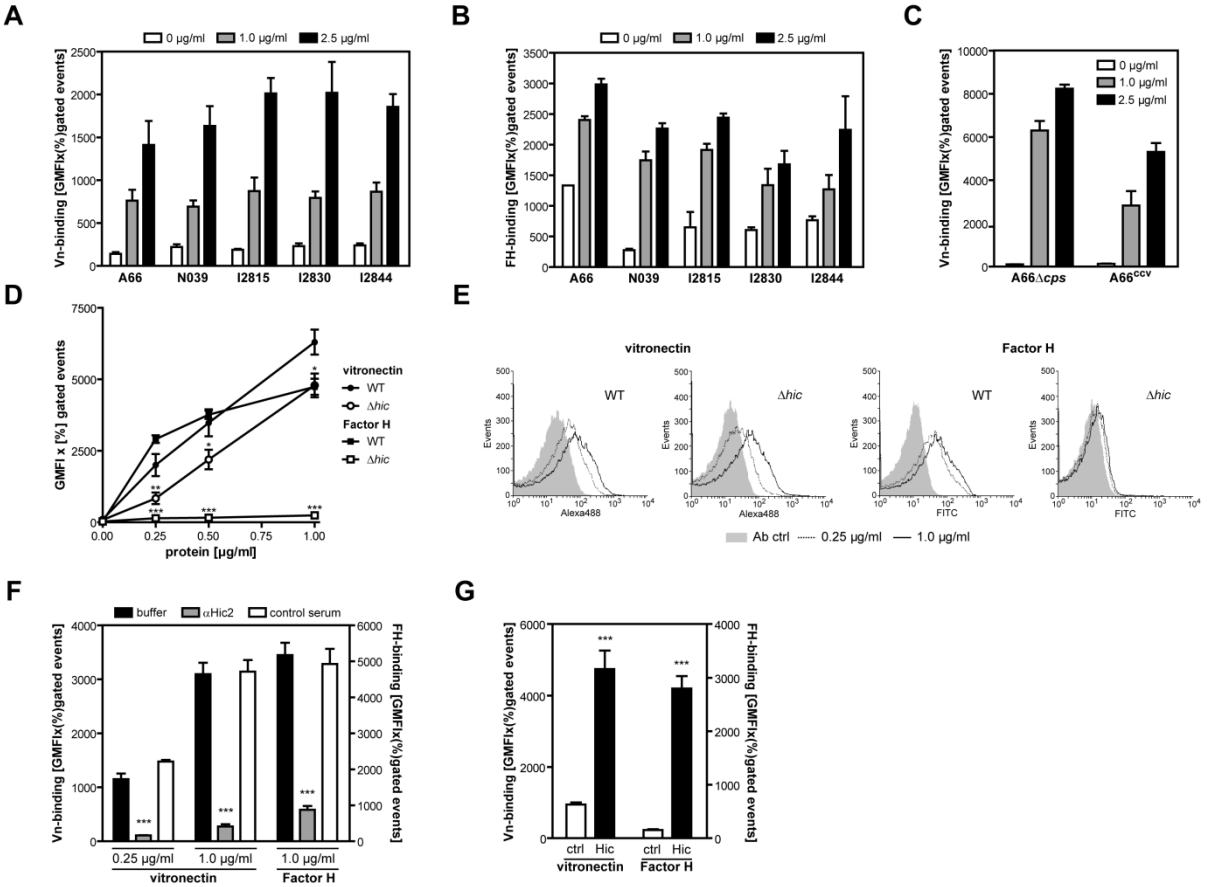
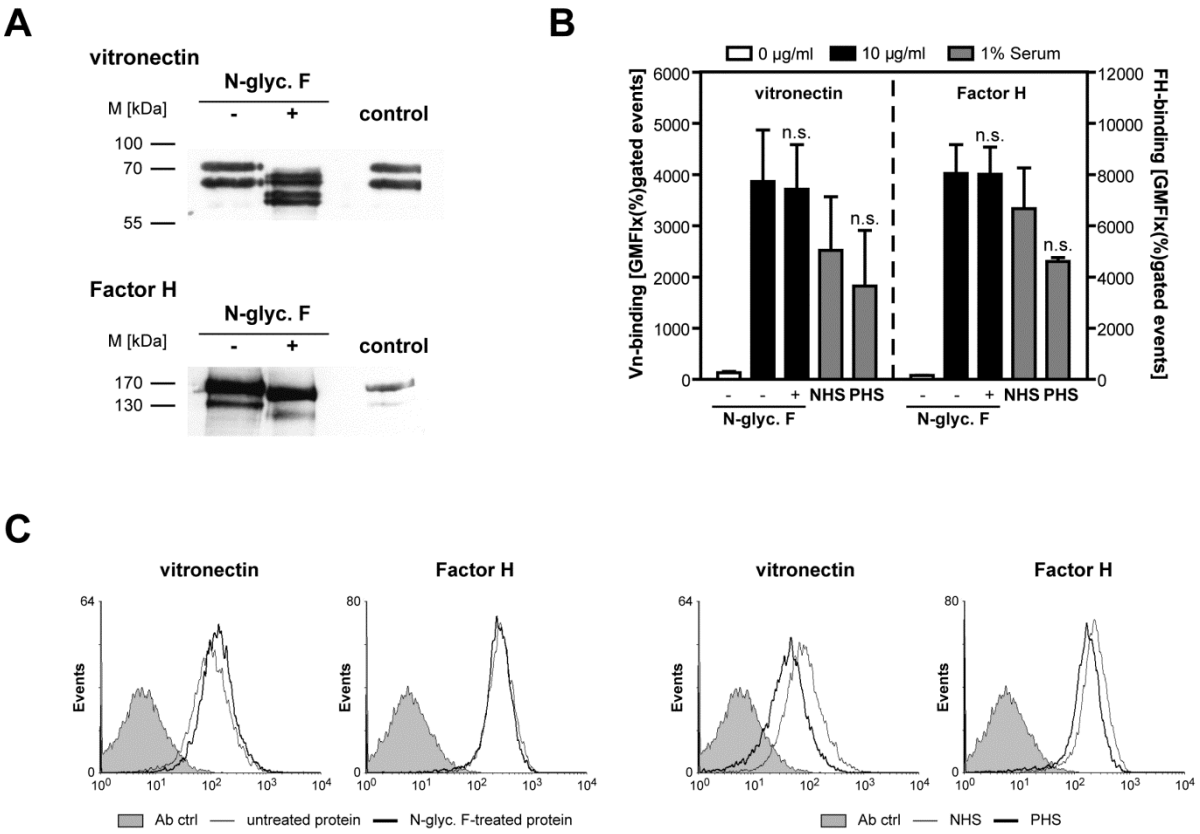


Figure 2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M [kDa]
-
+
control
100
70
55
Factor H
N-glyc. F
M [kDa]
-
+
control
170
130
B
0 µg/ml
10 µg/ml
1% Serum
Vn-binding [GMFIx(%)gated events]
FH-binding [GMFIx(%)gated events]
vitronectin
Factor H
n.s.
n.s.
n.s.
n.s.
- - + NHS PHS
N-glyc. F
- - + NHS PHS
N-glyc. F
C
vitronectin
Factor H
Events
Ab ctrl
untreated protein
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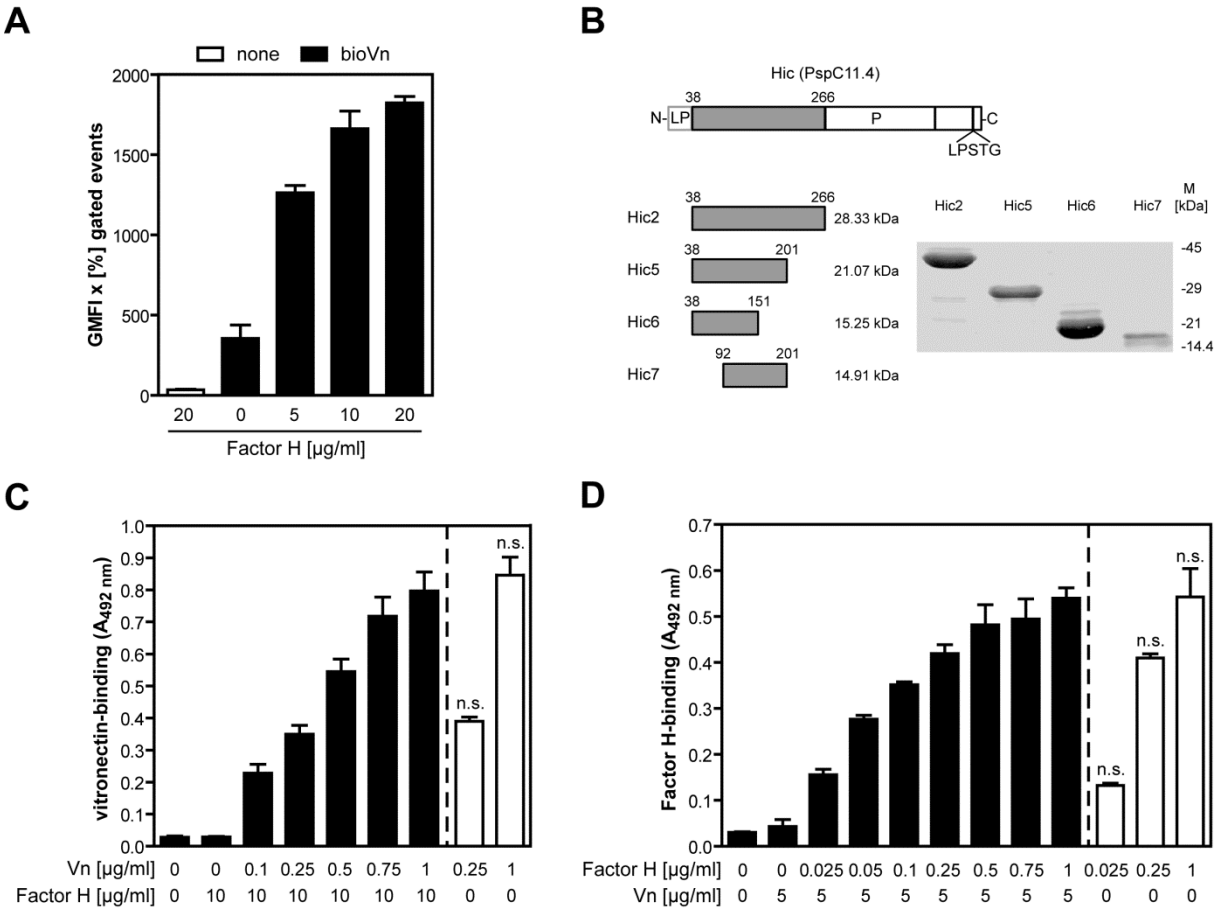
vitronectin
Factor H
Events
Ab ctrl
NHS
PHS

Figure 3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1500
1000
500
0
20 0 5 10 20
Factor H [µg/ml]
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Hic (PspC11.4)
38
266
N-
LP
P
C
LPSTG
Hic2
28.33 kDa
Hic5
21.07 kDa
Hic6
15.25 kDa
Hic7
14.91 kDa
M [kDa]
-45
-29
-21
-14.4
C
vitronectin-binding (A492 nm)
n.s.
Vn [µg/ml] 0 0 0.1 0.25 0.5 0.75 1 0.25 1
Factor H [µg/ml] 0 10 10 10 10 10 10 0 0
D
Factor H-binding (A492 nm)
n.s.
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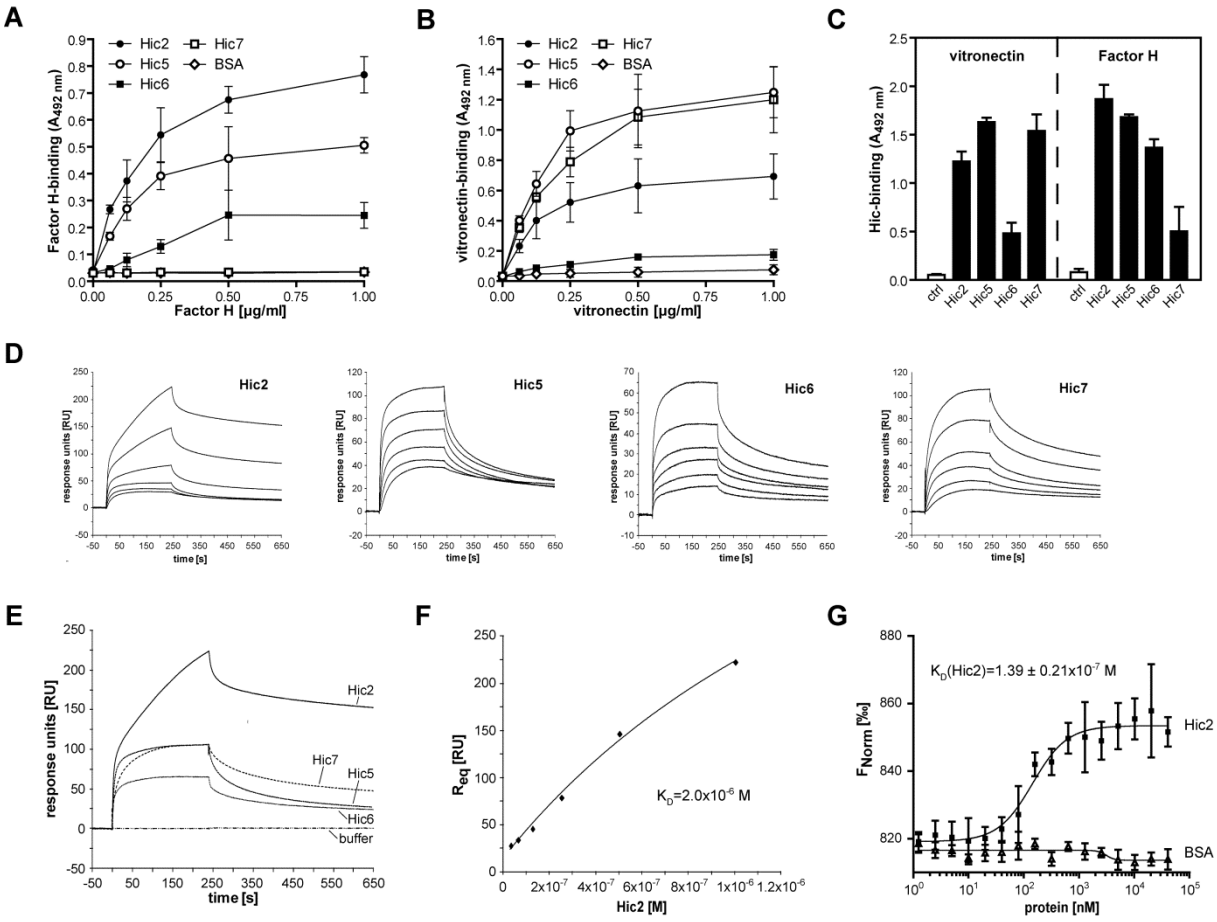
Figure 4


A
Hic2
Hic5
Hic6
Hic7
BSA
Factor H-binding (A492 nm)
Factor H [µg/ml]
B
vitronectin-binding (A492 nm)
vitronectin [µg/ml]
C
vitronectin
Factor H
Hic-binding (A492 nm)
ctrl
D
Hic2
Hic5
Hic6
Hic7
response units [RU]
time [s]
E
response units [RU]
buffer
time [s]
F
Req [RU]
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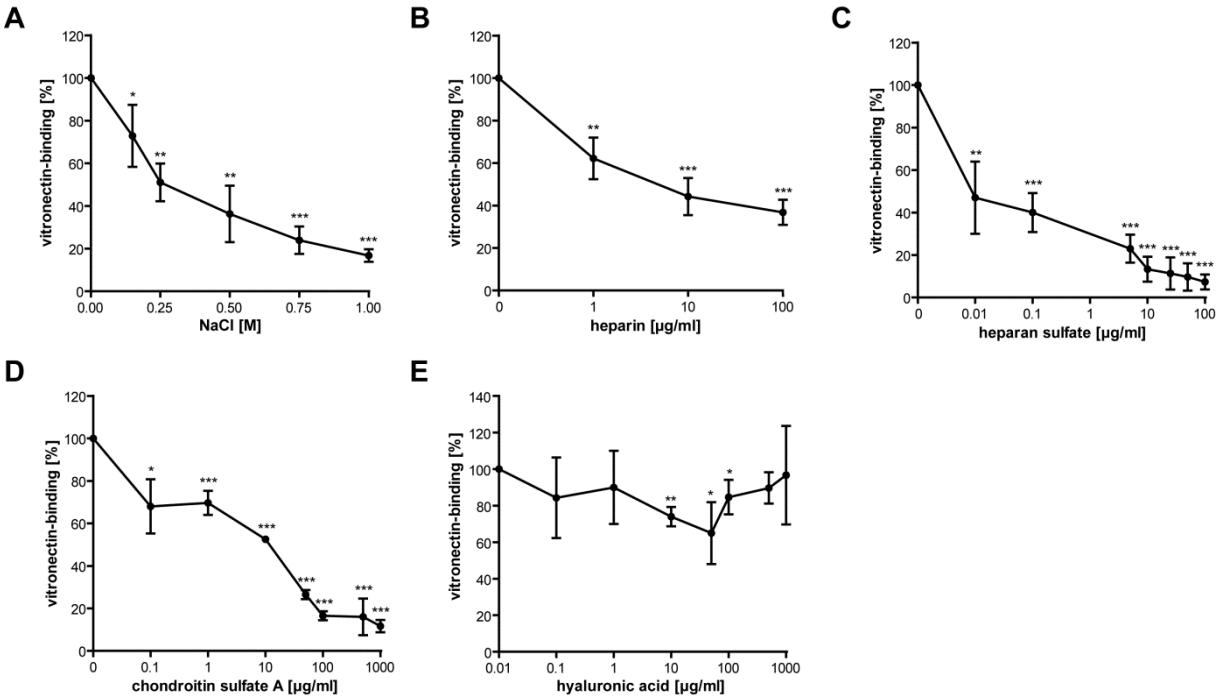
G
KD(Hic2)=1.39 ± 0.21x10-7 M
FNorm [‰]
protein [nM]

Figure 5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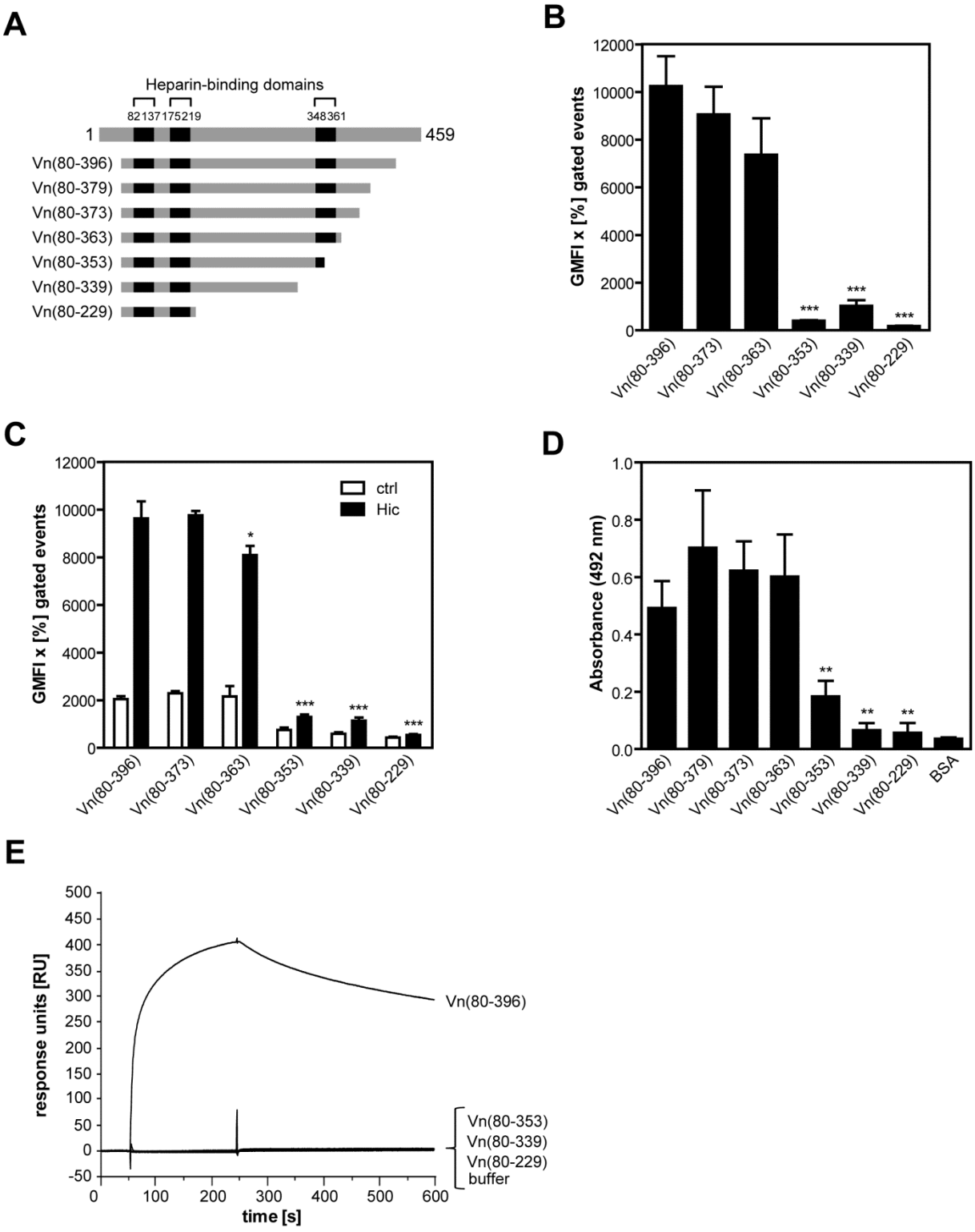
hyaluronic acid [µg/ml]

Figure 6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Heparin-binding domains
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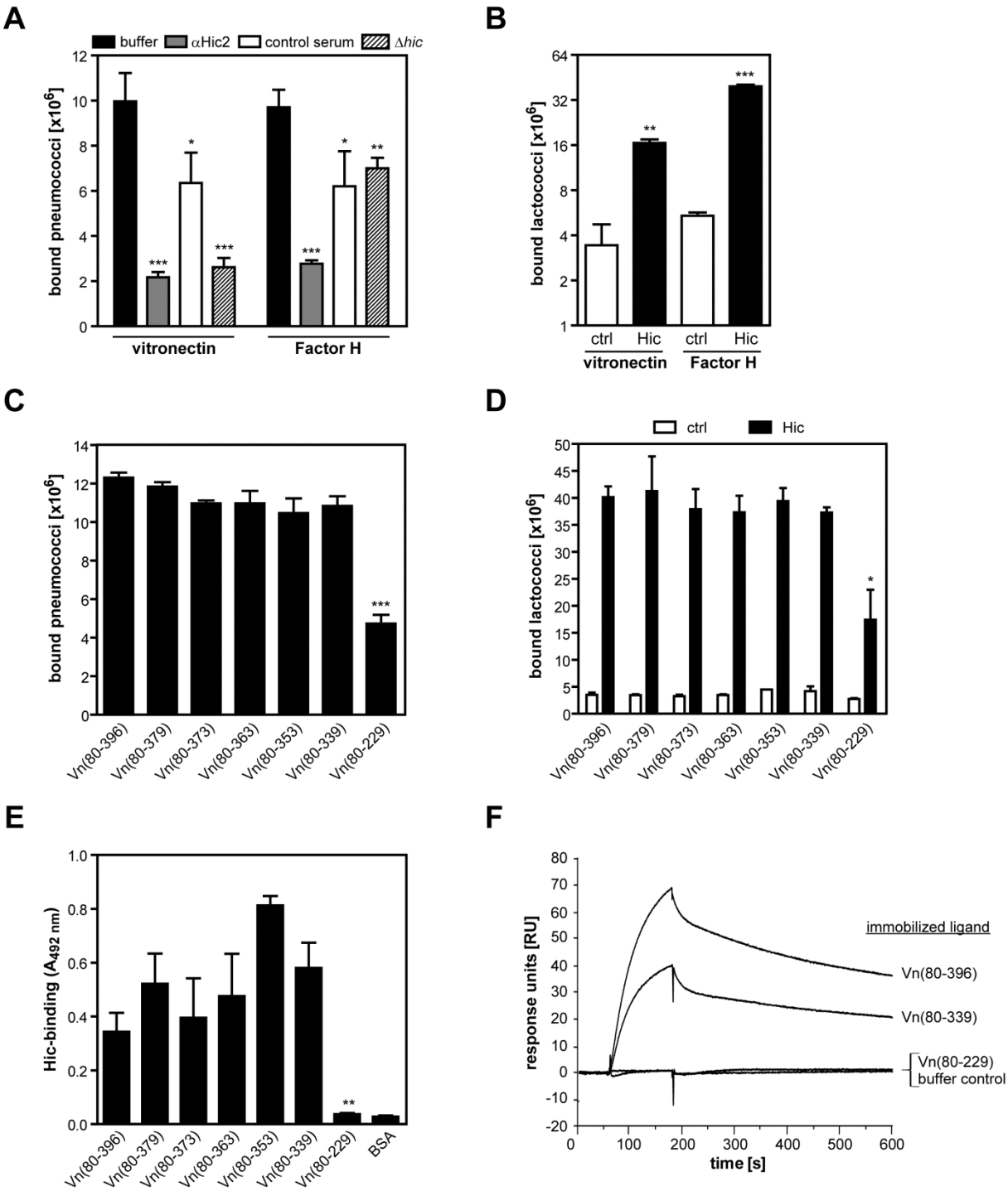
348 361
1
459
Vn(80-396)
Vn(80-379)
Vn(80-373)
Vn(80-363)
Vn(80-353)
Vn(80-339)
Vn(80-229)
B
GMFI x [%] gated events
12000
10000
8000
6000
4000
2000
0
***
***
***
Vn(80-396)
Vn(80-373)
Vn(80-363)
Vn(80-353)
Vn(80-339)
Vn(80-229)
C
GMFI x [%] gated events
ctrl
Hic
*
***
***
***
Vn(80-396)
Vn(80-373)
Vn(80-363)
Vn(80-353)
Vn(80-339)
Vn(80-229)
D
Absorbance (492 nm)
1.0
0.8
0.6
0.4
0.2
0.0
**
**
**
Vn(80-396)
Vn(80-379)
Vn(80-373)
Vn(80-363)
Vn(80-353)
Vn(80-339)
Vn(80-229)
BSA
E
response units [RU]
500
450
400
350
300
250
200
150
100
50
0
-50
0
100
200
300
400
500
600
time [s]
Vn(80-396)
Vn(80-353)
Vn(80-339)
Vn(80-229)
buffer

Figure 7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αHic2
control serum
Δhic
bound pneumococci [x10^6]
vitronectin
Factor H
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bound lactococci [x10^6]
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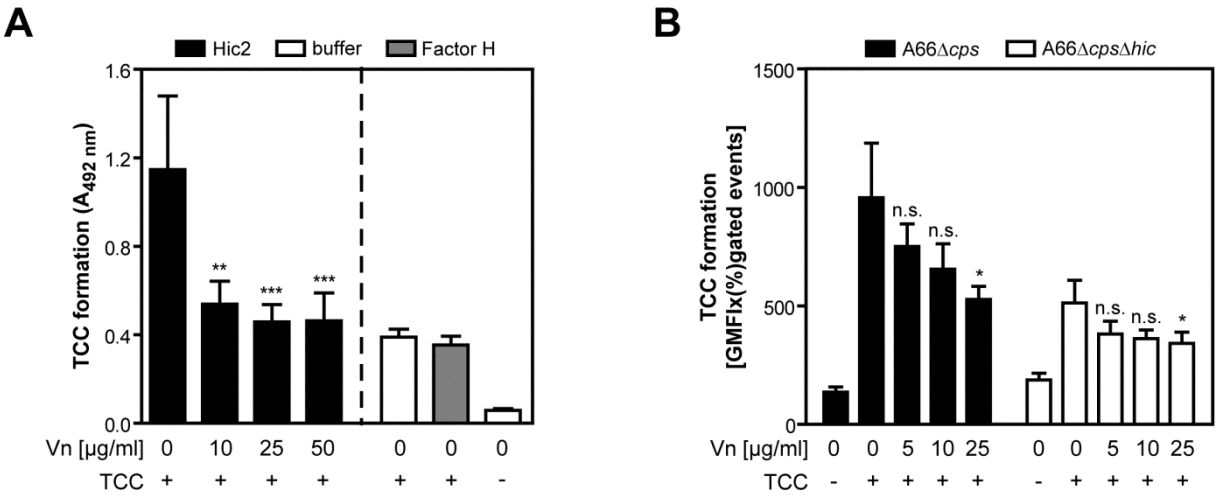
Hic
vitronectin
Factor H
C
bound pneumococci [x10^6]
Vn(80-396)
Vn(80-379)
Vn(80-373)
Vn(80-363)
Vn(80-353)
Vn(80-339)
Vn(80-229)
D
ctrl
Hic
bound lactococci [x10^6]
E
Hic-binding (A492 nm)
BSA
F
response units [RU]
time [s]
immobilized ligand
Vn(80-396)
Vn(80-339)
Vn(80-229)
buffer control

Figure 8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Hic2
buffer
Factor H
TCC formation (A492 nm)
1.6
1.2
0.8
0.4
0.0
**
***
***
Vn [µg/ml] 0 10 25 50 0 0 0
TCC + + + + + + -
B
A66Δcps
A66ΔcpsΔhic
TCC formation [GMFIx(%)gated events]
1500
1000
500
0
n.s.
n.s.
*
n.s.
n.s.
*
Vn [µg/ml] 0 0 5 10 25 0 0 5 10 25
TCC - + + + + - + + + +