Serotype 3 pneumococci sequester platelet-derived human thrombospondin-1 via the adhesin and immune evasion protein Hic

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Streptococcus pneumoniae serotype 3 strains emerge frequently within clinical isolates of invasive diseases. Bacterial invasion into deeper tissues is associated with colonization and immune evasion mechanisms. Thus, pneumococci express a versatile repertoire of surface proteins sequestering and interacting specifically with components of the human extracellular matrix and serum. Hic, a PspC-like pneumococcal surface protein, possesses vitronectin and factor H binding activity. Here, we show that heterologously expressed Hic domains interact, similar to the classical PspC molecule, with human matricellular thrombospondin-1 (hTSP-1). Binding studies with isolated human thrombospondin-1 and various Hic domains suggest that the interaction between hTSP-1 and Hic differs from binding to vitronectin and factor H. Binding of Hic to hTSP-1 is inhibited by heparin and chondroitin sulfate A, indicating binding to vitronectin, whereas PavB and Hic can bind simultaneously to hTSP-1. In conclusion, Hic binds specifically hTSP-1; however, truncation in the N-terminal part of Hic decreases the binding activity, suggesting that the full length of the α-helical regions of Hic is required for an optimal interaction.

The Gram-positive encapsulated Streptococcus pneumoniae (the pneumococcus) is typically a harmless colonizer of the human upper nasopharyngeal epithelium. In general, pneumococcal colonization occurs transiently and asymptptomatically. Importantly, pneumococci are facultative human pathogens, thus responsible for serious diseases ranging from mild local infections such as otitis media or sinusitis to more life-threat-
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To assess a direct interaction between pneumococcal Hic from serotype 3 strain A66 and hTSP-1, heterologously expressed His$_6$-tagged Hic proteins comprising either the factor H-binding domain (Hic5$^{AA38–201}$ and Hic6$^{AA38–151}$), the vitronectin-binding domain (Hic7$^{AA92–201}$), or both (Hic2$^{AA38–245}$) were used under static conditions in an ELISA (Fig. 1 A, C, E, and F). The purity of the recombinant Hic fragments was assessed by SDS-PAGE and subsequent Coomassie Brilliant Blue staining (Fig. 1D).

The results of the ELISA revealed a dose-dependent binding of all tested Hic peptides to immobilized hTSP-1 (Fig. 1E). The levels of Hic2 and Hic5 binding were similar when used in lower molecular ratios (related to hTSP-1), whereas Hic2 binding increased significantly at a molecular ratio of 1:5 in comparison to Hic5 (Fig. 1E). The Hic7 construct bound with lower efficiency to immobilized hTSP-1 compared with Hic2 when used in low molecular ratios and showed similar binding compared with Hic5 at high protein ratios. Hic6 showed the lowest hTSP-1 binding efficiency (Fig. 1E). Control experiments with immobilized factor H and recombinant Hic peptides verified previous findings (15). Vice versa, binding of soluble hTSP-1 to immobilized Hic or PspC3 SH13 (positive control) peptides was dose-dependent as well (Fig. 1F). In the range of 0.5–1 µg/ml hTSP-1, the glycoprotein exhibited affinity toward the Hic peptides in the following order Hic6 > Hic2 > Hic5 > Hic7, which changed to Hic2 > Hic6 > Hic5 > Hic7 (2.5–5.0 µg/ml hTSP-1) and Hic2 > Hic5 > Hic7 > Hic6 at high hTSP-1 concentrations (10–25 µg/ml hTSP-1). These data suggest that hTSP-1 preferentially binds to Hic2, which contains the complete predicted α-helical regions of the Hic molecule (Fig. 1B).

Human TSP-1 binds preferable to Hic2 as analyzed by SPR

To verify the results of the ELISAs and to conduct binding experiments under flowing condition, SPR experiments with immobilized hTSP-1 were performed. Soluble Hic fragments bound dose-dependently to immobilized hTSP-1 in a low micromolar range (Fig. 2A). The sensorgrams demonstrate a fast and stable association and a slow dissociation for Hic2 and Hic5 on a comparable level. For Hic6 a fast association and dissociation was observed. In contrast, Hic7 binding was characterized by a slower association and dissociation compared with the other Hic peptides. Strikingly, sensorgrams revealed the highest RU values of Hic7 binding to immobilized hTSP-1.
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[Graphs and images with data and molecular structures]

[Text descriptions of graphs and images]

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Figure 1. Natively purified Hic construct interact with hTSP-1. A, Hic peptide selection dependent on putative factor H-binding regions. Shown is the amino acid sequence of recombinant Hic2 (without His6 tag). Putative factor H-binding regions are highlighted in red, based on the publication of Jarva et al. (20). Recombinant Hic5/6/7 peptides are indicated with blue lines. B, secondary structure prediction for Hic. Secondary structure prediction for the recombinant Hic construct Hic2 (S. pneumoniae A66, residues 38–245) using Netsurfp and Jpret4. For Netsurfp a 50% threshold was set to determine the probability for α-helical regions. Matching predictions of both programs are shown as α-helices (yellow). C, schematic model of Hic of S. pneumoniae serotype 3 (A66) and heterologously expressed His6-tagged fragments. LP, leader peptide; P, proline-rich sequence; LPSTG, sortase anchoring motif. D, SDS-PAGE of heterologously expressed Hic fragments (Hic2, Hic5, Hic6, and Hic7) stained with Coomassie Brilliant Blue R250. Lane M, Fermentas-prestained protein ladder. E, dose-dependent binding of soluble pneumococcal Hic to immobilized hTSP-1 and human factor H (positive control). Human TSP-1 and factor H were coated on microtiter plates and, after blocking, incubated with increasing molecular ratios of recombinant Hic2 were analyzed by surface plasmon resonance spectroscopy. Sensorgrams show the dose-dependent binding of the human glycoproteins to immobilized Hic2. A CMS sensorchip was coated with recombinant Hic2, and the human proteins in PBS, 0.05% Tween 20 (pH 7.4) were used as analytes with a flow rate of 10 μl/min. The association and dissociation was observed, each of 300 s. The values of the control flow cell were subtracted from each sensorgram. Sensorgrams were iterated using a 1:1 ratio binding model, and the resulting constants are given in the figure.

Figure 2. Interactions of Hic part fragments with hTSP-1 under flow conditions. A, interactions of heterologously expressed pneumococcal Hic proteins with immobilized hTSP-1 were analyzed by surface plasmon resonance spectroscopy. Representative sensorgrams show the dose-dependent binding of the pneumococcal Hic constructs. A CMS sensorchip was coated with native hTSP-1, and the Hic constructs were used as analytes in PBS, 0.05% Tween 20 (pH 7.4) with a flow rate of 10 μl/min. The values of the control flow cells were subtracted from each sensorgram. Dissociation constant $K_D$ and $\chi^2$ values are calculated of at least three independent experiments. B, kinetics of the interaction between pneumococcal Hic and immobilized hTSP-1 or vitronectin. Interactions of natively purified hTSP-1 and vitronectin with immobilized Hic2 were analyzed by surface plasmon resonance spectroscopy. Sensorgrams show the dose-dependent binding of the human glycoproteins to immobilized Hic2. A CMS sensorchip was coated with recombinant Hic2, and the human proteins in PBS, 0.05% Tween 20 (pH 7.4) were used as analytes with a flow rate of 10 μl/min. The association and dissociation was observed, each of 300 s. The values of the control flow cell were subtracted from each sensorgram. Sensorgrams were iterated using a 1:1 ratio binding model, and the resulting $K_D$ and $\chi^2$ values are given in the figure.
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Table 1

| Fragment | $k_a^{1/2}$ (1/$M_\epsilon$)  | $k_d$ (1/$M_\epsilon$)  | $K_D$ (1/$M_\epsilon$)  | $R_{max}$ (RU)  | $\chi^2$  |
|----------|-----------------------------|------------------------|------------------------|-----------------|----------|
| Hic2_PBS | $5.069 \times 10^6 \pm 7.5 \times 10^4$ | $7.936 \times 10^{-4} \pm 8.8 \times 10^{-5}$ | $1.584 \times 10^{-9} \pm 2.5 \times 10^{-10}$ | $81.57 \pm 4.4$ | $9.5 \pm 2.1$ |
| Hic5_PBS | $5.351 \times 10^6 \pm 1.4 \times 10^4$ | $6.793 \times 10^{-4} \pm 2.4 \times 10^{-4}$ | $1.323 \times 10^{-9} \pm 5.7 \times 10^{-10}$ | $79.5 \pm 17.4$ | $9.0 \pm 3.6$ |
| Hic6_PBS | $8.814 \times 10^6 \pm 3.4 \times 10^4$ | $1.879 \times 10^{-3} \pm 6.0 \times 10^{-4}$ | $2.180 \times 10^{-9} \pm 1.9 \times 10^{-10}$ | $85.0 \pm 16.3$ | $14.3 \pm 3.1$ |
| Hic7_PBS | $2.161 \times 10^6 \pm 9.7 \times 10^4$ | $9.117 \times 10^{-3} \pm 1.7 \times 10^{-2}$ | $5.151 \times 10^{-7} \pm 9.7 \times 10^{-7}$ | $6165.8 \pm 11298$ | $69.5 \pm 21.8$ |

when using high protein concentrations and the lowest RU values at low protein concentrations confirming the results of the ELISA assays. To calculate the equilibrium dissociation constant $K_{D,Hic}$, kinetics were fitted to the 1:1 Langmuir binding model. The highest binding affinity was determined for Hic2 and Hic5 followed by Hic6 and Hic7 (Table 1). Unlike Hic 7, the $K_D$ was calculated in a nanomolar range for Hic2/5/6. Taken together, the results of the ELISA assays and SPR demonstrate that pneumococcal Hic interacts with hTSP-1, and binding efficiency correlates with the length of the $\alpha$-helical domain present in the various Hic peptides.

Interactions between pneumococcal Hic and hTSP-1 is inhibited by sulfated glycosaminoglycans and influenced by ionic forces

Heparin and chondroitin sulfate A are known to bind in the N-terminal domain of hTSP-1 and heparin additionally within the type I repeats (40, 41). The impact of both glycosaminoglycans and additional desulfated forms on the interaction of Hic with hTSP-1 was analyzed by measuring Hic2 binding to immobilized hTSP-1 in presence of increasing concentrations of heparin, chondroitin sulfate A, ODSH, or hyaluronic acid (Fig. 3A). The interaction of Hic2 and hTSP-1 was significantly diminished at a heparin concentration of 10 $\mu$g/ml and a chondroitin sulfate A concentration of 100 $\mu$g/ml (Fig. 3B). Interestingly, ODSH was able to diminish the interaction of Hic2 and hTSP-1 significantly at a concentration of 0.1 $\mu$g/ml (Fig. 3C). However, Hic binding was reduced by ODSH to $\sim$30% in contrast to heparin with $\sim$60% inhibition at a concentration of 1 mg/ml of the glycosaminoglycan. Hyaluronic acid inhibited Hic binding to hTSP-1 significantly only at a concentration of 1 mg/ml (Fig. 3D). The results of the competitive ELISA in the presence of increasing molarities of sodium chloride showed already a significant decrease in Hic2 binding at 62.5 mM (Fig. 3D). A physiological relevant salt concentration (125 mM) reduced the interaction of Hic2 and hTSP-1 by $\sim$20%.

Hic is involved in hTSP-1 recruitment to the pneumococcal surface

To analyze the impact of pneumococcal Hic for the recruitment of natively purified hTSP-1, S. pneumoniae A66Dcps and the isogenic hic mutant were incubated with increasing concentrations of platelet-derived hTSP-1. Flow cytometry analysis revealed a dose-dependent binding of hTSP-1 to pneumococci (Fig. 4A). Importantly, the isogenic hic mutant showed a significant reduction in hTSP-1 recruitment to the surface, resulting in a decrease in hTSP-1 binding up to 40%. Thus, these results indicate a contribution of Hic in the recruitment of soluble hTSP-1 to the cell surface of serotype 3 pneumococci.

To investigate the impact of Hic on hTSP-1 recruitment when exposed on a bacterial cell surface in the absence of other potential hTSP-1 binding candidates of S. pneumoniae, a Hic-expressing non-pathogenic Lactococcus lactis strain was employed in binding assays (15). The results of the flow cytometry assays showed a significant increase in hTSP-1 binding of Hic-expressing lactococci compared with the control lactococci harboring only the empty vector (Fig. 4B). These data confirm the adhesin Hic as a further hTSP-1 binding protein of serotype 3 pneumococci.

Competitive binding of hTSP-1 and vitronectin to pneumococcal Hic

The adhesin Hic of serotype 3 pneumococci exhibits vitronectin and factor H binding activity. It has been shown that factor H binds in the N-terminal part of Hic containing amino acids 38–92 (15). In contrast, vitronectin interacts with the central region of Hic comprising a domain between amino acids 151 and 201 and does not compete with factor H for binding to Hic (15). To investigate whether hTSP-1 and vitronectin or factor H bind simultaneously or compete for binding to immobilized Hic peptides, competitive ELISA assays were performed. The results demonstrate a dose-dependent inhibition of hTSP-1-binding in the presence of increasing molar ratios of vitronectin and vice versa (Fig. 5, A and B). Remarkably, the binding of hTSP-1 to immobilized Hic proteins was already inhibited in the presence of low molecular ratios of vitronectin (1:5, hTSP-1:vitronectin), whereas vitronectin binding was only significantly reduced in the presence of high molecular ratios of hTSP-1 (1:10, vitronectin:hTSP-1). These data indicate that hTSP-1 and vitronectin compete for binding to Hic and recognize similar domains within Hic. In addition, the data suggested that vitronectin has apparently a higher affinity to Hic compared with hTSP-1 in this approach. Competitive binding studies with soluble hTSP-1 and factor H demonstrated also a dose-dependent decrease in hTSP-1 binding to Hic by factor H (Fig. 5C). Human TSP-1 binding is inhibited already at low molecular ratios of factor H (1:0.5, hTSP-1:factor H) (Fig. 5, C and G). These results suggest that hTSP-1 binding involves also the factor H binding region of Hic. Factor H binding could not be inhibited by increasing molecular ratios of hTSP-1, which is probably due to the high affinity of factor H to pneumococcal Hic (Fig. 5, D and H). Based on the described direct interaction between hTSP-1 and factor H (Fig. 5F), binding of hTSP-1 was measured using an identical approach (Fig. 5E) (42, 43). An increased dose-dependent binding of hTSP-1 to Hic or factor H was detected. Importantly, hTSP-1 was not able to replace factor H from Hic (Fig. 5D). However, the increase in hTSP-1 binding in these assays could also be due to binding to the
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Serotype 3 pneumococcal Hic recruits matricellular hTSP-1

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The LPNTG-anchored adhesin PavB and the non-covalently attached surface protein PspC were shown to function as major Hic-binding partners of S. pneumoniae (21). To analyze whether Hic, PspC, and PavB bind to a similar region within Hic,2 binding to immobilized hTSP-1 in the presence of increasing molecular ratios of PavB (SSURE 1–5) and PspC (SH 13) was investigated by an ELISA (Fig. 6). The results revealed a significant reduction in Hic binding in presence of classical PspC, suggesting similar binding domains in Hic. Interestingly, PavB was not able to inhibit competitively binding of Hic2 to the glycoprotein hTSP-1; thus PavB and Hic differ in their binding behavior to hTSP-1.

Discussion

The ability of S. pneumoniae to trigger life-threatening diseases is implicitly linked to prior bacterial adhesion to human tissues. Preferentially, pneumococci interact with components of the extracellular matrix bound to host cell receptors (44). Among others, human hTSP-1 is a matricellular glycoprotein embedded in the complex network of the extracellular matrix and is exploited as bacterial attachment site (37). So far, the pneumococcal adhesins and virulence factors PavB and classical PspC proteins were shown to exhibit hTSP-1 binding activity (21). This study demonstrates that also the adhesin Hic, a PspC-like protein (PspC 11.4) of serotype 3 pneumococci, specifically binds hTSP-1.

Complementary protein-protein interaction studies under static and flow conditions demonstrated a dose-dependent binding of different N- and C-terminally truncated Hic peptides to purified hTSP-1. However, the ability to bind to immobilized hTSP-1 or to recruit soluble hTSP-1 was most efficient when the full-length of the α-helical regions of Hic was available.

Figure 3. Binding is charge-dependent and inhibited by sulfated glycosaminoglycans. A, structural formulas of sulfated and desulfated glycosaminoglycans. B-D, human TSP-1 (0.1 μg in 100 μl/well) was immobilized on microtiter plates (MaxisorpTM, Nunc) and incubated with a constant molecular ratio of recombinant Hic2 fragment (related to hTSP-1) in the presence of increasing concentrations of sodium chloride (0 – 0.5 M), heparin, chondroitin sulfate A, ODSH, or hyaluronic acid (0 – 1 mg/ml). Bound pneumococcal protein was detected using a polyclonal mouse anti-Hic antibody and a peroxidase-coupled secondary anti-mouse antibody. The mean values of at least three independent experiments are shown with error bars corresponding to S.D. The values of control wells without soluble overlay protein were subtracted from each measured value. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Figure 4. Hic recruits soluble human thrombospondin-1 to the bacterial cell surface. A and B, concentration-dependent binding of soluble hTSP-1 to S. pneumoniae (S. p.) A66Δcps, A66ΔcpsHic, and lactococci. Bacteria (2 × 10⁸) were incubated with increasing concentrations of hTSP-1 (0 – 25 μg/ml) in 100 μl of PBS. Binding of surface associated hTSP-1 was measured by flow cytometry using a specific polyclonal mouse anti-hTSP-1 antibody and secondary AlexaFluor® 488-conjugated anti-mouse IgG. Binding of hTSP-1 is shown as the geometric mean fluorescence intensity multiplied by the percentage of gated events (geometric mean fluorescence intensity (%) gated events). The mean values of at least three independent experiments are shown with error bars corresponding to S.D.* p < 0.05; **, p < 0.01; ***, p < 0.001.
Surface plasmon resonance analysis was examined using a mathematical iteration to a Langmuir binding model (1:1). The calculated dissociation constants were thereby in the nanomolar range (except for Hic7). Comparable dissociation constants were also reported for the pneumococcal hTSP-1 binding proteins PavB (SSURE1–5, $K_D: 5.55 \times 10^{-9}$ mol/liter) and PspC (SH13, $K_D: 1.10 \times 10^{-9}$ mol/liter) (21). The molecular mechanism of the Hic-hTSP-1 interaction is unknown so far. Nevertheless, further studies including microscale thermophoresis are needed to get a more detailed insight into the interaction of pneumococcal Hic and hTSP-1.

The staphylococcal hTSP-1 binding proteins Atl and Eap, as well the pneumococcal PavB and PspC, contain repeating structures that are essential for their hTSP-1 binding activity.
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The importance of Hic for the recruitment of hTSP-1 by pneumococci was demonstrated by flow cytometry. Acquisition of purified hTSP-1 to the hic mutant was significantly reduced. However, the deletion of Hic did not abolish completely hTSP-1 binding, suggesting additional hTSP-1 interaction partners on the surface of Hic-expressing pneumococci. A potential candidate is PavB, which possesses hTSP-1 binding activity and is also expressed by the serotype 3 A66 strain (data not shown) (21). The direct contribution of Hic in binding of hTSP-1 was further confirmed by using non-pathogenic lactococci heterologously expressing Hic. These Hic-expressing bacteria showed a significant enhanced capability to sequester soluble hTSP-1. Thus, the factor H and vitronectin binding adhesin Hic of S. pneumoniae further possesses hTSP-1 binding activity. A common scenario of human pathogens during colonization and infection is the simultaneous binding of the identical host factor by different surface-exposed adhesins. On the other hand, a single pneumococcal adhesin possesses multiple binding capacities for different human matrix proteins. Classical PspC, for instance, binds to the secretory component of the human polymeric immunoglobulin receptor, contributing to adherence and internalization of S. pneumoniae into host cells (46, 47). Additionally, C4BP, factor H, vitronectin, and hTSP-1 binding activity was proven for PspC, interfering pneumococcal immune evasion and adherence (17, 21, 48, 49).

To narrow down the binding site of hTSP-1 within Hic, hTSP-1 binding to Hic2 was tested in competitive binding assays with factor H and vitronectin. Both serum proteins inhibited hTSP-1 binding to immobilized Hic2 protein in a dose-dependent manner. Using peptide spot analysis, Jarva et al. (20) announced three putative factor H-binding domains within Hic comprising the Hic regions aa 39–57, 92–151, and 175–201 (Fig. 1A). However, binding studies with heterologously expressed Hic fragments showed a negligible interaction between factor H and the Hic7 construct (aa 92–201), whereby the factor H-binding region was located within aa 39–92 of Hic. Furthermore, Hic simultaneously binds vitronectin and factor

The heparin-binding domain of human matrix molecules like vitronectin, fibronectin, or thrombospondin-1 are involved in the interactions with different bacterial surface proteins (15, 17, 21, 45). The binding of classical PspC and non-classical PspC (Hic) to vitronectin occurs for instance via the C-terminal heparin-binding domain of vitronectin (15, 17). Furthermore, PavB and PspC binding to hTSP-1 was shown to be competitively inhibited by heparin. Similar, we were able to demonstrate that the interaction of pneumococcal Hic with hTSP-1 was affected by heparin. Also chondroitin sulfate A reduced Hic binding significantly, which is in accordance with previous results shown for classical PspC and hTSP-1 (21). Hence, previous studies, as well as our results in this study, support the hypothesis of a participation of the heparin binding domains of hTSP-1 (N-terminal domain, type I repeats) in the interactions with pneumococcal adhesins (Fig. 3, E–G). The minimal inhibition of Hic binding by desulfated glycosaminoglycans suggests the contribution of charged amino acids for the interaction with hTSP-1. Furthermore, increasing concentrations of sodium chloride decreased Hic binding to immobilized hTSP-1, suggesting the involvement of charged amino acids in the Hic-hTSP-1 interaction as previously shown for pneumococcal PavB and PspC. Similar, sodium chloride also inhibited the interaction between Hic and vitronectin (15). Positively charged amino acids of Hic could be located within the putative factor H-binding sites (20).

In contrast, Hic is, to our knowledge, the first bacterial hTSP-1 adhesin lacking repetitive domains. The heparin-binding domain of human matrix molecules like vitronectin, fibronectin, or thrombospondin-1 are involved in the interactions with different bacterial surface proteins (15, 17, 21, 45). The binding of classical PspC and non-classical PspC (Hic) to vitronectin occurs for instance via the C-terminal heparin-binding domain of vitronectin (15, 17). Furthermore, PavB and PspC binding to hTSP-1 was shown to be competitively inhibited by heparin. Similar, we were able to demonstrate that the interaction of pneumococcal Hic with hTSP-1 was affected by heparin. Also chondroitin sulfate A reduced Hic binding significantly, which is in accordance with previous results shown for classical PspC and hTSP-1 (21). Hence, previous studies, as well as our results in this study, support the hypothesis of a participation of the heparin binding domains of hTSP-1 (N-terminal domain, type I repeats) in the interactions with pneumococcal adhesins (Fig. 3, E–G). The minimal inhibition of Hic binding by desulfated glycosaminoglycans suggests the contribution of charged amino acids for the interaction with hTSP-1. Furthermore, increasing concentrations of sodium chloride decreased Hic binding to immobilized hTSP-1, suggesting the involvement of charged amino acids in the Hic-hTSP-1 interaction as previously shown for pneumococcal PavB and PspC. Similar, sodium chloride also inhibited the interaction between Hic and vitronectin (15). Positively charged amino acids of Hic could be located within the putative factor H-binding sites (20).

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H, and the binding domain for vitronectin comprises the region aa 151–201 of Hic (15). Human TSP-1 binding could be competitively inhibited in presence of vitronectin and factor H, supporting the idea that the complete α-helical regions of Hic are involved in the interaction with hTSP-1. Notably, hTSP-1 was not capable of reducing factor H binding to Hic, which can be explained by the high binding efficiency between factor H and Hic. Although a direct interaction between hTSP-1 and factor H is described, hTSP-1 may still have bound to the vitronectin-binding site within Hic. In contrast to interactions of Hic with vitronectin or factor H, the interplay between hTSP-1 and the pneumococcal adhesin seems to exhibit a new feature.

Although the hTSP-1 binding domains in bacterial hTSP-1 adhesins were narrowed down, the binding domain(s) of various hTSP-1 adhesins in the host protein have to be explored. To define similarities or differences in binding to hTSP-1, competitive inhibition experiments were conducted. Hic and classical PspC binding to immobilized hTSP-1 was decreased by chondroitin sulfate A and heparin, whereas PavB binding was inhibited only by heparin (21). In addition, PavB was not able to diminish the interactions between hTSP-1 and pneumococcal Hic. This suggests that PavB and classical PspC or Hic differ in their mechanism toward human thrombospondin-1. In contrast, Hic and PspC target identical or similar regions in hTSP-1 as suggested by a competitive inhibition of Hic binding to hTSP-1 and vice versa.

In conclusion, this study identified an additional important adhesive function of Hic and suggests that the three identified pneumococcal hTSP-1 adhesins differ in their mechanism to interact with hTSP-1. Interestingly, although Hic and classical PspC share only minor sequence homology, the polymorphic proteins within a family share a similar binding behavior, as already shown for interactions with human vitronectin (15, 17). Taken together, the PspC-like protein Hic of S. pneumoniae represents an important virulence factor and adhesin with multiple functions toward human ECM and plasma proteins including hTSP-1.

**Experimental procedures**

**Reagents and antibodies**

Human factor H and the polyclonal rabbit anti-human vitronectin antiserum were obtained from Complement Technology. Goat anti-human factor H IgG was purchased from Calbiochem (Merck Millipore). The ELISA HRP substrate O-phenylenediamine dihydrochloride and the secondary HRP-coupled goat anti-rabbit IgG were provided by DAKO (Germany). Secondary HRP-coupled goat anti-mouse IgG was ordered from Jackson ImmunoResearch Laboratories. Secondary Alexa Fluor 488-conjugated anti-mouse IgG was provided by Abcam. Paraformaldehyde, nisin, isopropyl β-D-thiogalactopyranoside, chondroitin sulfate A, hyaluronid acid, and Bradford Reagent were purchased from Sigma-Aldrich. Heparin (potassium salt) was obtained from ICN Biomedicals. ODSH was provided as a solution (50 mg/ml) by ParinGenix Inc. Protein marker was purchased from Fermentas (Thermo Fisher Scientific). Bovine serum albumin, Coomassie Brilliant Blue R250, and Tween 20 were provided by Roth. The thrombin receptor activator peptide 6 (TRAP-6) was obtained from Bachem. Anti-Hic2 IgG as well as polyclonal anti-TRAP-1 IgG was generated by intraperitoneal immunization of mice as described (15, 38). Specificity of the anti-Hic2 IgG against individual Hic peptides was tested in an ELISA format (Fig. 7C). The specificity of the anti-hTSP-1 IgG was evaluated using further human ECM and plasma proteins by dot blot and Western blotting analysis (Fig. 7, A and B).

**Bacterial strains and culture conditions**

*S. pneumoniae* A66Δcps and its isogenic hic mutant were cultivated on blood agar plates (Oxoid) supplemented with appropriate antibiotics (50 μg/ml kanamycin or 1 μg/ml chloramphenicol) at 37 °C and 5% CO₂ or in liquid Todd-Hewitt-Broth (Roth) enriched with 0.5% yeast extract (Roth) (THY) to mid-log phase (A₆₀₀ = 0.35–0.40). Recombinant *L. lactis* MG1363 strains were cultured as described recently (15). Briefly, lactococci were cultivated on M17 agar plates or in M17 broth (Oxoid) supplemented with 0.5% glucose and 5 μg/ml erythromycin at 30 °C under static conditions. By adding 1 μg/ml nisin to exponential phase grown bacteria (A₆₀₀ = 0.55–0.60) Hic protein expression was induced for 1.5 h. Recombinant *Escherichia coli* BL21 strains were grown on LB medium plates at 30 °C or in liquid LB medium in presence of 50 μg/ml kanamycin to mid-log phase (A₆₀₀ = 0.80) on an environmental shaker (Table 2).

**Isolation and purification of human thrombospondin-1 and vitronectin**

Human thrombospondin-1 was isolated from thrombin-activated human platelets and purified via heparin affinity chromatography as described (38). Purification of monomeric vitronectin from human plasma occurred using heparin affinity chromatography as published (50).

**Heterologous expression and affinity chromatographic purification of Hic fragments**

Recombinant Hic (GenBank™ accession no. AAG16729.1) part fragments used in this study have been described earlier (Fig. 1A and Ref. 15). N-terminally His₉-tagged proteins were purified from *E. coli* BL21 cell lysates via Ni²⁺ affinity chromatography with the Protino Ni-TED prepacked column kit after induction of protein expression with 1 mM isopropyl β-D-thiogalactopyranoside for 3 h at 30 °C. After purification according to the manufacturer's instructions (Macherey-Nagel), the proteins were

| Table 2 |
| --- |
| **Bacterial strains used in this study** |
| **Strain** | **Characteristics** | **Reference** |
| *S. pneumoniae* | | |
| PN456 | A66Δcps (Δgprg:kan') | Ref. 19 |
| PN457 | A66Δpic:Hic (ΔHic::erm') | Ref. 19 |
| *L. lactis* | | |
| LL16 | MG1363 pMSP3545 | Ref. 15 |
| LL17 | MG1363 pMSP3545-PspC11.4' | Ref. 15 |
| *E. coli* BL21 | | |
| pET-28TEV-Hic2 | | Ref. 15 |
| pET-28TEV-Hic5 | | Ref. 15 |
| pET-28TEV-Hic6 | | Ref. 15 |
| pET-28TEV-Hic7 | | Ref. 15 |
dialyzed (12–14-kDa molecular mass cutoff) against PBS (pH 7.4) at 4 °C overnight. The protein concentration was determined using the Bradford protein assay. The stability and purity of expressed proteins was monitored by SDS-PAGE and Coomassie Brilliant Blue staining.

**ELISA**

The interaction between recombinant pneumococcal Hic protein derivatives and native hTSP-1 was investigated by ELISAs. Cavities of microtiter plates (96-well, Maxisorp™; Nunc, Thermo Fisher Scientific) were coated with 0.1 μg/well hTSP-1 or human factor H as control in PBS (pH 7.4) overnight at 4 °C. The plates were washed three times with washing buffer (PBS, pH 7.4, 0.05% Tween 20), and the wells were blocked with blocking buffer (PBS, 0.1% Tween 20 supplemented with 1% BSA) for 1 h at room temperature. The washed wells were then incubated with increasing molecular ratios of recombinant Hic protein diluted in blocking buffer for 1 h at room temperature. Binding of Hic proteins was measured using a polyclonal anti-Hic IgG derived from mice (1:500 in blocking buffer) followed by incubation with a secondary goat anti-mouse IgG coupled to HRP (1:1000 in blocking buffer). As control, binding of Hic
peptides to the blocking solution or BSA alone was evaluated (Fig. 8). Vice versa, microtiter plates were coated with heterologously expressed Hic proteins and PspC3 SH13 as control in equimolar amounts related to Hic7 (0.5 μg/well). After washing with washing buffer the wells were saturated with blocking buffer followed by incubation with increasing concentrations of hTSP-1 (0–25 μg/ml) in blocking buffer. Binding of hTSP-1 was determined using a specific polyclonal mouse anti-hTSP-1 (1:500 in blocking buffer) antibody and a HRP-coupled second-ary anti-mouse antibody (1:1000 in blocking buffer).

In competition assays binding of hTSP-1 at a constant concentration (5 μg/ml) or vitronectin (1 μg/ml) to immobilized Hic peptides was measured in the presence of increasing molecular ratios of vitronectin (related to hTSP-1) or hTSP-1 (related to vitronectin). Glycoprotein binding was detected either with polyclonal mouse anti-hTSP-1 IgG (1:500 in blocking buffer) or polyclonal rabbit anti-human vitronectin antiserum (1:1000 in blocking buffer) followed by incubation with a secondary HRP-coupled anti-mouse IgG and a secondary alkaline phosphatase-coupled goat anti-rabbit IgG.

In a similar approach, binding of a constant concentration of hTSP-1 (2.5 μg/ml) or factor H (1 μg/ml) to immobilized Hic2 was measured in the presence of increasing molecular ratios of factor H (related to hTSP-1) or hTSP-1 (related to factor H). Binding of human proteins was detected using either polyclonal mouse anti-hTSP-1 IgG (1:500 in blocking buffer) or polyclonal goat anti-human factor H IgG (1:500 in blocking buffer) followed by incubation with a secondary HRP-coupled goat anti-mouse IgG or HRP-coupled rabbit anti-goat IgG. Inhibition assays, binding of Hic2 to immobilized hTSP-1 (0.1 μg/well) was monitored in the presence of increasing concentrations of sodium chloride (0–0.5 M), heparin, chondroitin sulfate A, ODSH, or hyaluronic acid (each 0–1 mg/ml) in blocking buffer. To detect binding of Hic, a polyclonal mouse anti-Hic IgG and a secondary HRP-coupled goat anti-Hic IgG were used. As control, binding of heparin to Hic2 was evaluated by dot blot analysis (Fig. 3).

In competition assays with pneumococcal hTSP-1 binding proteins, a constant molecular ratio of Hic2 to immobilized hTSP-1 (0.1 μg/cavity) was used in the presence of increasing molecular ratios of PavB SSURE1–5 or PspC3 SH13 (related to Hic2). Detection of Hic2 binding occurred as described. O-Phe-nylendiamine dihydrochloride was used as HRP substrate, and color reaction was measured at an absorbance of 492 nm using a FLUOstar Omega Fluorometer (BMG Labtech). The values of control wells without soluble overlay protein were subtracted from each measured value in ELISA experiments.

**SPR spectroscopy**

Direct protein-protein interactions between native hTSP-1 and His6-tagged pneumococcal Hic derivate were performed under flow conditions by SPR using a BiacoreT100 optical bio-

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**Figure 8. Interaction between Hic2 and BSA.** A, microtiter plate (Maxisorp™; Nunc) coated with blocking solution containing increasing concentrations of BSA followed by incubation with Hic2. Binding of Hic2 was detected using a polyclonal anti-Hic2 IgG followed by a secondary HRP-coupled goat anti-mouse IgG. The results are illustrated as mean values of one experiment with duplicates. B, interactions of hTSP-1, factor H, and BSA with immobilized Hic2 were analyzed by surface plasmon resonance spectroscopy in manual runs. Therefore, a CM5 sensorchip was coated with native Hic2, and kinetics were conducted in PBS, 0.05% Tween 20 (pH 7.4) with a flow rate of 10 μl/min. The values of the control flow cells were subtracted from each sensorgram. C, binding of Hic2 (10 μg/ml) to immobilized BSA and factor H. Bound Hic was detected using a polyclonal anti-Hic2 IgG and a secondary alkaline phosphatase-coupled goat anti-mouse IgG and NBT/BCIP as substrate.
Serotype 3 pneumococcal Hic recruits matricellular hTSP-1

sensor (GE Healthcare). Human thrombospondin-1 was covalently immobilized as ligand on a NHS/EDC-activated carboxymethyl dextran (CM5) sensor chip as described (38). The final value received after immobilization for hTSP-1 was 1400 RU. The control flow cell was treated similar without adding a ligand. Binding studies were performed in PBS containing 0.05% Tween 20 at 25 °C using a flow rate of 10 μl/min. Both association and dissociation of analytes were observed for 300 s. Each interaction was measured in at least three independent kinetic assays. Vice versa, hTSP-1 and vitronectin binding to immobilized Hic2 was analyzed using a CM5 sensor chip immobilized with Hic2 as described before (Fig. 2B) (15). The data were analyzed using BiacoreT100 evaluation software (version 2.0.1.1).

Flow cytometric analysis

The recruitment of hTSP-1 to viable pneumococci or lactococci was detected by using a flow cytometry-based analysis. Briefly, a suspension containing 2 × 10⁶ bacteria was incubated with native purified hTSP-1 in a volume of 100 μl for 30 min at 37 °C and 5% CO₂ in 96-well plates (U-bottom; Greiner Bio-One). After washing with PBS, 0.5% FCS, hTSP-1 binding was analyzed using a polyclonal mouse anti-hTSP-1 IgG (1:500 in PBS, 0.5% FCS) followed by a secondary Alexa Fluor 488-conjugated anti-mouse IgG. Finally, bacteria were washed and fixed with 1% paraformaldehyde in PBS, 0.5% FCS overnight at 4 °C, and fluorescence intensity was measured using a FACSCalibur™ flow cytometer and CellQuestPro 6.0 (Becton Dickinson) for data acquisition. 50,000 bacteria were detected using log forward and log side scatter dot plots containing a gating region to exclude lager aggregates of bacteria and debris. Data analysis was performed using Flowing software 2.5.0 (Turku Centre for Biotechnology, Turku, Finland). The results of human protein binding to bacteria are shown as the geometric mean fluorescence intensity (GMFI) of the gated bacterial population multiplied by the percentage of labeled bacteria.

Statistical analysis

All data are reported as means ± S.D. Statistical analysis was performed using the unpaired two-tailed Student’s t test. Platelet assays were analyzed with paired Student’s t test. In all analysis a p value of <0.05 was considered statistically significant.

Author contributions—U. B. wrote the paper, performed experiments, and supervised J. H.; T. P. K. supervised U. B. and wrote the paper; K. K. and J. H. executed experiments; S. K. contributed to conception of the study; H. S. was the supervisor of K. K. and was involved in experimental design; and S. H. wrote the paper and was the project leader. All authors reviewed the results and approved the final version of the manuscript.

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