Characterization of the interaction of the pneumococcal surface protein SpsA with the human polymeric immunoglobulin receptor (hpIgR)

Christine Elm, Manfred Rohde, Jean-Pierre Vaerman, Gursharan S. Chhatwal & Sven Hammerschmidt

Department of Microbial Pathogenicity, GBF-National Research Centre for Biotechnology, 38124 Braunschweig, Germany

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Background & objectives: The polymeric immunoglobulin receptor (pIgR) is produced by mucosal epithelial cells and plays a crucial role in mucosal immunity. At the basolateral surface of mucosal cells, the pIgR binds predominantly polymeric immunoglobulins, such as dimeric IgA and polymeric IgA (pIgA) and mediates their transport across the polarized cells. This results in apical release of secretory component (SC), either free or bound covalently to IgA, forming secretory IgA (SIgA). The choline-binding protein (Cbp) SpsA, also called PspC and CbpA, has been shown to interact with the pIgR. A hexapeptide motif in SpsA was identified as the minimal binding motif required for binding specifically to pIgR and SC. The present study was carried out to show that the hexapeptide motif in SpsA is crucial for the interaction of pneumococci and pIgR-expressing cells.

Methods: Streptococcus pneumoniae were cultured to mid-log phase. Calu-3 cells and MDCK epithelial cells, stably transfected with the hpIgR cDNA in pCB6 were used in in vitro infection experiments. Pneumococcal adherence to and invasion of epithelial cells were assayed.

Results: By the use of the N-terminal domain of SpsA and SpsA201, which exhibits a single amino acid substitution in the pIgR-binding motif, in vitro assays indicated the association of the identified hexapeptide motif, located between amino acid 198 and 203 in SpsA, with pneumococcal adherence to and invasion of hpIgR-expressing cells.

Interpretation & conclusion: The present findings demonstrated not only the crucial role of the hexapeptide of SpsA, not only for the SpsA-pIgR interaction, but also for adherence and invasion of hpIgR-expressing cells.

Key words Choline binding protein - polymeric IgA receptor - Streptococcus pneumoniae

Streptococcus pneumoniae is an important pathogen colonizing the upper and lower respiratory tract of humans and capable to cause relatively harmless local infections as well as life-threatening pneumonia and meningitis1. Dissemination of pneumococci starts at the nasopharynx after penetration of epithelial cells. Recently, the Cbp SpsA was identified as the bacterial adhesin of the pIgR. The latter is synthesized as an integral membrane protein in the rough endoplasmic reticulum of mucosal epithelial cells and comprises a pIgA-binding extracellular region, a membrane spanning domain and a cytoplasmic C-terminal tail2. The receptor is sorted to the basolateral pole of mucosal epithelial cells and binds there non-covalently to pIgA. The complex is endocytosed into vesicles which are translocated to and fuse with the apical surface of the cells. A proteolytic cleavage of the extracellular portion of the pIgR, also known as secretory component (SC), then results in release of SC, either free or covalently coupled to pIgA, forming SIgA3,4. Among known Cbps, the SpsA protein
exhibits a unique interaction with free SC or the SC portion of pIgR and SIgA. The pathogenic significance of this interaction is underlined by the fact that SpsA provides a mechanism for adherence to nasopharyngeal pIgR-expressing cells. In addition to the efficient colonization, the interaction was also associated with translocation of pneumococci across an epithelial barrier by adopting in a reverse way the pIg receptor transcytosis machinery\(^5\). In addition, a pneumococcal mutant with deficient expression of SpsA displayed a reduced ability to colonize the nasopharynx of rats\(^6\) and showed a significant decrease of nasopharyngeal colonization in pIgR-KO mice\(^5\). A hexapeptide motif YRNYPT was identified as the minimal pIgR/SC-binding motif using synthetic peptide technology\(^7\). The present study shows that this hexapeptide is crucial for the interaction of pneumococci with the pIgR, and for their in vitro adherence to and invasion of epithelial cells expressing the hpIgR.

**Material & Methods**

**Bacterial strains and cell culture:** S. pneumoniae were cultured in Todd-Hewitt-broth (Oxoid, Basingstoke, England) supplemented with 0.5 per cent yeast extract (THY) to mid-log phase or grown on blood agar (Merck). Calu-3 cells (human lung epithelium; ATCC HTB-55) and MDCK (Madin-Darby Canine Kidney) epithelial cells, stably transfected with the hpIgR cDNA in pCB6\(^6\), were used in in vitro infection experiments. Calu-3 and MDCK-hpIgR cells were cultured in Eagle’s Minimum Essential Medium supplemented with 10 per cent foetal calf serum, 2 mM glutamine, penicillin G (100 IU/ml), and streptomycin (100 µg/ml; all from GIBCO BRL) at 37°C under 5 per cent CO\(_2\) for 4 h. The medium for Calu-3 cells was further supplemented with 1 mM sodium pyruvate and 0.1 mM non-essential amino acids.

**Proteins and antisera:** Escherichia coli M15[pREP4] (Qiagen, Hilden, Germany) was used as host for recombinant pQE expression plasmids and cultured at 37°C on Luria-Bertani (LB) agar or grown on LB-agar containing 100 µg/ml ampicillin. Expression of His-tagged fusion proteins was induced with 1 mM isopropyl-β-D-thiogalactopyranoside after the culture reached an OD\(_{600}\) of 0.8 and continued to grow at 30°C for 4 h. The Histagged fusion proteins were purified by chromatography under native conditions on Ni-nitrilotriacetic acid resins according to the manufacturer (Qiagen).

SIgA and free SC were purified as described earlier from milk from humans. Antiserum against SpsA was generated by Eurogentec (Sart-Tilman, Belgium). Rabbits were immunized subcutaneously with 50 µg of purified SpsA-derivate encoded by pQSH2 in 1 ml of 1:1 emulsion of buffer and complete Freund’s adjuvant and boosted with 50 µg in incomplete Freund’s adjuvant at days 14, 28, and 56.

**Epithelial cell adherence and invasion assay:** Pneumococcal adherence to and invasion of epithelial cells were assayed in 24-well plates (Greiner, Germany). Confluent epithelial cells (2 × 10\(^5\)) were inoculated with 5 × 10\(^6\) pneumococci and incubated in Dulbecco’s Minimal Medium-HEPES at 37°C and 5 per cent CO\(_2\) for 4 h. Subsequently, the cells were rinsed several times with PBS to remove unbound bacteria. Numbers of adherent and invasive pneumococci were measured by microscopy. Extracellular bacteria, adhering to epithelial cells, were incubated for 15 min with a rabbit anti-pneumococcal antiserum, followed by fixation in 3.7 per cent paraformaldehyde and visualized by FITC-labelled goat anti-rabbit Ig (Dianova). After this, intracellular (invasive) pneumococci were stained with the same anti-pneumococcal antiserum followed by TRITC-labelled goat anti-rabbit Ig (Dianova). Adherence (green bacteria) and invasion (red bacteria) were scored on at least 50 cells. Each experiment was repeated at least five times; results were expressed as mean ± standard deviation.

**Results**

**pIg receptor-binding domains of pneumococcal SpsA protein:** Binding of human pIgR and SIgA to the surface-displayed Cbp SpsA is mediated by SC. Our previous studies had mapped the binding region to the N-terminal part of SpsA. A hexapeptide YRNYPT was identified as the minimal SC-binding motif. The gene encoding the SpsA protein has a mosaic structure and two major clades of the protein exist\(^9\). The hexapeptide occurs twice in SpsA of most bacterial serotypes, at positions 125 and 287, whereas only one copy is present, at position 198 to 203, in SpsA of serotype 1 strains. For investigation of the SpsA-pIgR interaction in in vitro assays, functional domains of the SpsA protein were expressed as Histagged fusion proteins. The SpsA proteins selected were expressed by pQSH2, which expressed the mature
N-terminal part of SpS from amino acids 37 to 283, and pQSM1, which expressed a functional domain of SpS from amino acid 159 to 324 and includes the proline rich sequence (aa 284 to 324). Cloned sequences were derived from a spsA gene of a serotype one strain. Therefore, in both SpS-derivates the hexapeptide occurs only once (Fig. 1). To validate the function of the hexapeptide in in vitro assays, the tyrosine at position 201 was selected for aspartic acid substitution. The resulting protein encoded by pQSH2 was designated SpS201. Immunoblot analysis indicated that binding of SC to SpS201 is completely abolished (Fig. 1).

Expression of human plgR by human epithelial cells: In order to investigate the role of the SpS-plgR interaction for pneumococcal invasion, the expression of the human plgR (hpIgR) was investigated among different respiratory cell lines, i.e., A549, HEp-2, Calu-3 cells, which were compared by immunoblot analysis. MDCK cells exhibiting a stable expression of hpIgR (MDCK-hpIgR) were used as a positive control cell line. Immunoblot analysis of cells with anti-SC antiserum showed production of the plgR in the MDCK-hpIgR and Calu-3 cells, and not in A549 and HEp-2 cells (Fig. 2).

SpS-dependent adherence to and invasion of hpIgR-expressing cells: Previous studies showed that Streptococcus pneumoniae is able to adhere to and invade eukaryotic cells by using different adhesin-receptor interactions. In order to demonstrate the effect of the SpS-hpIgR interaction for the pathogenesis of S. pneumoniae, the adherence to and invasion of hpIgR-expressing lung epithelial Calu-3 and MDCK-plgR cell lines was investigated for wildtype and spsA-mutant pneumococci. Numbers of adherent and invasive pneumococci were measured by microscopy. When using SpS-KO pneumococci in infection experiments, the bacteria substantially lost their ability to adhere to and invade Calu-3 and MDCK-hpIgR cells (Fig. 3). These results indicate that pneumococcal invasion occurs in a hpIgR-dependent manner as shown for the human Detroit nasopharyngeal cells. The ability of spsA-deficient pneumococci to adhere to Calu-3 cells is substantially reduced and the ability to invade these cells is completely abolished. These data, therefore, suggest that expression of the hpIg receptor is sufficient to mediate both adherence and invasion of pneumococci.
and that the SpsA-hpIgR interaction is early involved in pneumococcal invasion.

**Inhibition of pneumococcal adherence to pIgR-expressing cells:** Previous studies and our data revealed that the SpsA-pIgR interaction provides a common mechanism for internalization. However, the contribution of the identified SpsA hexapeptide motif in the infectious process was not shown. In order to investigate whether the identified hexapeptide pIgR-binding motif(s) are crucial for adherence and invasion of pneumococci, *in vitro* infection assays were performed using hpIgR-expressing epithelial cells and SpsA-derivates. Blocking experiments were conducted using the N-terminal His-tagged SpsA-derivate SH2, encoded by pQSH2, and the mutated SpsA201 with a critical Tyr/Asp amino acid substitution at position 201. The N-terminal part of SpsA inhibited adherence of pneumococci to the cells. In addition, also anti-SpsA antibodies substantially reduced adherence and invasion of pneumococci. In contrast, the recombinant mutated SpsA201 protein did not reduce adherence and invasion of pneumococci to Calu-3 cells and MDCK-hpIgR cells.

**Discussion**

The surface of *S. pneumoniae* is decorated with at least nine different Cbps. SpsA, also designated CbpA and PspC, is one of the best characterized pneumococcal surface proteins. The protein was independently identified by different groups. SpsA was identified by its ability to bind free SC and SIgA4. CbpA was identified as the most abundant Cbp in a *pspA*-mutant strain and shown to be responsible for binding of pneumococci to eukaryotic cells6. In addition to its function as SC-binding protein, this protein was recently reported to bind human complement Factor H10. The gene encoding the bacterial adhesin for SC/hpIgR has a mosaic structure, and comparison of the alleles revealed that modular domains have contributed to gene diversity during evolution9. However, the minimal SC-binding motif was localized to a highly conserved region within the two different clades of the adhesin. A hexapeptide YRNYPT was identified to mediate binding of pneumococci to SC in human SC and SIgA7. This hexapeptide motif is present once in one of the two clades and twice in the other clade.

The role of the SpsA/CbpA-pIgR interaction for pneumococcal pathogenesis was addressed in *in vivo* and *in vitro* studies. The *in vitro* studies demonstrated that the SpsA/CbpA-pIgR interaction provides a mechanism not only for colonization, but also for invasion. The interaction is associated with the translocation of pneumococci across nasopharyngeal cells by co-opting the receptor transcytosis machinery4, albeit in a reverse apical to basolateral direction.

Our data now demonstrate the crucial role of the hexapeptide of SpsA, not only for the SpsA-pIgR interaction, but also for adherence to and invasion of hpIgR-expressing cells. The functional domain of SpsA blocked the pneumococcal invasion of hpIgR-expressing cells, whereas SpsA201, with a single amino acid substitution at position 201 within the hexapeptide motif, did not strongly affect the invasion by pneumococci. However, the pIgR-dependent invasion of human respiratory epithelial cells by *S. pneumoniae* was claimed to be a limited phenomenon, that would be restricted in cell type- and bacterial strain-specific manners11.

*In vivo* studies using a model of colonization demonstrated a significant loss of nasopharyngeal colonization in rats for the pneumococcal *cbpA*-mutant6. A further study apparently observed a significantly reduced ability of pneumococci to colonize pIgR-KO mice5. In contrast, our biochemical analysis indicated that the interaction between pIgR/SC and pneumococcal CbpA/SpsA was restricted to human pIgR/SC. Binding assays indicated that SpsA binds only to SC and SIgA of human origin, but not to SC and SIgA from rabbit, rats, and mice7. These data are further supported by the fact that human, but not rabbit pIg receptor are able to promote bacterial entry in stably transfected MDCK cells5. The mechanisms underlying these *in vivo* effects are, therefore, unclear in the light of the species-specific binding demonstrated *in vitro*. Therefore, it will be important to localize the `domain´ within pIgR which is interacting with the pneumococcal SpsA/CbpA. The determination of this `domain´ could help to elucidate whether a genetic diversity of the binding site in pIgR molecules among various mammals is responsible for the observed effects.
References


Reprint requests: Dr Sven Hammerschmidt, Research Centre for Infectious Diseases, University of Wurzburg, D-97070, Wurzburg, Germany e-mail: S.Hammerschmidt@mail.uni-wuerzburg.de