The pneumococcal eukaryotic-type serine/threonine protein kinase StkP co-localizes with the cell division apparatus and interacts with FtsZ \textit{in vitro}

Carmen Giefing, Kira E. Jelencsics, Dieter Gelbmann, Beatrice M. Senn and Eszter Nagy

Intercell AG, Vienna Biocenter 3, A-1030 Vienna, Austria

The importance of serine/threonine phosphorylation in signalling and regulation of gene expression in prokaryotes has been widely recognized. Driven by our interest in StkP (the pneumococcal serine/threonine kinase homologue) for vaccine development, we studied its cellular localization. We found that the C-terminally located PASTA (penicillin-binding protein and serine/threonine kinase associated) domains, but not the N-terminal kinase domain of StkP, were located on the surface of live pneumococcal cells grown \textit{in vitro} and were also accessible to antibodies during pneumococcal infection in mice and man. Most importantly, we discovered, by immunofluorescence microscopy, that StkP co-localized with the cell division apparatus. StkP and FtsZ, the prokaryotic tubulin homologue, co-localized at mid-cell in most cells. Formation and constriction of the ring-like structure of StkP followed the dynamic changes of FtsZ in dividing cells. This pattern resembles that of the ‘late’ divisome protein penicillin-binding protein 2X. The lack of StkP in gene deletion mutants did not disturb FtsZ ring formation, further suggesting that StkP joins the divisome after the FtsZ ring is assembled. We also present evidence that StkP binds and phosphorylates recombinant FtsZ \textit{in vitro}; however, we could not detect changes in the phosphorylation of FtsZ in a \textit{stkP} deletion strain relative to wild-type cells. Based on its cell-division-dependent localization and interaction with FtsZ, we propose that StkP plays a currently undefined role in cell division of pneumococcus.

\textbf{INTRODUCTION}

Despite the availability of antibiotics and efficacious polysaccharide-based vaccines \textit{Streptococcus pneumoniae} (pneumococcus) remains a major threat to mankind, killing more than 1.5 million people annually worldwide (CDC, 2007; O’Brien et al., 2009). This Gram-positive pathogen is a frequent colonizer of the human host, especially during childhood, and causes invasive diseases such as pneumonia, sepsis and meningitis, primarily when the balance between the bacterium and the host is altered, typically upon a viral infection (O’Brien et al., 2000; Peltola & McCullers, 2004). Improved prophylaxis by broad-coverage vaccines is highly warranted. Conserved antigens that play crucial roles in the pneumococcal life cycle seem to be ideal targets for protective antibodies, since the risk of escape mutations due to immune pressure or negative selection is unlikely.

In a previous study, we identified such conserved protein antigens by applying a genomic-scale approach using human serum antibodies obtained from donors exposed to pneumococcus (Giefing et al., 2008). The eukaryotic type serine/threonine kinase protein, StkP, was selected as the lead vaccine candidate based on conservation and protection against lethal sepsis and pneumonia induced by different pneumococcal serotypes (Giefing et al., 2008).

Since pneumococcus is not a particularly toxic pathogen, it wins the battle against its host mainly by a rate of multiplication that is higher than its rate of elimination. Therefore, increased fitness and highly adaptable growth that is responsive to environmental signals in the host is important in its pathogenesis. Previously it was thought that signal transduction in prokaryotes is mainly mediated by two-component signal transduction systems comprising a histidine kinase and a response regulator protein (Stock

\textbf{Abbreviations:} PASTA; penicillin-binding protein and serine/threonine kinase associated; PBP, penicillin-binding protein; RuBPS, ruthenium(II)–tris(bathophenanthroline disulfonate).
In the past years, serine/threonine phosphorylation has been widely recognized also to regulate various cellular processes in prokaryotes (Wang et al., 2002). Serine/threonine protein kinases have been identified in a wide range of bacterial species (Shi et al., 1998). Members of a family of Gram-positive serine/threonine kinases comprise an N-terminal eukaryotic-like kinase domain and C-terminal reiterated penicillin-binding protein and serine/threonine kinase associated (PASTA) domains that are suggested to bind stem peptides of unlinked peptidoglycan (Jones & Dyson, 2006).

Unlike streptomyces, mycobacteria, myxococci and cyanobacteria (Petrickova & Petricek, 2003; Wang et al., 2002; Av-Gay & Everett, 2000; Inouye et al., 2000; Thakur & Chakrabarti, 2006), S. pneumoniae, like other streptococci, possesses only a single eukaryotic-type serine/threonine kinase. StkP and its corresponding phosphatase PhpP form a functional pair and are involved in dynamic phosphorylation–dephosphorylation of proteins on serine and threonine residues (Novakova et al., 2005; Osaki et al., 2008). Based on our previous gene deletion studies, StkP seems to have an important function in bacterial multiplication: in addition to the drastic decrease in in vitro growth and virulence, electron microscopic analysis revealed an elongated cell shape instead of the typical coccoid form and a less prominent septum formation in the ΔstkP mutant strain (Giefing et al., 2008). StkP plays an important role in virulence and competence (Echenique et al., 2004; Giefing et al., 2008) and stkP deletion mutants are more sensitive to various stress conditions, such as elevated temperature, oxidative stress, osmotic pressure, acidic conditions and antibiotics (Saskova et al., 2007; Giefing et al., 2008). Deletion of stkP homologues in other bacterial species was also associated with defects in growth and septation, decrease in virulence and increased sensitivity to cell wall stress (Rajagopal et al., 2003; Jin & Pancholi, 2006; Kristich et al., 2007).

These observations prompted us to study StkP expression and localization in the context of bacterial growth and cell division. This is a complex and highly organized process that is initiated in nearly all bacteria by polymerization of the prokaryotic tubulin homologue, FtsZ, into a ring-like structure at mid-cell. This FtsZ ring functions as a scaffold for a group of conserved proteins that participate in the formation of the division septum, the so-called divisome (den Blauwen et al., 2008; Errington et al., 2003; Goehring & Beckwith, 2005). The major events of the division process in S. pneumoniae are consistent with those in the rod-shaped model organisms Escherichia coli and Bacillus subtilis and they involve the initiator proteins FtsZ and FtsA and the later division proteins DivIB, DivIC, FtsL, FtsW, PBP1A and PBP2X (Le Gouellec et al., 2008; Fadda et al., 2007; Lara et al., 2005; Noircier-Savoye et al., 2005; Morlot et al., 2003, 2004; for review see Zapun et al., 2008).

In this study, we characterized the pneumococcal StkP by defining its cellular localization. We found growth-stage-dependent surface staining for the PASTA domains on live bacteria and proved their accessibility to antibodies during pneumococcal disease. Most importantly, we discovered that StkP shows cell-division-dependent localization that follows the dynamic changes in the FtsZ architecture. Moreover, StkP interacts with recombinant FtsZ via its kinase domain in vitro, resulting in phosphorylation of FtsZ.

**METHODS**

**Bacterial strains and growth conditions.** TIGR4 (serotype 4) and PJ1324 (serotype 6B) strains were provided by Birgitta Henriques-Normark (Swedish Institute for Infectious Disease Control) and 4DS2341-94 (serotype 4) strain by Eddie Ades (CDC, USA). ΔstkP mutant strains were generated as described previously (Giefing et al., 2008). Bacteria were cultured in Todd–Hewitt broth supplemented with 0.5% yeast extract at 37 °C in an atmosphere of 5% CO₂.

**Cloning and expression of recombinant pneumococcal proteins.** The following StkP sub-fragments were generated from PJ1324 genomic DNA: aa 1–273 (StkP-N), aa 273–659 (StkP-C) and aa 345–659 (StkP-PASTA₁–₄) (Fig. 1). FtsZ (aa 2–419), PcsB (SP2216) and PsPA (SP0117) were cloned from TIGR4 genomic DNA. All genes were cloned into pET28b(+) vector (Novagen) and expressed with a C-terminal His-tag in E. coli BL21 Star (Invitrogen). Proteins were bound to Ni-Sepharose beads (GE Healthcare) and eluted with 500 mM imidazole in 20 mM NaH₂PO₄, 0.5 mM NaCl, pH 7.4. Proteins from the insoluble fraction were solubilized in 8 M urea in 50 mM Tris/HCl, pH 8.

**Generation of hyperimmune mouse and rabbit sera.** C3H/HeNHsd mice (female, 6–10 weeks; Harlan Winkelmann) were immunized three times at 14 day intervals subcutaneously (into the flank) with 50 μg recombinant FtsZ, StkP-FL, StkP-N, StkP-C, StkP-PASTA₁–₄, PcsB or PsPA proteins and hyperimmune sera were collected on day 35. New Zealand white rabbits (Charles River) were immunized at days 0, 28, 42 and 56 with 250 μg StkP-PASTA₁–₄ recombinant protein and CFA/IFA as adjuvant and terminally bled on day 70.

**Surface staining of bacteria.** Bacteria from different growth phases (according to OD₆₅₀ and growth curves) were collected by centrifugation, and stained with StkP-FL, StkP-N, StkP-C and StkP-PASTA₁–₄ specific hyperimmune sera and FITC-labelled goat anti-mouse IgGs (Dako), as described previously (Giefing et al., 2008). Surface binding antibodies were detected by using FACScan (Becton Dickinson). Data were collected and analysed using CELLQUEST (Becton Dickinson) and WinMDI version 2.7. (Flow Cytometry Application, Joseph Trotter).

**Western blotting.** Proteins were separated on 4–20% gradient SDS-PAGE gels under reducing conditions and blotted to nitrocellulose membranes using a dry-blotting system (Invitrogen). Blocking and incubation with primary (1:1000 dilution) and horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution) were done in 5% milk in PBS containing 0.1% Tween 20 for 1 h at room temperature. Membranes were viewed with chemiluminescent substrate (Chemiglow; Alpha Innotech) and a CCD camera (FluorChem SP; Alpha Innotech).

**ELISA.** ELISA was performed according to standard protocols (Dryla et al., 2007). Recombinant proteins (1 μg ml⁻¹ in carbonate buffer) were coated on Maxisorb plates (Nunc) and human sera were used in a 1000–5000-fold dilution range. For peptide ELISA biotin-tagged 20 aa long peptides (overlapping by 5 aa) were coated on streptavidin
pre-coated ELISA plates (Nunc) at a concentration of 1 μg ml⁻¹ in PBS and tested with human sera diluted 200–1000-fold.

**Human serum samples.** The sera used here were obtained in a previous study (Giefing et al., 2008).

**Bacterial challenge experiments in mice.** All animal experiments were carried out according to Austrian Law (BGB1 Nr. 501/1989). In serum transfer experiments (10 mice per group), mice received 150 μl serum intraperitoneally 1–3 h before intraperitoneal challenge with 10⁵ cfu. S. pneumoniae strain PJ1324 and were monitored for survival for 14 days. For neutralization, sera were pre-incubated with 5 or 50 μg recombinant StkP-PASTA1–4 for 1 h prior injection. P-values were calculated by using the Mantel–Cox test.

**Immunofluorescence microscopy.** Bacteria were grown till OD₆₅₀ 0.4 and then fixed with 3 % paraformaldehyde in 30 mM sodium phosphate pH 7.5 for 1 h at 4 °C, washed three times with 100 mM glycine in PBS, and resuspended in 50 mM EDTA in 20 mM Tris/HCl (pH 7.5). Bacteria were transferred to microscope slides (Poly-Prep Slides, Sigma) and permeabilized with 0.1 % Triton X-100 in PBS. Slides were blocked with 2 % BSA in PBS overnight. Anti-StkP rabbit and anti-FtsZ mouse sera were diluted 1:50 and anti-rabbit-Cy2 and anti-mouse-Texas Red antibodies (JacksonImmuno) were diluted 1:200 1 % BSA–PBS. Slides were blocked with 2 % BSA in PBS at room temperature. Anti-StkP rabbit and anti-FtsZ mouse sera were diluted and stained with DAPI for 1 h. Slides were mounted with Eukitt (Fluka) and visualized with a Zeiss Axiovert 200M microscope with a Zeiss LSM510META confocal laser-scanning unit, an AxioCam HRc (Zeiss) objective (Zeiss). Images were prepared with Zeiss ZEN Browser version 4.2.0.121 and with Adobe Photoshop CS4 software to adjust brightness and contrast of the Cy2-StkP and DAPI staining.

**In vitro binding assays.** In an ELISA-based assay, plates were coated with an equimolar amount of rStkP-N (1 μg), rStkP-PASTA1–4 (1.1 μg) or BSA (2.2 μg) per well in carbonate buffer pH 9 overnight at 4 °C and blocked with 2 % BSA in PBS at room temperature. Following washing with PBS plus 0.1 % Tween, FtsZ was added at 15, 30, 60, 125 or 250 ng per well in 1 % BSA–PBS for 2 h at 37 °C. After washing, bound FtsZ was detected with specific antibodies. Binding was expressed as OD₄₀₅ values multiplied by 1000.

**Kinase assay.** In vitro kinase assay was performed according to the method described by Chaba et al. (2002). Recombinant StkP-FL, StkP-N, StkP-PASTA1–4 and/or bacterial lysate (all 1 μg) were incubated with recombinant FtsZ in kinase buffer containing 2 μCi [γ-³²P]ATP, 50 mM NaCl, 10 mM MnCl₂, 50 mM Tris/HCl, pH 7.5, for 30 min at room temperature. The reaction was stopped by addition of 6 × SDS sample buffer, proteins were separated in 12 % SDS-PAGE gels that were stained with a 400 nM solution of ruthenium(II)–tris(bathophenanthroline disulfonate) (RuBPS) as described previously (Rabilloud et al., 2001). Fluorographs were scanned with FluorImager 595 (GE Healthcare) at a resolution of 100 μm. Gels were scanned with Phosphor Imager SI (Molecular Dynamics), resolution 200 μm.

**Immunoprecipitation.** FtsZ specific antibodies coupled to Protein G agarose beads (Pierce) were incubated with pneumococcal lysate from wild-type and stkP deletion mutant strains. Beads were extensively washed with IgG binding buffer (Pierce) and bound material was eluted first by addition of IgG elution buffer (Pierce) (eluate 1) and then by incubating the beads in SDS sample buffer to at 95 °C for 5 min (eluate 2). The fractions were analysed by Western blotting using FtsZ-, phospho-serine- and phospho-threonine-specific antibodies.

**RESULTS**

**The C-terminal PASTA domains of StkP are surface exposed**

StkP was reported previously to be located in the membrane fraction of pneumococcus (Novakova et al., 2005; Pallova et al., 2007; Osaki et al., 2009) and was detected by us on the bacterial surface (Giefing et al., 2008). In order to determine which part of the protein is extracellular, we generated hyperimmune mouse sera by immunization with recombinant proteins representing the N-terminal kinase domain (StkP-N), the C-terminal half of the protein (StkP-C) and the repetitive PASTA domains (StkP-PASTA1–4) (Fig. 1).

**Fig. 1.** Graphical representation of StkP structure. Predicted domains of StkP: serine/threonine kinase catalytic domain (S/TKc) aa 11–259, transmembrane domain (TM) aa 345–364 (http://cmr.jcvi.org, according to Sonnhammer et al., 1998) and four PASTA domains aa 366–643. The immunogenic epitope identified by ANTIGENome screens is displayed as a horizontal line (Giefing et al., 2008). Recombinant StkP subdomain proteins expressed in this study are displayed below the full-length StkP (StkP-FL): StkP-N, aa 1–273; StkP-C, aa 273–659; and StkP-PASTA₁–₄, aa 345–659.

Surface expression of StkP was then investigated by staining live wild-type pneumococcal cells with domain-specific antibodies and analysing these by flow cytometry. We detected surface signals with the same intensity using hyperimmune sera generated with the full-length recombinant StkP, StkP-C or StkP-PASTA1–4 while antibodies specific for the N-terminal half of the protein containing the kinase domain (StkP-N) did not bind to live bacteria (Fig. 2a). We also observed that the surface staining signal was most intensive in the middle of the exponential growth phase (OD₆₂₀ ~ 0.4), while in earlier and later growth stages,
the staining was less pronounced or undetectable (Fig. 2b). This variable surface accessibility was not the result of growth-phase-dependent expression of StkP, since immunoblot analysis of total bacterial lysates detected equal amounts of protein (Fig. 2c).

Based on these data we concluded that the kinase domain is located intracellularly, while the PASTA domains are exposed extracellularly on pneumococci grown in vitro.

**PASTA domains are surface accessible in vivo and are recognized by the immune system**

Virulent pneumococcal strains express a polysaccharide capsule that might interfere with binding of antibodies to surface proteins in vivo. Therefore, we performed immunization and challenge studies to address whether the PASTA domains of StkP were also accessible in vivo during disease. In active immunization studies, we observed the same level of protection with the StkP-PASTA1-4 as with the full-length StkP, while StkP-N was ineffective (Giefing et al., 2008 and unpublished data). We also wanted to test more directly the surface accessibility of the PASTA domains in passive vaccination studies. We found that transfer of hyperimmune rabbit sera specific for StkP-PASTA1-4 protected most of the animals from septic death, suggesting that the PASTA domains were accessible for binding to cognate antibodies. Specificity was confirmed by the loss of protection upon addition of recombinant StkP-PASTA1-4 to the serum before passive immunization (Fig. 3a).

To further substantiate these findings, we then tested whether human serum antibodies induced by pneumococcus in its natural host would also preferentially recognize the PASTA domains. We reported earlier that antibodies against StkP were induced during childhood, most likely as a consequence of repeated exposure to pneumococcus (colonization in the nasopharynx), and were also present in healthy adults and convalescent patients (Giefing et al., 2008; unpublished data). Using the same serum samples collected in this previous study, we measured immune reactivity of the recombinant proteins StkP-N, StkP-C and StkP-PASTA1-4 by ELISA. We found that all sera that contained IgG specific for StkP recognized the StkP-C and StkP-PASTA1-4 proteins with equal reactivity, but the kinase domains not at all, suggesting that StkP-specific antibodies are induced only by the PASTA domains in vivo (Fig. 3b).

To address the question of whether all PASTA domains are equally immunogenic and accessible to the immune system during infection, we performed an ELISA-based epitope mapping of the PASTA domain region with synthetic overlapping peptides and human sera. We found that the most C-terminal part of the last PASTA domain displayed the highest antibody-binding capacity, although immune reactive peptides were also found within the second and third PASTA domains (peptides 8 and 11) (Fig. 3c). Human sera from healthy controls that did not react with
recombinant StkP also did not react with any of the peptides. These data suggested that at least the three C-terminal PASTA domains are seen by the human immune system and therefore are likely to be located extracellularly during human infection as also observed in murine models of pneumococcal disease (Fig. 3a and Giefing et al., 2008).

**StkP co-localizes with the cell division apparatus**

Previously we reported that the **stkP** gene deletion mutant pneumococcus displayed an altered growth phenotype and elongated cell shape based on electron microscopic analysis (Giefing et al., 2008). These observations, as well as the presence of PASTA domains in penicillin-binding proteins (PBPs) described as cell division proteins, prompted us to determine the staining pattern for StkP within pneumococcal cells. Staining for FtsZ, the prokaryotic homologue of tubulin localized at the division septum, and simultaneously for DNA is the most suitable way to visualize cell cycle events in bacteria. Therefore we performed triple-staining of pneumococcal cells with DAPI and StkP-specific rabbit and FtsZ-specific mouse hyperimmune sera. Confocal microscopic analysis revealed a characteristic staining pattern for StkP that was completely overlapping.

---

**Fig. 3.** PASTA domains of StkP are accessible in vivo. (a) Mice received rabbit hyperimmune sera (rs HI) generated with StkP-PASTA$_{1-4}$ or PBS (negative control) prior to lethal challenge with strain PJ1324 intraperitoneally and were monitored for 14 days. To neutralize StkP-specific antibodies, sera were pre-incubated with 5 or 50 µg recombinant protein StkP-PASTA$_{1-4}$ before administration, as indicated. (b) Sera from patients (P) and children (C) were analysed by ELISA at a 1:1000 serum dilution using recombinant StkP-N, StkP-C and StkP-PASTA$_{1-4}$ as coating antigens. (c) Peptide ELISA using sera from patients and healthy individuals with high anti-pneumococcal titre was performed at a 1:200 serum dilution with synthetic overlapping peptides. The geometric means of ELISA units calculated for the healthy adult and patient donor groups are shown. The alignment of the tested peptides to the C-terminal part of StkP is shown below the plot.
with that of FtsZ in the majority of cells, and showed equatorial localization around the centrally positioned DNA in single (not dividing) (Fig. 4a, i) and already duplicated (Fig. 4a, vi) daughter cells. This septal localization of StkP was maintained in all cells, but the dynamics of forming a ring-like structure by StkP in dividing cells were delayed relative to those of the FtsZ ring. After duplication and separation of the chromosomes, FtsZ and StkP remained co-localized at the future division site (Fig. 4a, ii) or the StkP ring was still maintained at the septum between two newly forming daughter cells, while constriction of FtsZ had already started (Fig. 4a, iii). We also found another pattern when FtsZ established new rings at the duplicated equatorial sites, the future sites of cell division, while StkP either remained at the original division site as a constricted small ring after the FtsZ ring collapsed (Fig. 4a, iv) or followed FtsZ to the new duplicated equatorial sites, and its ring-like localization at the original septal site disappeared (Fig. 4a, v).

Based on this observed staining pattern, we concluded that StkP is a cell division protein. The dynamics of the ring-like structure formation suggested that StkP is likely to be a late divisome protein as its localization to the division site overlapped with or followed that of FtsZ, as schematically illustrated in Fig. 4b. By analysing bacteria in stationary phase, we found that the characteristic mid-cell localization of StkP and FtsZ disappeared and the StkP signal formed a circle around the cells, which is consistent with diffuse membrane localization (Fig. 4c).

Importantly, we found that the characteristic ring-like FtsZ staining pattern was unchanged in ΔstkP cells we generated previously (Giefing et al., 2008) (Fig. 5a). Based on Western blot analysis of total bacterial lysates, we could not detect differences in expression levels of FtsZ in stkP deletion strains in three different genetic backgrounds (Fig. 5b).

The localization pattern described for StkP in this study resembled that of the high molecular mass PBP2X, the only pneumococcal protein that possesses PASTA domains besides StkP and which is characterized as a late divisome protein (Morlot et al., 2003). Although the similarity between PASTA domains of PBP2X and StkP is limited at

**Fig. 4.** Localization of StkP relative to FtsZ during the progression of cell division. (a) Pneumococcal cells (4DS2341-94 wild-type strain) were triple-stained with anti-StkP rabbit serum/Cy2-labelled anti-rabbit IgG and anti-FtsZ mouse serum/Texas Red-labelled anti-mouse IgG; DNA was counterstained with DAPI. The merged pictures show the overlay of StkP and FtsZ staining. Representative pictures were selected and sorted according to six different phases of the cell division (i–vi). Five hundred and sixty labelled cells were counted and the proportion of cells displaying each pattern was calculated. *Due to the lack of cell wall staining, doublets or already-separated cells (i and vi) could not be clearly distinguished. (b) Based on the original pictures, the observed localization pattern of StkP (green) and FtsZ (red) and the proposed time-lapse of formation of septum and cell wall (dark blue) during growth of pneumococcal cells are depicted. (c) Pneumococci from exponential (OD₆₀₀~0.5) and late stationary (overnight culture) growth phases were stained with anti-StkP rabbit serum/Cy2-labelled anti-rabbit IgG and DAPI.
the primary sequence level (max. 35% aa similarity), it has been suggested that these domains are structurally and functionally related (Jones & Dyson, 2006). The lack of signal in the ΔstkP mutants stained with StkP-specific hyperimmune sera excluded cross-reactivity between StkP and PBP2X and confirmed the specificity of staining (Fig. 5a).

**StkP binds and phosphorylates FtsZ in vitro**

The observed temporal co-localization of StkP and FtsZ evoked the question whether direct interaction between these proteins occurs. Therefore we measured binding of recombinant forms of these proteins in an *in vitro* assay. ELISA plates were coated with StkP-N or StkP-PASTA1–4 protein and FtsZ binding was detected with FtsZ-specific anti-serum. We observed very good binding of FtsZ to the N-terminal kinase domain, while the signal derived from the PASTA domains was low (Fig. 6). Similar binding activity was detected when the experiment was performed with FtsZ-coated plates and StkP-N was detected with specific antibodies (data not shown).

In subsequent experiments, we tested whether this interaction could result in phosphorylation of FtsZ. In an *in vitro* kinase assay using [γ-32P]ATP and recombinant forms of StkP and FtsZ, we observed that the full-length StkP, as well as the N-terminal domain (StkP-N), phosphorylated FtsZ (Fig. 7a, left panel). We also detected autophosphorylation of StkP based on the radioactive labelling of both the full-length protein and the N-terminal fragment, while no phosphorylation of StkP-PASTA1–4 was seen. Interestingly, this autophosphorylation was reduced in the presence of FtsZ, suggesting a preferential use of FtsZ as substrate. Phosphorylation of StkP-N and FtsZ was reduced in the presence of pneumococcal lysate possibly due to the presence of phosphatases (Fig. 7a, right panel). To control for equal protein loading gels were stained with RubPS (Fig. 7b).

However, FtsZ purified from wild-type and *stkP* gene deletion cells by immunoprecipitation with specific antibodies reacted to the same extent with anti-phosphothreonine antibodies (Fig. 7c). No signal was detected with anti-serine antibodies (data not shown).

**DISCUSSION**

Driven by our interest in StkP for vaccine development (StkP is currently being tested as a component of a protein-based vaccine in phase one for safety and immunogenicity in humans), we studied the surface exposure of its domains as this is relevant for binding of antibodies induced by immunization. By using domain-specific hyperimmune sera in a flow cytometry-based staining assay, we determined that the C-terminally located PASTA domains...
but not the N-terminal kinase domain of StkP were surface-accessible to antibodies on live wild-type pneumococcal cells grown in vitro. Since the total amount of StkP did not change from early logarithmic till late stationary phases, we concluded that either the interaction of StkP with the cell wall or masking by the polysaccharide capsule was subject to changes allowing surface detection mainly in the mid-exponential phase. To exclude the possibility of an in vitro artefact, we confirmed the in vivo accessibility of the PASTA domains based on the protective effect of PASTA-domain-specific hyperimmune serum in a lethal pneumococcal challenge model. Here, we also demonstrated that human serum antibodies induced by StkP during pneumococcal infections exclusively recognize the PASTA domain region. Within this approximately 35 kDa portion of StkP, the most immunogenic region was the most C-terminal 25 aa stretch located in the last (fourth) PASTA domain, as determined by epitope mapping (Fig. 1), and also observed in genomic peptide libraries used for antigen identification (Giefing et al., 2008). Since epitopes with lower immune reactivity were also detected within the second and third PASTA domains, it is likely that these three C-terminal PASTA domains are visible to the immune system during infection.

The previously observed decreased growth rate and reduced septum formation in the ΔstkP strains (Giefing et al., 2008) together with the growth-phase-dependent surface expression of StkP pointed towards a potential role in bacterial multiplication. Bacterial cell division is directly linked to the dynamic process of peptidoglycan remodelling (for review see Zapun et al., 2008; Scheffers & Pinho, 2005; Goehring & Beckwith, 2005). The presence of PASTA domains in PBPs, enzymes involved in the synthesis and processing of peptidoglycan stem peptides, as well as in serine/threonine kinases evokes a question: what is the common pathway that these proteins are involved in? Although the similarity between PASTA domains of PBPs and serine/threonine kinases is limited at the primary sequence level, it has been suggested that structurally these
domains are related and capable of binding peptidoglycan precursors (Jones & Dyson, 2006). Since all high molecular mass PBPs in pneumococcus, including the PBP2X that possesses two PASTA domains, have been shown to have a cell-cycle-dependent localization and are associated with the cell division apparatus (Morlot et al., 2003, 2004), it was tempting to address this question for StkP. Staining pneumococci with StkP-specific antibodies revealed a characteristic staining pattern that completely overlapped with that of FtsZ in most cells. Formation and constriction of the StkP ring-like structure revealed by immunofluorescence microscopy followed that of FtsZ, and as soon as the septal ring of StkP started to collapse, it rebuilt at the duplicated equatorial sites of the daughter cells. Cells of overnight cultures did not display the ring-like pattern of StkP, but showed circular staining around the bacteria suggesting diffuse membrane localization. The staining pattern we observed for StkP (as illustrated in Fig. 4b) in dividing cells of growing cultures resembles that of PBP2X, a transpeptidase important for septal peptidoglycan synthesis (Morlot et al., 2003). The correct localization of PBPs relative to FtsZ in pneumococci requires PBP3, a carboxypeptidase that degrades the substrates of high molecular mass PBPs and which is normally excluded from the future division site (Morlot et al., 2004). In Staphylococcus aureus and E. coli, the localization of the PBP2X homologues, PBP2 and FtsI (PBP3), is disrupted by treatment with cell-wall-synthesis inhibiting antibiotics (Pinho & Errington, 2005; Wang et al., 1998) and recruitment of FtsI to the division site requires FtsW, a putative translocase of peptidoglycan precursors (Mercer & Weiss, 2002). Based on these data, it was hypothesized that the localization of PBPs employs a dual mechanism: first, PBPs are recruited to the division site via protein–protein interactions with division proteins and then this cell wall synthesis complex is uncoupled from the other cell division proteins and localizes through substrate (peptidoglycan) recognition (Scheffers & Pinho, 2005). As we could show in this study that StkP binds and interacts with FtsZ directly via its kinase domain, we propose a similar localization mechanism for StkP to that depicted for the PBPs. In this model, StkP would localize to the septum via interaction with FtsZ and subsequently through recognition of unlinked peptidoglycan via its PASTA domains (depicted in Fig. 8).

The four PASTA domains in StkP have a low amino acid sequence identity among them (22–68 %) and also compared to the two PBP2X domains (18–24 %). It was previously suggested that PASTA domains in eukaryotic-type kinases recognize different types of unlinked peptidoglycan by low affinity and play a role in sensing the concentration of these substrates (Yeats et al., 2002). Recently it has been demonstrated in B. subtilis that the eukaryotic-like serine/threonine kinase PrkC can signal muropeptides released from growing bacteria in the local environment and can induce germination (Shah et al., 2008). Thus, based on the surface localization of the PASTA domains, it is possible to speculate that StkP might also be able to detect extracellular peptidoglycan components, and thus signal about the growth status in a bacterial population.

Fig. 8. Schematic model of the extracellular and intracellular localization of StkP. StkP and its domains (PASTA, TM, kinase) are shown in pink embedded in the cytoplasmic membrane (grey) and the cell wall (green). Unlinked peptidoglycan precursors are shown as dark green ovals.
The dynamic remodelling of peptidoglycan and cell wall has to be well co-ordinated with the events of cell division. It is relevant in this context, which StkP was shown to phosphorylate GlmM, which catalyses the first step in biosynthesis of UDP-N-acetylglucosamine, a peptidoglycan precursor (Novakova et al., 2005). In addition, phospho-peptide analysis of wild-type and stk1 deletion mutant group B streptococcal strains suggested that Stk1 phosphorylates the cell division protein DivIVA (Silvestroni et al., 2009). Phosphorylation of FtsZ in an stk1-overexpressing strain was also demonstrated in vivo, but could not be confirmed in vitro using purified proteins (Silvestroni et al., 2009). It is noteworthy that orthologues of DivIVA and FtsZ were also identified as substrates of the serine/threonine kinases PknA and PknB from Mycobacterium tuberculosis (Thakur & Chakraborti, 2006; Kang et al., 2005). We could show in vitro phosphorylation of FtsZ by StkP using recombinant proteins. However, in stkP deletion strains, the phosphorylation status of FtsZ was unchanged compared with the parent wild-type strain. Thus, the in vivo consequences of the observed interaction of recombinant StkP and FtsZ remain unknown.

In conclusion, StkP emerges as a novel player in pneumococcal cell division. We propose a model (depicted in Fig. 8); according to this, StkP, localized to the divisome by interaction with FtsZ and peptidoglycan, is involved in the regulation of cell division and bacterial growth.

ACKNOWLEDGEMENTS

The authors would like to thank Manuel Zerbs, Sonja Prustomersky, Michael Schunn, Barbara Maierhofer, Josef Gottmann, Christopher Gerner and Edith Bayer for technical help; and Birgitta Henriques-Normark and Eddie Ades for providing bacterial strains.

REFERENCES


and protein phosphatase of *Streptococcus pneumoniae* and identification of kinase substrates. *FEBS J* 272, 1243–1254.


Edited by: T. den Blaauwen