Pneumococcal phosphoglycerate kinase interacts with plasminogen and its tissue activator

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Summary

Streptococcus pneumoniae is not only a commensal of the nasopharyngeal epithelium, but may also cause life-threatening diseases. Immuno-electron microscopy studies revealed that the bacterial glycolytic enzyme, phosphoglycerate kinase (PGK), is localised on the pneumococcal surface of both capulated and non-capulated strains and colocalises with plasminogen. Since pneumococci may concentrate host plasminogen (PLG) together with its activators on the bacterial cell surface to facilitate the formation of plasmin, the involvement of PGK in this process was studied. Specific binding of human or murine PLG to strain-independent PGK was documented, and surface plasmin resonance analyses indicated a high affinity interaction with the kringle domains 1–4 of PLG. Crystal structure determination of pneumococcal PGK together with peptide array analysis revealed localisation of PLG-binding site in the N-terminal region and provided structural motifs for the interaction with PLG. Based on structural analysis data, a potential interaction of PGK with tissue plasminogen activator (tPA) was proposed and experimentally confirmed by binding studies, plasmin activity assays and thrombus degradation analyses.

Keywords

Angiostatin, plasminogen, phosphoglycerate kinase, Streptococcus pneumoniae, tissue-type plasminogen activator

Introduction

Infectious diseases caused by Streptococcus pneumoniae (pneumococcus) represent a major and increasing life-threatening fact in both industrialised and developing countries. As commensals, pneumococci colonise the upper respiratory tract of humans and mediate local infections, e.g., otitis media. More important, S. pneumoniae is known to cause severe systemic diseases like pneumonia and meningitis in children, elderly patients and immune compromised people (1). Increasing antibiotic resistances state reason for the current intensive efforts in vaccine-based prevention strategies and lead to maintenance of high mortality and morbidity rates of pneumococcal infections (1, 2). Pneumococci express different surface molecules mediating interactions with host cell receptors, extracellular matrix proteins, and components circulating in the vascular system (3, 4). Binding and activation of the major fibrinolysis protein plasminogen (PLG) has been extensively investigated during the last decade. Its recruitment on the bacterial surface provides an effective precaution to subvert the activity of the serine protease plasmin. As proteolytic cofactor, surface-bound plasmin enables pneumococci to spread intercellularly and disseminate in deeper tissue sites and within the vascular system (5, 6).

PLG is a 92 kDa pro-enzyme of the broad-spectrum protease plasmin, which counteracts the blood coagulation cascade. It is composed of a pre-activation peptide (~8 kDa), five homologous disulfide-bonded triple-loop kringle structures (K1–5, 65 kDa) and a serine-protease domain (7). The first four kringle domains...
of PLG act as an inhibitory molecule for angiogenesis and are, therefore, termed angiostatin (8), whereas the last kringle domain together with the enzymatic domain is known as mini-plasminogen (mini-PLG) (9). PLG is converted into plasmin by eukaryotic plasminogen activators (urokinase-type plasminogen activator, uPA and tissue-type plasminogen activator, tPA) (10). These activators initiate the conversion of PLG to plasmin, which hydrolyses polymerised fibrin strands into soluble fibrin degradation products. Increased levels of tPA, urokinase-type plasminogen activator receptor (uPAR), plasminogen activator inhibitors 1 and 2 (PAI-1, PAI-2), and also uPA have been reported in the cerebrospinal fluids of patients suffering from pneumococcal meningitis (11).

A variety of bacterial metabolic enzymes, such as enolase and glyceraldehyde-3-phospho dehydrogenase (GAPDH), have been characterised as surface located proteins, called non-classical surface proteins (4). These proteins exhibit "moonlighting" functions including adherence to various host components like fibronectin (12) and laminin (13) and as receptors for PLG recruitment (6, 14). The phosphoglycerate kinase (PGK) was identified as an additional glycolytic enzyme bearing moonlighting function on the surface of S. agalactiae (15). The mechanism of cell wall secretion and the mode of surface anchorage remains an unsolved question. The pneumococcal PGK represents a monomeric two-domain protein of 41.9 kDa that mediates transfer of anhydride phosphate of 1,3-biphospho-D-glycerate (1,3-BPG) to Adenosin 5'-diphosphate magnesium salt (MgADP), yielding 3-phospho-D-glycerate and the phosphoglycerate dehydrogenase (3-PGA) and Adenosin 5’-triphosphate magnesium salt (MgATP) within glycolytic metabolism (16). The N-terminal domain contains the substrate binding site followed by an ATP-binding site and the catalytic active centre in the C-terminus (17). The present study provides insights in the moonlighting functions of pneumococcal PGK by biophysical and structural analyses.

Bacterial strains and culture

Streptococcus pneumoniae serotype 2 strain D39, corresponding capsule-deficient mutant (D39Δcps; [18]) and serotype 35A pneumococci (ATCC11733) were used for electron microscopic studies and amplification of the pgk-gene for expression cloning. Pneumococcal strains were cultured at 37°C in Todd-Hewitt broth (Oxoid, Hampshire, UK) supplemented with 0.5% yeast extract (THY) to mid log phase or were grown on blood agar plates (Becton Dickinson, Heidelberg, Germany). Escherichia coli M15[pREP4] (Qiagen, Hilden, Germany) was used as host strain for recombinant pQE expression plasmids (Qiagen) and was cultured at 37°C on Luria-Bertani (LB) agar or grown on LB agar containing 100 µg/ml ampicillin and 50 µg/ml kanamycin.

Cloning, expression and protein purification of recombinant PGK

Expression and purification of PGK was performed as described in (19). In brief, the pgk gene from S. pneumoniae D39 (Acc. No: YP_815953.1) was amplified with oligonucleotides (5’-GGGGATCTTGGGCAAAATCTTAGTTAAAGAG-3’) and (5’-CCCCGTCGACTTTTCTGAAGGCTCGAAG-3’) (MWF Biotech, Ebersberg, Germany) and ligated into the pQE30 plasmid (Qiagen) generating pQE30PGKw7 in E. coli M15. After induction of protein expression with 1 mM IPTG, E. coli cells were lysed mechanically using a French Pressure Cell (SLM-Aminco). Purification of recombinant PGK was performed by affinity chromatography according to standard protocols recommended by Qiagen.

Protein dot spot analysis

Recombinant PGK proteins, purified human or murine PLG, PLG derivatives and tissue-type plasminogen activator (tPA) were immobilised onto a nitrocellulose membrane. After blocking with 10% skimmed milk, the membrane was incubated with 40 µg of PLG or 40 µg PGK protein for 4 hours (h) at 4°C. After three wash steps with PBS (phosphate-buffered saline, 80 mM NaHPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl, pH 7.4), supplemented with 0.05% Tween 20, the membrane was incubated for 1 h at room temperature with PLG-specific antibody from goat or PGK-specific antibody raised in rabbit followed by incubation with horse radish peroxidase-conjugated secondary antibodies recognising goat or rabbit for 1 h at room temperature. Signal detection was performed with 1 mg/ml 4-chloro-1-naphthol and 0.1% H₂O₂ in PBS or enhanced chemiluminescence (Pierce, Rockford, IL, USA).

Surface plasmon resonance analyses

The association and dissociation reactions of human and murine PLG to recombinant PGK were analysed in the BIACore optical biosensor (BIACore 2000 system, GE Healthcare, Freiburg, Germany) using CM5 sensor chips. Covalent immobilisation of PLG

Material and methods

Proteins and antibodies

Polyclonal antibodies recognising recombinant pneumococcal PGK were raised in rabbits and were purchased commercially (Pineda, Berlin, Germany). Human PLG was purchased from Sigma-Aldrich (St. Louis, MO, USA), tPA, uPA and thrombin (derived from bovine plasma) were purchased from Haemochrom Diagnostica (Essen, Germany). Protein fractions containing kringle domains 1–3 or 1–4 (angiostatin) from PLG, mini-plasminogen (mini-PLG) and murine plasminogen were gratefully obtained from K.T. Price (University of Giessen, Germany). Anti-PLG antibody from goat was purchased from Affinity Biologicals (GAPG-IG; Affinity Biologicals, Ancaster, ON, Canada), HRP-conjugated polyclonal rabbit anti goat antibody from Dako (PO449, 0.5 g/l; Dako, Glostrup, Denmark).

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was performed using a standard amine coupling procedure essentially described previously (20). Binding analyses was performed in HBS BIACore-running buffer (10 mM Hepes, 150 mM NaCl, and 0.05 % Tween 20 (pH 7.4) without EDTA at 20°C using a flow rate of 30 µl/minute (min). PGK was used as analyte in concentrations of 4.0 µM, 2.0 µM, 1.0 µM and 0.5 µM. The affinity surface was regenerated with 10 µl of 20 mM NaOH. Binding was assayed at least in duplicates using independently prepared sensor chips. Parameters of binding kinetics were analysed from raw data of the BIACore sensograms suitable for analysis using the kinetic models included in the BI Ae valuation software version 3.0. The experimental data were fitted globally using a 1:1 Langmuir kinetic. For each evaluation a minimum of four data sets were analysed.

Peptide spot array

The peptide spot array was performed essentially as described (19, 20). The highly conserved 398 amino acid sequence of the pneumococcal PGK was subdivided into 16mers with offset of four residues. Based on the sequences of three peptide spots, which were recognised to be positive for PLG binding, a systemic mutational analysis was performed. Peptides were synthesised containing alanine amino acid exchanges at each position of the original peptide sequence. For assaying PLG binding, membranes were treated as described previously (20). For protein overlay the membrane was incubated with 50 µg human PLG for 4 h at room temperature. Detection of binding was performed with PLG-specific antibodies and horse radish peroxidase conjugated secondary antibody for 1 h each at room temperature. For visualisation of binding signals, the membrane was subjected to enhanced chemiluminescence reaction (Super Signal West Pico, Thermo Scientific, Ulm, Germany) following the instructions of the manufacturer. In order to exclude unspecific binding signals, the membrane was treated equally without the step of PLG incubation. A specific washing procedure based on a stepwise incubation with dest. H₂O, N,N-dimethylformamid and 8 M urea-containing buffer allowed a repetition of binding studies.

PGK crystallisation, data collection and processing

Crystals for X-ray analysis were prepared as previously described (21) using 30% PEG 4000, 0.2 M MgCl₂ and Tris-HCl at pH 8.6 in the presence of AMP-PNP (adenyl-imidodiphosphate) (100 mM) and 3-PGA (3-phospho-D-glyerate) (100 mM). Crystals belonged to the monoclinic space group P2₁ with unit-cell parameters a = 40.35 Å, b = 78.23 Å, c = 59.03 Å and β = 96.35°. X-Ray diffraction data sets were collected using an ADSC210 CCD detector (λ = 0.933 Å) on beamline ID 14–2 at 100 K in the ESRF facility of Grenoble. Collected data from cryo-protected crystals were processed using IMOSFLM (22) and scaled with SCALA, (Collaborative Computation Project, 1994). Electron density maps for model building were calculated using conventional 2Fo-Fc and Fo-Fc coefficients with PHENIX suite (24). Intensive model building was performed with COOT (25). Excellent density maps were obtained for the PGK structure except for the two last residues of the polypeptide chain. Electron density corresponding in shape and size to AMP-PNP was observed in maps calculated in the final stages of model building and refinement. Four glycerol molecules used as cryoprotectant were found on the protein molecular surface involved in crystal packing interactions. The refinement converged to the final values of R = 0.14 and R_free = 0.19 at 1.78 Å resolution with 404 waters. Structural validation was done with a Ramachandran plot from PHENIX, it shows that over 98% of the non-glycine, non-proline residues are found in the most favored regions, with no residues in disallowed regions.

PGK structure determination and refinement

PGK structure was solved by the molecular replacement method using the MOLREP program from the CCP4 package with the Thermotoga maritima PGK structure (PDB code 1VPE) as the initial model (23). Electron density maps for model building were calculated using conventional 2Fo-Fc and Fo-Fc coefficients with PHENIX suite (24). Intensive model building was performed with COOT (25). Excellent density maps were obtained for the PGK structure except for the two last residues of the polypeptide chain. Electron density corresponding in shape and size to AMP-PNP was observed in maps calculated in the final stages of model building and refinement. Four glycerol molecules used as cryoprotectant were found on the protein molecular surface involved in crystal packing interactions. The refinement converged to the final values of R = 0.14 and R_free = 0.19 at 1.78 Å resolution with 404 waters. Structural validation was done with a Ramachandran plot from PHENIX, it shows that over 98% of the non-glycine, non-proline residues are found in the most favored regions, with no residues in disallowed regions.

Modelling of PGK in complex with human kringle structures

Initial structures of human Kringle 2 (PDB code 1B2I), Angiostatin (PDB code 1K10), human tissue-type plasminogen activator (tPA, PDB code 1TPK) and urokinase plasminogen activator (uPA, PDB code 219A) were manually oriented facing their Kringle binding sites to the PGK PLG binding site and uploaded to Rosetta Dock Server (RDS) (26). A total of 1,000 solutions were kept from each docking and filtered to select those containing the interactions involving Lys14, Lys15 and Arg53 residues. For each pair of structures a contact-overlap, based on counting common contacts, was calculated and arranged in a similarity matrix. The Average Linkage clustering algorithm was applied to the matrix using the standard Euclidean distance as a classification criterion and a maximum of 30 clusters were retained (MATLAB, The MathWorks Inc., 7.11.0; Natick, MA, USA, 2010). The most stable pose according to its ISM binding energy (see below) within the most populated cluster was selected as the docking pose.

Selected docking poses were minimised in vacuum with the AMBER 03 force field (27, 28) during 10,000 steps (the first 1,000 with the steepest descent method and the rest with the conjugated gradient) solely to alleviate the possible clashes that may be left after the docking program. All calculations were done with AMBER 11 package (29). The resulting structures where then processed with ISM (30). In brief, ISM calculates the binding energy according to the following equation:

\[ \Delta G_{\text{binding}} = \Delta G_{\text{vdW}} + \Delta G_{\text{elec}} + \Delta G_{\text{desolv}} + \Delta G_{\text{apso}} + \Delta G_{\text{Hb}} \]

where \( \Delta G_{\text{binding}} \) represents van der Waals interactions calculated through the 12–6 Lennard-Jones potential; \( \Delta G_{\text{elec}} \) accounts for charge-charge electrostatic interactions modelled with a sigmoidal dielectric screening function; \( \Delta G_{\text{desolv}} \) and \( \Delta G_{\text{apso}} \) are the desolv-
Electron microscopic visualisation

Pre-embedding labelling of *S. pneumoniae* serotype 35A and 2 D39 wild-types and capsule-deficient mutant was performed as described before (14) using 100 µg/ml PGK-specific IgG antibody (protein A-sepharose purified) and a 1:10 dilution of the stock solution of 15 nm protein A gold-nanoparticles. After fixation in 1% glutaraldehyde bacteria were adsorbed onto carbon-coated Formvar grids, washed in TE buffer and distilled water before air drying. Samples were examined in a Zeiss EM910 transmission electron microscope at calibrated magnifications and at an accelerating voltage of 80 kV. Images were recorded digitally with a SlowScan CCD-Camera (ProScan, 1024x1024, Scheuring, Germany) with ITEM-Software (Olympus Soft Imaging Solutions, Münster, Germany).

For colocalisation studies, pre-embedding labelling of surface-displayed PGK and recruited plasminogen was performed with capsule-deficient mutant of *S. pneumoniae* D39. After fixation of bacteria with 1% formaldehyde directly in THY culture medium, bacteria were harvested by centrifugation (5,000 rpm, 10 min). For preincubation with PGK, 5 x 10⁸ bacteria were incubated for 30 min at room temperature with 100 µg purified PGK protein followed by three washing steps with PBS. PGK-preincubated and non-preincubated bacteria were washed in PBS containing 10 mM glycine for quenching of free aldehydes. After washing in PBS, bacteria were resuspended in 200 µl of plasminogen-gold nanoparticles (15 nm in size), incubated for 30 min at 30°C and washed twice with PBS. Then, bacteria were resuspended in 200 µl of a 1:25 solution in 100 mM PBS of rabbit anti-PGK antibodies (1.8 mg/ml IgG, generated by rabbit immunisation with recombinant *S. pneumoniae* PGK), incubated for 30 min at 30°C and washed twice with 100 mM PBS. Bound antibodies were detected with protein A/G gold nanoparticles (10 nm in size) by incubation for 30 min at 30°C. As control bacteria were incubated with 1:10 dilution of PGK-preimmune serum followed by incubation with 15 nm protein A/G gold particles. After washing twice in 100 mM PBS bacteria were fixed with 2% glutaraldehyde at room temperature for 15 min and washed in PBS and TE (Tris 20 mM, 1 mM EDTA, pH 6.9). After resuspension in TE-buffer, bacteria were placed onto butvar-coated nickel grids (300 mesh), left for 10 min to absorb to the plastic film, washed in distilled water and air dried. Samples were observed in a Zeiss Merlin field emission scanning electron microscope (FESEM) at an acceleration voltage of 15 kV. Imaging was performed with the high-efficiency SE2 Everhart-Thornley detector. Contrast and brightness of EM pictures were adjusted using the software program Adobe Photoshop CS5.

Immobilisation of recombinant PGK to Dynabeads®

Covalent immobilisation of recombinant PGK, human PLG and BSA (bovine serum albumin, Carl Roth GmbH, Karlsruhe, Germany) to carboxylated polystyrene Dynabeads® (My OneTM Carboxylated acid, 1.0 µm in size; Invitrogen, Carlsbad, CA, USA) was performed as described by the manufacturer. In brief 280 µg recombinant PGK protein or PLG or BSA was incubated with 660 µl beads (~6 x 10⁹ beads) over night at 4°C, followed by blocking with BSA (Roth). Protein coupling was visualised by SDS-PAGE.

Plasmin activity assay

Plasmin activity was determined as described in Fulde et al. (51) using the chromogenic substrate D-valyl-leucyl-lysine-p-nitroanilide dihydrochloride (S-2251, Fluka). In brief, 10 µl of PGK-coated Dynabeads® (~5 x 10⁸ beads) were incubated for 30 min with 50 µg PLG. After three washing steps with PBS using a magnet, the beads were incubated with 600 ng tPA or 600 ng uPA. In parallel, 5.0 µg of PGK-coated Dynabeads® were incubated with 10 µg of tPA or uPA for 30 min. After three washing steps with PBS, the beads were incubated with 3.0 µg PLG. Plasmin activity was determined in time intervals of 5.0 min up to 60 min in triplicates using the Varioscan Flash ELISA Reader (Thermo Scientific) at 405 nm. Measurement started immediately after adding 30 µl of plasmin substrate S-2251 (Haemochrom Diagnostica). For evaluation of plasmin activity, background values of all samples without activator or PLG was determined and mean values was subtracted from tPA-, uPA-, and PLG- incubated samples at each time point of measurement. BSA-coated beads were applied with and without PLG incubation and activator in order to determine background plasmin activity. PLG-coated beads were incubated with tPA or uPA as positive controls.

Degradation of fibrin matrix

Solid fibrin matrix was produced on transwell insets (falcon, membrane with 3 µm pores) for photographic visualisation and on cover slips for electron microscopy as described in Fulde et al. (52). In brief, 100 µl of 50 mg/ml plasminogen-depleted fibrinogen (Haemochrom Diagnostica) was incubated with 3.0 µl of 1.0 KU/ml thrombin over night at 37°C. After PLG incubation, ~2.5 x 10⁷ PGK coated Dynabeads® were incubated with aggregated fibrin matrix and plasmin formation was induced by adding 600 ng tPA or uPA. To exclude unspecified background degradation, BSA-coated Dynabeads® were incubated with plasminogen as indicated above and incubated with the aggregated fibrin matrix with and without 600 ng tPA or uPA. As positive control, PLG-coated Dynabeads® were incubated with fibrin matrix together with 600 ng tPA. Dissolution of fibrin was determined by eye and documented photographically in a time series of two hours for up to 24 h.
Electron microscopic evaluation of dissolved fibrin matrices was performed as described in Fulde et al. (52). In brief, dissolution of fibrin matrices by plasminogen-pretreated PGK-coated beads was initiated by adding 600 ng tPA as plasminogen activator and incubated for 3 h at 37°C. Dissolution was stopped by adding 2% glutaraldehyde in PBS. As control, fibrin thrombi were incubated with PLG-preincubated PGK-coated beads without adding a plasminogen activator. BSA-coated Dynabeads® were used as further control after preincubation with PLG either with or without tPA. To visualise the intact fibrin matrix, aggregated fibrin thrombi were incubated for 3 h with PBS alone. After fixation, samples were washed with TE-buffer (20 mM TRIS, 1 mM EDTA, pH 7.0) followed by dehydrating in a graded series of acetone (10, 30, 50, 70, 90, 100%) on ice for 15 min for each step. Samples were then subjected to critical-point drying with liquid CO₂ (CPD 30, Bal-Tec, Leica Microsystems, Wetzlar, Germany). Dried samples were covered with a gold film by sputter coating (SCD 500, Bal-Tec, Leica Microsystems, Wetzlar, Germany). Dried samples were examined in a Zeiss Merlin field emission scanning electron microscope (FESEM) at an acceleration voltage of 15 kV. Imaging was performed with the high-efficiency SE2 Everhart-Thorley detector and Inlens-SE detector in a 50:50 ratio. Contrast and brightness of images was adjusted by applying Adobe Photoshop CS5.

Computational sequence alignment

Amino acid sequences were aligned using MegAlign software tool provided by DNASTAR Lasergene 7.1 program according to the Jotun Hein Alignment with a gap penalty of 11 and a gap length penalty of 3.

Statistical evaluation

All experiments were performed in at least three independent assays, each in triplicate and the data were expressed as mean ± standard deviation. Statistical significances were analysed by the two-tailed unpaired Student’s t-test; p-values of < 0.05 were considered as statistically significant.

Results

PGK is expressed on the bacterial surface

A prerequisite for environmental interactions is the exposition of pneumococcal binding proteins on the bacterial surface. After generation and purification of antibodies specifically recognising pneumococcal PGK, surface expression was visualised by transmission electron microscopy using immune gold labelling technique. PGK was detected in the polysaccharide matrix of the capsule-deficient serotype 2 derivative D39cps using 1:1 Langmuir binding algorithm indicated a nanomolar binding affinity for the interaction of PGK with human PLG (KD = 1.3 x 10⁻⁹ M, Figure 2C). The parameters describing binding dynamics are presented in Table 1. Scanning electron microscopy was performed after incubation of D39cps bacteria with PLG-gold nanoparticles (15 nm in size) in parallel to detection of surf-
face-exposed PGK with specific antibodies and gold-labelled protein A/G (10 nm in size). Electron microscopic visualisation detected clusters of both signals, indicating *in vivo* colocalisation of PGK and PLG (Figure 2D, arrowheads). Preincubation of bacteria with PGK increased the amount of surface associated PGK-PLG complexes (Figure 2D, +PGK in two magnifications). The control indicates only marginal unspecific background signals after incubation with PGK-preimmune serum and 15 nm gold particles (control). The colocalisation studies confirmed localisation and reassociation of PGK on the bacterial surface and its function as PLG binding protein.

**PGK-mediated plasmin activity and fibrin degradation**

Plasmin activity analyses were conducted in order to determine conversion of PGK-bound PLG to plasmin. Plasmin-mediated cleavage of the chromophor S-2251 was monitored in time intervals of 5 min (Figure 3A). After incubation of PGK-coated Dynabeads* with PLG, supplementation of either tPA (line with rhombus) or uPA (line with x) resulted in an exponentially enhanced plasmin activation reaching a plateau after 25 min with tPA (1.27 ± 0.12) and after 40 min with uPA (1.2 ± 0.21) (Figure 3A). In contrast, only minor activity was determined in the absence of an activator (PGK-PLG, line with dots). A weak linear increasing plasmin activity was observed after PLG incubation of BSA-coated Dynabeads* in the presence of either tPA (dotted line with triangles) or uPA (broken line with squares) indicating minor unspecific PLG-binding to BSA-coated beads. Both, PGK-coated Dynabeads* and BSA-coated Dynabeads* showed no residual plasmin activity without PLG-incubation in presence of either tPA or uPA.

**Figure 2: Biochemical characterisation and electron microscopic visualisation of PGK-plasminogen interaction.** A) Blot overlay with human PLG after immobilisation of recombinant PGK on nitrocellulose at indicated concentrations in absence or presence of the ATP-analagon AMP-PNP as stabilising substrate. PLG binding was detected with PLG-specific antibodies and peroxidase-conjugated secondary antibodies. Without PLG incubation, no unspecific binding of antibodies was detected (control). B) Blot overlay with recombinant PGK after immobilisation of human PLG. Binding was detected with PGK-specific antibodies and peroxidase-conjugated secondary antibodies. Immunoblot with PGK-specific antibodies indicated no unspecific antibody recognition (control). C) Surface plasmon resonance (SPR) measurements of PGK interaction with human PLG. PLG was immobilised on a BIAcore™ CMS chip and recombinant PGK was used as analyte. Changes in Plasmon resonance are shown as relative response units (RU). PGK was injected in concentrations of 4.0 µM, 2.0 µM, 1.0 µM and 0.5 µM. Sensograms demonstrate concentration dependent binding of PGK to human PLG and were used for evaluation of binding kinetics. The blank run was subtracted from each sensogram. BIAcore results representing the association rate constant ($k_a$), the dissociation rate constant ($k_d$), the affinity constant ($K_D$) and the Chi² values are shown in Table 1. D) Field scanning electron microscopy (FESEM) of PGK-PLG colocalisation. D39cps bacteria were preincubated with recombinant PGK protein and incubated with PGK-gold nanoparticles (15 nm). Surface associated PGK was detected with specific antibodies and protein A/G gold nanoparticles (10 nm). Visualisation by FESEM on the two upper pictures (+ PGK in two different magnifications) detected both nanoparticles in clusters on the bacterial surface, indicating colocalisation of PGK and PLG (arrow heads). Similar colocalisation clusters but less amount of PGK-detecting nanoparticles were visualised on bacterial surface without PGK preincubation (-PGK). Only marginal signals were detected after incubation of bacteria with preimmune serum and 15 nm gold nanoparticles in a control experiment (ctrl). Arrows mark unspecific background binding of PGK nanoparticles (10 nm) and arrow heads point to gold particles (15 nm).
uPA (Figure 3A). In contrast, PLG-coated beads exhibited similar plasmin activity in presence of either tPA or uPA as PGK-coated beads after PLG in presence of tPA or uPA (resulting in absorbance at 405 nm of 1.77 ± 0.05 (with tPA) and 1.695252 ± 0.03 (uPA, data not shown)).

Incubation of synthetically generated fibrin aggregates with PGK-coated Dynabeads® after PLG incubation in presence of tPA or uPA resulted in a stepwise dissolution of the solid, white fibrin matrix into clear liquid. Photographic illustration of fibrin aggregates generated on 24-well transwell insets indicated the start of visible fibrin dissolution 4 h after incubation with tPA or uPA as activator (Figure 3B, PKG-Dynabeads® + PLG + tPA and PGK-Dynabeads® + PLG + uPA). After 6–8 h the process of fibrin degradation has reached the bottom of the transwell inset. The complete dissolution of the turbid fibrin matrix into a clear solution was monitored after 24 h of incubation (Figure 3B, PGK-Dynabeads®, red frame). Fibrin dissolution was also observed with PLG-coated Dynabeads® in the presence of tPA as positive control (Figure 3B, PLG-Dynabeads®, green frame). In contrast no visible fibrin dissolution was monitored after incubation of the fibrin meshwork with PGK-coated Dynabeads® alone or without an activator and after incubation with BSA-coated Dynabeads®.

Electron microscopic visualisation of fibrin degradation at three different magnifications by PLG-preincubated PGK-Dyna-
Plasminogen binding is species-unspecific

The pathogenic potential of S. pneumoniae is not restricted to humans, but also to other mammals such as horses, dolphins, and guinea pigs (31). Results of dot spot analyses using human and murine PLG as ligand (Figure 4A) or analyte (Figure 4B), respectively, demonstrated a similar binding strength of pneumococcal PGK to both, human and murine PLG. Moreover, after immobilisation of murine PLG onto CM5-Sensorchips, SPR analysis revealed a concentration-dependent binding of pneumococcal PGK (Figure 4C). The respective dissociation constant of \( K_D = 4.69 \times 10^{-7} \text{ M} \) was lower compared to the dissociation constant obtained for the interaction between PGK and human PLG \( (K_D = 1.3 \times 10^{-6} \text{ M}) \) (Figure 4C, D and Table 1). Comparison of single kinetic parameter indicated similar association rates of \( 8.39 \times 10^{3} \text{ M}^{-1} \text{ s}^{-1} \) for human PLG and \( 7.98 \times 10^{3} \text{ M}^{-1} \text{ s}^{-1} \) for murine PLG, but a higher dissociation rate for the interaction of PGK with murine PLG \( (1.06 \times 10^{-5} \text{ s}^{-1} \) for human PLG and \( 3.75 \times 10^{-3} \text{ s}^{-1} \) for murine PLG, Table 1). These results show that interaction of pneumococcal PGK with murine PLG occurs specific but with lower affinity and indicate a species-independent interaction.

Identification of angiostatin as PGK-ligand

Cleavage of PLG with elastase results in generation of different PLG fragments including the N-terminal kringle domains 1–3 and 1–4 representing angiostatin and the C-terminal mini-PLG containing kringle domain 5 and the enzymatic domain (8) (Figure 5A). Dot spot overlay analyses demonstrated a dose-dependent binding of full-length PLG, angiostatin (ANG), and kringle domains 1–3 (Kringle 1–3) to pneumococcal PGK (Figure 5B). Furthermore, immunoblots confirmed that PLG-specific polyclonal antibodies recognised all immobilised PLG fragments (Figure 5B, control 1) but no unspecific binding to PGK was detected (Figure 5B, control 2). In contrast, no PGK binding was detected using mini-PLG as analyte (Figure 5A, B). Therefore the binding site for pneumococcal PGK in PLG could be narrowed down to the first three kringle domains of PLG representing angiostatin.

Localisation of plasminogen binding sites in PGK

In order to identify the PLG binding sites within the PGK protein, a spot membrane-based peptide array representing the whole amino acid sequence of pneumococcal PGK was conducted. The PGK peptides were 16 amino acids in length with an offset of four amino acids. Overlay analyses with human PLG indicated the presence of two quaternary groups of peptide spots (BS1: GKKVLVRVDFNVPLKD, BS2: RAILFSHLGRVKEESD) with increasing PLG binding intensity (Figure 6A). Sequence mapping

Figure 3: C) Electron microscopic visualisation of fibrin thrombi generated on cover slips after incubation for 3 h with PLG-preincubated PGK-coated Dynabeads® and tPA (PGK-beads, +PLG, +tPA). Three different magnifications for each incubation set up are illustrated. Size bars of pictures at left hand site represent 20 µm, of pictures in the mid position 10 µm and on the right hand site 2.0 µm. The first row depicts the intact aggregated fibrin matrix after 3 h of incubation in PBS as control (ctrl). No fibrin dissolution is visible after incubation of the fibrin thrombi with PLG-preincubated BSA-coated Dynabeads® in presence of tPA (BSA-beads, +PLG, +tPA) or with PLG-preincubated PGK-beads without activator (PGK-beads, +PLG, +tPA).
indicated that the amino acid motifs “GKKV” (BS1) and “RAIL” (BS2) are crucial for PLG binding. The importance of the single amino acids in PLG binding was investigated in a second peptide array comprising the 16mers of BS1 and BS2 with a systematical alanine exchange at each subsequent amino acid position. After PLG overlay, a significant reduction of binding signal was detected for two peptide spots with substitutions of the lysines in BS1 (peptide no. 3 and 4) resulting in the peptide sequence “GAKVLVRDFNVPLKLD” (no. 3) and “GKAVLVRDFNVPLKLD” (no. 4, Figure 6B, C). A further loss of binding signal was identified in BS2 after alanine substitution of an arginine (peptide no. 19) resulting in the peptide sequence “AAILESHLRVKEESD” (no. 19, Figure 6B, C). The peptide spot array analyses detected two putative PLG binding sites in the amino-terminal domain of PGK and identified two lysines of BS1 and an arginine in BS2 as crucial for interaction.

Structural determinants of PLG binding by PGK

Three-dimensional structure of pneumococcal PGK was solved by X-ray crystallography at 1.78 Å resolution (Table 2). PGK folds into two globular domains, the N-domain and C-domain, consisting of residues 1–191 and 192–398, respectively (Figure 7A). The 10 C-terminal residues cross back to the N-terminal domain, via a short helical segment leaving the Ca-atoms of the N- and C-terminal residues only 8 Å apart. Each domain folds as a α/β domain consisting of five parallel β-strands (in N-domain), and four parallel β-strands (in C-domain); both sheets are sandwiched between four long α-helices, two to a side. In addition, C-domain has two antiparallel β-strands above the C-termini of the parallel β-strands. A search for structurally PGK related proteins performed by PDBe server identified the PGKs from *Geo-bacillus stearothermophilus* (PDB code 1PHP, Z score of 20.0 and rmsd of 1.33 Å for 387 Ca-atoms) and from *Bacillus anthracis* (PDB code 3B2B, Z score of 19.7 and rmsd of 1.31 Å for 382 Ca-atoms) as the most closely related known three-dimensional (3D) structures.

The binding site for the ATP-analog AMP-PNP is located in the C-domain. The nucleotide is oriented with the adenine ring distal to the N-domain and the triphosphate moiety adjacent to the interdomain cleft and stabilised by many polar interactions, some of them mediated by water molecules (Suppl. Figure S1, available online at www.thrombosis-online.com). The 3-phosphoglycerate binding site is located at the active site of the N-terminal domain. This site was not occupied in our pneumococcal PGK structure.

Two PLG binding sites, BS1 (presenting the 16GKKV motif) and BS2 (presenting the 16RAIL motif), were identified by spot membrane-based peptide array. Crystal structure of pneumococcal PGK reveals that both sites are indeed placed in the same locus defining a single PLG binding site. This region is located in the N-terminal domain at the opposite side of the catalytic site and presents a large number of well-paired surface charges (Figure 7B). Besides the Lys14, Lys15, and Arg53 from BS1 and BS2, PLG binding site presents other positively charged residues (Lys12) and negatively charged residues (Asp10, Glu101, Glu108, Asp109, Asp144, and Glu170) (Figure 7B). This paired charge distribution has been also observed for other PLG binding proteins such as enolase (32). Sequence analysis among PGK from different species suggests that the regions that we im-

![Figure 4: Dot spot analyses and surface plasmon resonance measurements of PGK-plasminogen interactions.](image)

**Table 1: BIAcore parameter of binding dynamics.**

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<th>Ligand</th>
<th>Analyte</th>
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<th>$k_d$ (1/s)</th>
<th>$K_D$ (M)</th>
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plicate in the PLG binding are not conserved in bacterial PGKs other than streptococci (Suppl. Figure S2, available online at www.thrombosis-online.com).

A computational model of the PGK:Kringle complex has been generated based on our PGK crystal structure and the reported three-dimensional structures of kringles (see Methods). This model (Figure 7C) faces the PGK PLG binding site, with the lysine-binding site of K2 and reveals a perfect charge complementarity between the pneumococcal protein and human kringle structure. Interestingly, PGK:Kringle complex reveals that Lys14 residue from PGK would play a central role by interacting with both Trp residues of the Lysine-binding site through a cation–π interaction and making a salt bridge interaction with Glu59 from kringle (Figure 7C). Around this central interaction many polar and electrostatic interactions are predicted between PGK and kringle structures. Very recently the crystal structure of inactive full-length human PLG comprising the Pan-apple (PAp) domain, five kringle domains (K1–5), and a serine protease (SP) domain, has been reported (33). Interestingly, the interaction of K2 with the SP domain observed in human PLG is extremely close to that predicted for the PGK:K2 complex (Suppl. Figure 3, available online at www.thrombosis-online.com). In both cases a Lys residue is inserted in the Trp hydrophobic cavity of kringle, while establish a salt bridge interaction with acidic residues from the kringle. The central interaction mediated by the Lys residue is complemented by many salt bridge interactions between kringle and SP/PKG charged residues (Suppl. Figure S3, available online at www.thrombosis-online.com).

Binding of PGK to tissue-type plasminogen activator

Besides PLG, other human-derived proteins involved in fibrinolysis like PLG activators comprise kringle domains. Two PLG activators serve as early mediators of fibrinolysis and degrade fibrin. Tissue-type plasminogen activator (tPA) possesses two such structural units (34) and urokinase plasminogen activator (uPA) present one kringle module (35) (Suppl. Figure S5A, available online at www.thrombosis-online.com). Binding of tPA has already been reported to some non-streptococcal species, whereas no streptococcal binding to tPA and no bacterial binding to uPA has been demonstrated so far (36). The 3D structures of kringle 2 domain of tPA (34) and angiostatin (kringle domains 1 to 3) (37) have been reported. Structural superimposition of these structures on modelled PGK:K2 complex (Figure 8A) and molecular dynamic simulations (Suppl. Figure S6, available online at www.thrombosis-online.com) reveal that despite structural differences between these modular proteins, all of them could be fitted on the PLG binding site found in PGK. To rule out this hypothesis dot blot binding analyses with immobilised human PLG, tPA and uPA were performed. Our results confirmed a dose-dependent binding of pneumococcal PGK to PLG and tPA but not to uPA (Figure 8B).

Figure 5: Dot spot analyses of PGK interaction with human plasminogen different plasminogen derivatives. A) Schematic model of PLG domain structure and used PLG derivatives. The PAN-apple domain is shown in red, the kringle domains are depicted in green, and the serine protease domain is shown in violet (K, Kringle; PAN, PAN-apple (Pap)-domain; ANG, angiostatin, miniPLG, mini-plasminogen). B) Dot Spot analyses of PGK binding to different PLG derivatives. Pneumococcal PGK was immobilised in different amounts ranging from 10.0 µg down to 0.16 µg onto a nitrocellulose membrane and human plasminogen (PLG) and the plasminogen derivatives angiostatin (ANG), representing kringle domains 1–3 and 1–4 (K1–3; K1–4), mini-plasminogen (miniPLG), representing the last kringle domain (K5), and the enzyme domain, and kringle domains 1–3 (Kringle 1–3) were used as analytes in overlay studies. Detection of binding signal was performed with PLG-specific antibodies. Control 1 represents equal detection intensity of the different PLG derivatives by PLG-specific antibodies. Control 2 shows no unspecific background binding of PLG antibodies to PGK.
Figure 6: Determination of plasminogen binding sites in PGK by peptide spot array. A) PLG-overlay of PGK-peptide spot array. A peptide spot membrane of the 398 amino acid sequence of PGK divided into overlapping peptides of 16 amino acids in length with an offset of four amino acids was analysed for PLG binding. Twenty-five peptides were immobilised in one line. Binding of PLG was detected in spots 1 to 4 (BS1, first box) representing the peptide sequence from 1 to 28 and in spots 11 to 14 (BS2, second box) representing the peptide sequence from 41 to 68. The crucial four amino acids were marked in bold. Reactivity of other spots was due to unspecific binding of plasminogen-antibodies and secondary antibodies. B) Alanine-amino acid exchange analyses of PLG-binding sites BS1 and BS2 of PGK. PLG overlay analyses of a peptide spot membrane representing a systematic alanine amino acid exchange of the 16mer peptide sequences BS1 and BS2 at each single amino acid position. Alanine exchange at position 3 and 4 of BS1 and at position 19 representing amino acid 2 of BS2 leads to loss of PLG-binding activity. C) Amino acid sequences of the immobilised peptide spots after alanine exchange. Two putative PLG binding sequences were identified BS1: GKKVLVRVDFNVPLKDLQ, BS2: RAILFSLHGRVKEEDSLQ with the lysines of BS1 (in bold) and the arginine in BS2 (in bold) as crucial amino acids.

In addition to structural analysis, a sequence alignment between tPA and uPA was carried out to explain the observed differences in binding activity for tPA and uPA (Suppl. Figure S5B, available online at www.thrombosis-online.com). Interestingly, one of the two conserved Trp residues forming the binding site in PLG kringle domains and in tPA (Trp235 in PLG and Trp72 in tPA) is substituted by Val123 in uPA. Of special relevance is a substitution of Asp57 (tPA) by Arg108 (uPA) (Suppl. Figure S5C, available online at www.thrombosis-online.com) as this Asp residue is involved in the critical salt-bridge interaction with Lys residue of PGK. This interaction, observed in the crystal structure of inactive PLG and also in our modelled complexes between K2:PGK and tPA:PGK, would not be possible between PGK and uPA. In summary, differences in uPA binding-site conformation together with the presence of Arg108 residue would impair the interaction with PGK.

Plasmin activity analysis was performed with PGK-coated Dynabeads®, preincubated with tPA. Subsequent incubation with PLG resulted in a fast and substantial plasmin generation (PGK-tPA + PLG, line with triangles, Figure 8 C) reaching an absorbance of 1.06 ± 0.02 after 60 min of incubation. In contrast, only marginal plasmin activity was monitored after incubation of PGK-coated beads with uPA prior to PLG supplementation (PGK-uPA + PLG, line with x, Figure 8 C). Only minor conversion of PLG into plasmin was determined using PGK-beads after incubation with tPA (PGK-tPA, broken line with dots) or uPA (PGK-uPA, broken line with rhombus) alone. Plasmin activity was absent after incubation of tPA- or uPA- preincubated BSA-beads, indicating no unspecific binding of plasminogen activators to the BSA-coated beads (data not shown). These results confirm the direct interaction between PGK and tPA and demonstrate the effective conversion of PLG into plasmin by tPA bound to PGK protein.
Table 2: Data collection and refinement statistics.

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Values in parenthesis correspond to the highest resolution shell. \( R_{merge} = \sum (I - <I>) / \Sigma I \).

Discussion

Interaction of *S. pneumoniae* with host derived PLG has been characterised as powerful virulence mechanism facilitating dissemination within the host tissue and transmigration of tissue barriers by subversion of the proteolytical potential (5, 6). Our work reveals that the pneumococcal glycolytic enzyme PGK has to be classified as an additional “moonlighting protein” that is expressed on the surface and interacts with host proteins of the fibrinolytic system. PLG binding by bacterial PGK has also been reported for oral streptococci (*S. anginosus* and *S. oralis*) (38), and *S. agalactiae* (39, 40). In accordance with our former studies on enolase, electron microscopic analysis demonstrates surface localisation and surface reassociation of PGK. Furthermore, FESEM visualised colocalisation of PGK with PLG, confirming PGK as PLG binding protein. SPR-binding analyses of the interaction between the pneumococcal enolase and PLG suggested a conformational change resulting in a two-step dissociation (20), while SPR evaluation of PGK-PLG interaction followed a Langmuir 1:1 binding model. The calculated dissociation constant of PGK-PLG binding of \( K_D = 1.3 \times 10^{-9} \) M was in similar high-affinity range compared to the dissociation constants reported for the interaction of the pneumococcal enolase with PLG (\( K_D = 0.55 \times 10^{-9} \) M and \( K_D = 86.3 \times 10^{-9} \) M) (20) and for *S. pyogenes* enolase SEN with PLG (41, 42). Determination of a dissociation constant of \( 4.69 \times 10^{-7} \) M for the binding of murine PLG to PGK confirmed the results of dot blot binding studies and indicated a species-unspecific interaction, but with lower affinity. Although computational analyses of amino acid sequence homology revealed similarity of 78.8% between human PLG (Ac.no. : AAA36451.1) and murine PLG (Ac.no. AAA50168.1), the reduced binding affinity to murine PLG could be the result of residual differences in the amino acid sequence.

Structural determination of PGK indicated that PLG-binding site is located at the N-terminal part of PGK in opposite location of the catalytic site, thereby most probably unaffected conformational changes or enzymatic activities. This is also confirmed by the Langmuir-1:1 binding model applied for SPR analyses, representing PGK-PLG interaction without conformational changes.

In contrast to several PLG binding proteins i.e. eukaryotic enolase that interact with PLG lysine binding sites via C-terminal lysine residues, internal lysine-containing binding motifs have been characterised as major binding sites for several bacterial PLG-binding proteins including pneumococcal enolase (5) and the M-protein of group A streptococci (PAM) (43). Alignment of peptide sequences of the pneumococcal PGK with enzymes of other oral streptococci like *S. oralis* and *S. anginosus* and with *S. agalactiae* revealed up to 98% sequence identity (Suppl. Figure S4, available online at www.thrombosis-online.com), implying similar biochemical binding mechanisms. Recently, lysine-rich PLG binding sites were identified via peptide mapping in the PGK protein of *S. agalactiae* (40). Interestingly, the pneumococcal PGK peptide sequences which constitute highest homology to the identified PLG-binding sites of *S. agalactiae* PGK do not react with PLG in a similar peptide spot array. Nevertheless, a structural overlay of the PGKs of *S. pneumoniae* and *S. agalactiae* indicated a localisation of the binding site (12KKESKNDNDE13) from GBS (40) within the PLG binding region formed by the pneumococcal PLG-binding sites BS1 and BS2. Therefore, the results of peptide spot arrays and structural mapping point to a similar binding chemistry of pneumococcal and group B streptococcal PGK and illustrate the crucial contribution of the identified BS1 and BS2. Lastly, Boone and Tyrrell suggested a glutamic residue as crucial for PLG binding of *S. agalactiae* PGK (40). However, this position is occupied by a pro-
Figure 7: Crystal structure of pneumococcal PGK and modelled interaction with plasminogen. A) Crystal structure of the PGK protein. The N-domain (residues 1–191) is depicted in green, C-domain (192–398) in blue, glycerol molecules and AMP-PNP are depicted in sticks. Mg\(^{2+}\) ions are drawn as green spheres. B) Location of the PLG binding site on PGK molecular surface. PGK (brown) is oriented as in panel A. Paired charged distribution is found in the PLG binding site. Negatively charged residues (Asp10, Glu101, Asp109, Asp144 and Glu170) are depicted in red and positively charged residues (Lys12, Lys14, Lys15 and Arg53) in blue. C) Computational model of PGK in complex with plasminogen K2 domain. Residues involved in the predicted interactions between PGK (wheat colour) and K2 (green) are drawn as sticks. Polar interactions are represented as dotted lines.

line in both the \textit{S. pneumoniae} PGK and in the \textit{S. anginosus} PGK and by a serine in \textit{S. oralis} (Suppl. Figure S4 marked in blue, available online at www.thrombosis-online.com), all of them reported to bind PLG (40). These data indicate that the amino acid at position 133 is not likely critical for PLG binding.

PLG is known to interact with its ligands via pre-formed lysine-binding sites within the N-terminally located triple-disulfide-bonded kringle domains (7). Reported structures of different kringle domains (44–48) delineate the basic characteristics of the binding pocket (named lysine-binding site) of kringles (49, 50), which consists of a groove at the surface of the molecule, formed by two tryptophan side chains, flanked by charged residues. In contrast to the interaction of the \textit{S. canis} M protein SCM with mini-PLG (51, 52), dot spot results demonstrate binding of the pneumococcal PGK to angiotatin, which represents the kringle domains 1–3. Likewise, interaction of two internal peptide sequences (a1 and a2) of the \textit{S. pyogenes} M protein PAM to kringle domain 2 of plasminogen has been reported (53). Crystal structure of pneumococcal PGK shows the presence of a patch of exposed lysine-rich residues that might mediate interaction with the PLG K2 as observed for the interaction of K2 with the SP domain in the crystal structure of inactive PLG (35).

Our structural model for the interaction between PGK and K2 (Figure 7C and Figure 8A) pointed to a potential interaction with other kringle-containing proteins such as PLG activators tPA and uPA. Overlay analyses confirmed binding of tPA to pneumococcal PGK but not for uPA. This differential binding has been also observed in non-streptococcal bacteria (\textit{E. coli} S-fimbria, \textit{Haemophilus} and \textit{Neisseria}) which bind tPA but not uPA (54). This is, to our knowledge, the first report of a tPA binding to \textit{S. pneumoniae}. It is worth noting that up to now none of the tested streptococci (\textit{S. pyogenes}, \textit{S. equisimilis} and group G streptococci) showed binding for the PLG activators (55), indicating a unique binding of \textit{S. pneumoniae} to tPA. The interaction with the amino-
terminal kringle domains may allow a conformational opening and enhanced activation of the proteolytic active center of PLG. It has been demonstrated that recruitment of plasmin activity to the pneumococcal surface facilitates degradation of fibrin, thrombin and complex extracellular matrix, thereby providing enhanced dissemination through tissue barriers and entry into the vascular system (5). In contrast to other streptococcal species, pneumococcal plasminogen binding proteins are not acting as direct plasminogen activators. But consistent with all pneumococcal PLG binding proteins identified so far, results of plasmin activity analysis indicated that PLG binding to PGK promotes conversion of PLG into plasmin via host-derived tPA or uPA. A remarkable and unique prop-
property of PGK is the recruitment of tPA, which also leads to enhanced activation of plasminogen and subsequently to an effective degradation of fibrin thrombi. The synergistic immobilisation of both PLG and the host-derived activator tPA to the bacterial surface might promote the efficiency and speed of enzymatic PLG conversion via tPA at the local side of pneumococcal infection and may translocate fibrinolytic cascade directly to the bacterial surface in order to increase the resulting degradation activity, which may facilitate bacterial spread within host.

Considering that fibrinolysis also contributes to complex inflammatory processes, our results describe a new scenario in which this important human pathogen can subvert the host fibrinolytic machinery during the initial phase of bacterial colonisation enhancing both dissemination through tissue barriers and entry into the vasculature.

Acknowledgements

We thank Astrid Droegge for technical assistance and Ina Schleicher (HZI, Braunschweig) for the preparation of probes for electron microscopy. We are also grateful to Uwe Schubert (Justus-Liebig University, Giessen) for purification of PLG and various derivatives and thank Andy Polok, Andreas Raschka, and Sandra Koch for technical assistance. The authors thank the ID14–2 beamline at the ESRF facility of Grenoble for access to synchrotron radiation.

What is known about this topic?

- *Streptococcus pneumoniae* binds human plasminogen via several surface-exposed binding proteins, but does not express an endogenous plasminogen activator.
- Recruitment of plasminogen to pneumococcal surface promotes conversion of plasminogen into proteolytic active plasmin.
- Bacterial-surface-bound plasmin activity leads to dissolution of fibrin thrombi and to degradation of extracellular matrix proteins of the host.

What does this paper add?

- We demonstrate that phosphoglycerate kinase (PGK) is indeed exposed on pneumococcal surface colocalising with plasminogen. We provide kinetic evaluation of interaction with human and murine plasminogen, detect two linear plasminogen binding sites and localise the crucial amino acids of PGK contributing to plasminogen binding.
- We present the high-resolution crystal structure of pneumococcal PGK revealing localisation of the plasminogen-binding sites in one surface exposed region of the N-terminal domain and determine the first four kringle domains of plasminogen, named angiostatin, as binding domains required for interaction with PGK.
- Kringle-based interaction between PGK and tPA was predicted by structural modelling and confirmed experimentally by binding studies and plasmin activity assays.

Conflicts of interest

None declared.

References


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