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Biological functions of GCS3, a novel plasminogen binding protein of *Streptococcus dysgalactiae* ssp. *equisimilis*

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Abstract

Increasing awareness of the relevance of *S. dysgalactiae* ssp. *equisimilis* as a human pathogen motivates the analysis of its pathomechanisms. One of the mechanisms that increases infectivity and dissemination of several streptococcal species is the recruitment and subsequent activation of host plasminogen on the streptococcal surface. This study identified GCS3 as a novel plasminogen binding M-protein of *S. dysgalactiae* ssp. *equisimilis* and revealed a difference in the mode of binding as compared to the plasminogen binding protein PAM of *Streptococcus pyogenes*. In contrast to PAM, GCS3 did not bind to the kringle 1-3 region of plasminogen. Despite this difference GCS3 exerts the same function of recruiting plasminogen to the streptococcal surface, which can be activated by streptokinase and host plasminogen activators to serve as a spreading factor. Moreover, we demonstrate a role of GCS3 in plasminogen dependent streptococcal adherence to human pharyngeal cells (cell line Detroit 562) that indicates an additional function of the protein as an adhesin in the oral cavity.

Introduction

Streptococcus pyogenes is a dominant cause for pyogenic streptococcal infections in human. However, a considerable part of these infections is caused by *Streptococcus dysgalactiae* subsp. *equisimilis* (herein abbreviated *S. equisimilis*). Strains of this species carry Lancefield group C or G polysaccharides that are also found in a number of other streptococcal species that infect animals or humans (for reference see: Facklam, 2002; Reißmann et al., 2010). *S. equisimilis* causes a similar spectrum of disease as *S. pyogenes* comprising suppurative throat and skin infections and is isolated from life–threatening invasive infections in human with an alarming frequency (Broyles et al., 2009; Cohen-Poradosu et al., 2004; Ekelund et al., 2005; Nohlgard et al., 1992; Sylvetsky et al., 2002)

Although genetically distinct species (Facklam, 2002), *S. pyogenes* and *S. equisimilis* share several virulence factors (Davies et al., 2007), including the surface anchored M proteins (Bisno et al., 1987; Broyles et al., 2009; Pinho et al., 2006; Reißmann et al., 2010; Simpson et al., 1987) of which more that 150 are known to date. M proteins are coded by the *emm* genes, which are specific for either streptococcal species. They contribute to the resistance of streptococci to phagocytosis as well as to host colonization (for reference see: Nitsche-Schmitz et al., 2007a). Although other plasminogen binding factors exist on the surface of *S. pyogenes* such as the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and α-enolase (Bergmann et al., 2001; Bergmann et al., 2004; Pancholi and Fischetti, 1998; Pancholi and Fischetti, 1993), M proteins have evolved that confer to certain strains the ability to bind substantial quantities of plasminogen. One of the best studied plasminogen binding M proteins is PAM (Berge and Sjöbring, 1993; Walker et al., 2005; Wistedt et al., 1998). The plasminogen binding domain of PAM displays a high degree of variability at the amino acid level, likely due to host selective immune pressure since antibodies against this region are opsonic (Sanderson-Smith et al., 2006a). However, despite the high sequence variability plasminogen binding is retained, indicating that it has an important function on the surface of

S. pyogenes (Sanderson-Smith et al., 2006a; Sanderson-Smith et al., 2006b). Indeed, the ability of *S. pyogenes* to bind plasminogen has been linked to a higher propensity to cause invasive disease, since isolates from invasive infections bound more plasminogen than those from non-invasive disease (McKay et al., 2004). Moreover, human plasminogen transgenic mice showed an increased susceptibility to *S. pyogenes* infection (McKay et al., 2004; Sun et al., 2004).

Plasminogen is the 92 kDa pro-enzyme form of plasmin, a key component of the mammalian fibrinolytic system. It circulates in the human plasma and extracellular fluids at a concentration of approx. 2 μM and is composed of an N-terminal pro-peptide followed by 5 kringle domains and a catalytic C-terminal serine protease domain (Parry et al., 2000). Proteolytic cleavage of the N-terminal 8 kDa peptide transforms the original Glu-plasminogen into the pre-activated form Lys-plasminogen. The kringle domains contain lysine-binding sites that allow binding to fibrinogen and to plasminogen receptors. The pro-enzyme is activated by the specific plasminogen activators urokinase (uPA) and tissue-type plasminogen activator (tPA) of the host, which cleave between the fifth kringle domain and the catalytic domain at the bond between Arg_{560} and Val_{561} to generate the two-chained plasmin, which is held together by inter-chain disulfide bridges. This activated broad range serine protease is crucial for blood clot dissolution and exerts additional functions in cell migration and tissue remodeling (Lähteenmäki et al., 2001; Parry et al., 2000). The ability to bind plasminogen on their outer surface is not unique for streptococci, but observed for several other bacterial species. Recruitment of its proteolytic activity is considered as a way for bacteria to increase their dissemination (Lähteenmäki et al., 2001). The importance of this mechanism is underlined by the fact that some human pathogenic streptococcal species like *S. pyogenes* and *S. equisimilis* express streptokinase, which is a potent and specific activator for human plasminogen. Moreover, plasminogen is readily activated by human tPA or uPA when bound

112 to the *S. pyogenes* surface. Plasminogen binding M proteins have also been identified in *S.*
113 *equisimilis* strains, namely MLC36 and MLG72. These proteins like PAM of *S. pyogenes*
114 bind to a fragment of plasminogen that comprises the kringle domains 1 to 3 (Ben Nasr et al.,
115 1994). Although it has not been studied sufficiently, it is likely that plasminogen binding
116 plays a similar important role in infections with *S. equisimilis* as it does for the virulence of *S.*
117 *pyogenes*. We have recently discovered the plasminogen binding activity of the *S. equisimilis*
118 M protein GCS3 and provide evidence for its diverse roles during infection.

Results

Absorption of plasminogen from human plasma by *S. dysgalactiae* ssp. *equisimilis*

The ability of different *S. equisimilis* strains to bind plasminogen was determined by incubating the bacteria with human plasma and eluting bound proteins under acidic condition. The amount of plasminogen in the eluates was visualized by SDS-PAGE and immunoblot analysis, using specific antibodies against human plasminogen. Strains C90 and C107 as opposed to strain G35 bound clearly detectable amounts of a protein that had the mobility of a 100 kDa protein in SDS-PAGE (Fig. 1A). The immunoblot identified this protein as plasminogen (Fig. 1B). In a binding experiment with ^{125}I-labeled human plasminogen (14 ng, 100,000 cpm), strain C90 and C107 bound 78% and 51% of the added plasminogen, respectively, indicating that the strains have a similar affinity for this protein as the PAM expressing GAS strain (A158) that bound 61% (Fig. 1C). Sequencing of the *emm* genes revealed that strain C107 (*emm* type *stc36*) carries the plasminogen binding M protein MLC36 (Ben Nasr et al., 1994). The *emm*-gene of strain C90 (*emm* type *stc*839) encodes for the M-protein GCS3. Binding of plasminogen to this M protein had not been shown previously.

GCS3 is a plasminogen binding factor of C90

To investigate the role of GCS3 in plasminogen binding, GST-fusion proteins of MLC36 (GST-MLC36) and GCS3 (GST-GCS3) were examined for plasminogen binding in a dot blot that was probed with human ^{125}I-plasminogen (25 µg, 1.8×10^8 cpm). Both GCS3 and MLC36 showed concentration-dependent binding of plasminogen. No binding was seen with the control M proteins GST-M6.1, GST-M3.1, which excluded interaction with the GST-tag. The hexa-histidine tagged protein His_6-GRAB served as an additional negative control (Fig. 2A).

Concentration-dependent interaction between tag free GCS3 and human plasminogen was observed in surface plasmon resonance measurements. An apparent dissociation constant of K_D = 18 nM (Fig. 2B) could be determined based on the Langmuir model of a 1:1 interaction. Thus, GCS3 binds to plasminogen with a similar affinity as PAM (K_D = 1 nM) (Berge and Sjöbring, 1993). To study the ability of GCS3 to recruit plasminogen to the streptococcal surface, the protein was heterologously expressed on the surface of *S. gordonii* strain GP1221. Measuring binding of ^{125}I-plasminogen (14 ng, 100,000 cpm) revealed a significantly increased binding of plasminogen to the GCS3 expressing recombinant *S. gordonii* (SGO GCS3). This presents further evidence that GCS3 is a streptococcal plasminogen receptor (Fig. 2C).

GCS3 binding site in human plasminogen

The *S. pyogenes* protein PAM and *S. equisimilis* proteins MLC36 and MLG72 (Ben Nasr et al., 1994; Wistedt et al., 1998) bind to the lysine-binding sites in a fragment of plasminogen that consists of the kringle domains 1 to 3 (kringle 1-3). To test whether GCS3 utilizes the same contact site for plasminogen binding the following competition experiments were performed. Binding of full-length ^{125}I-plasminogen (14 ng, 100,000 cpm) or ^{125}I-kringle 1-3 (14 ng, 100,000 cpm), respectively to *S. equisimilis* strain C90 and to the PAM expressing *S. pyogenes* strain A158 was measured. Furthermore, the inhibitory effect of unlabeled kringle 1-3 (20 µg) was tested (Fig. 3A). Although full-length ^{125}I-plasminogen bound to both C90 and A158, the 3400fold molar surplus of unlabeled kringle 1-3 did only interfere with binding to A158 indicating that GCS3 of strain C90 bound to a different contact site on the full length protein. This was supported by the observation that binding of ^{125}I-kringle 1-3 to C90 (18%) was low, as compared to A158 (42%)(Fig. 3A). Like binding of ^{125}I-plasminogen to A158, interaction with C90 can be inhibited by ε-aminocaproic acid (EACA) in a concentration-dependent manner (Fig. 3B). Increased binding of ^{125}I-plasminogen due to expression of

GCS3 on the surface of *S. gordonii* can also be abolished by addition of EACA in concentrations above 1 μM. In summary the experiment indicates that the lysine analogue EACA inhibits the interaction between human plasminogen and GCS3.

Plasminogen activation and biological activity on the surface of *S. dysgalactiae* ssp. *equisimilis*

Inhibition experiments with kringle 1-3 indicated that the mode of the plasminogen interaction differs between PAM and GCS3. To investigate whether GCS3-bound plasminogen, like PAM-bound plasminogen, remains prone to activation, its ability to cleave the chromogenic substrate S-2251 was measured. Incubation of C90 or SGO GCS3 with human plasma (Fig. 4) or purified human plasminogen (data not shown) in the presence of streptokinase led to an increased cleavage of S-2251 as compared to the negative control SGO WT (Fig. 4A). Similarly, addition of the host plasminogen activator uPA, activated the plasminogen that was bound to the GCS3 bearing strains (Fig. 4B). Biological activity of the activated plasmin(ogen) was examined by measuring the degradation of immobilized ^{125}I-fibrinogen (Fig. 5A). In absence of streptokinase the plasminogen-coated strains SGO GCS3 and SGO WT did not cause considerable proteolysis. In contrast, during incubation with *S. equisimilis* strain C90, degradation of fibrinogen was observed, probably due to the production of streptokinase. Addition of streptokinase increased the fibrinogen degradation, which was about twice as high with the GCS3 bearing strains as with the GCS3-negative SGO WT. This indicated a contribution of the GCS3-bound plasmin(ogen) to fibrinogen digestion. Immunoblot analysis of a fibrinogen solution that was incubated with plasminogen pre-incubated strains C90 and SGO GCS3 in the presence of streptokinase revealed that degradation occurs also in solution (Fig. 5B). Plasminogen and streptokinase dependent degradation of fibrin by SGO GCS3 was visualized by scanning electron microscopy (Fig. 6). The results demonstrate that GCS3-bound plasminogen can be activated by streptokinase or

193 the host plasminogen activator uPA. The activated plasmin(ogen) degrades fibrinogen and its
194 coagulated form, fibrin. Interestingly, at the same conditions that were sufficient for
195 degradation of fibrinogen (Fig. 5D), we could not detect a proteolytic activity of the activated
196 plasmin(ogen) against fibronectin (Fig. 5C), suggesting a certain specificity for the
197 coagulation factor.
198

199 ## Adherence to host cells

200 Previous work suggested a role of plasminogen that is bound to the surface of *S. pyogenes* by
201 enolase and GAPDH in the colonization of human pharyngeal cells. Plasminogen acted as a
202 bridging molecule between the streptococcal proteins and Detroit 562 cells which are known
203 to express human α-enolase on their surface and bind Lys-plasminogen, preferentially
204 (Pancholi *et al.*, 2003). In an infection assay *S. equisimilis* C90 showed an increased
205 adherence to Detroit 562 cells when pre-coated with Lys-plasminogen (Fig. 7A). In order to
206 study the role of GCS3-bound plasminogen in adherence to these pharyngeal host cells, they
207 were infected with SGO GCS3 and SGO WT both in the presence or absence of Lys-
208 plasminogen. In both cases SGO WT showed low binding to the host cells while SGO GCS3
209 showed higher numbers of adherent bacteria. The increase in adherence due to expression of
210 GCS3 was observed in absence of plasminogen, but was amplified 3-fold by the pre-
211 incubation of SGO GCS3 with Lys-plasminogen (Fig. 7B). This result indicates that
212 plasminogen bound by M protein GCS3 adds to the adhesion to Detroit 562 cells and thus
213 could promote colonization of pharyngeal cells. Plasminogen dependent increase in adhesion
214 of GSC3 expressing streptococci was not observed with HEp-2 cells (Fig. 7C), indicating that
215 is has a certain cell type specificity.

Discussion

A deciding influence of the interaction between human plasminogen and streptococcal M proteins like PAM on the pathogenesis of *S. pyogenes* infections has been demonstrated previously (Sanderson-Smith et al., 2008). Activation of M protein-bound plasminogen is considered as important for dissemination and invasion of *S. pyogenes*, since plasmin has a broad range protease activity and possibly aids in degrading host tissue. It is becoming increasingly clear that the M proteins of *S. dysgalactiae* ssp. *equisimilis*, which are found in the great majority of strains that infect humans (Broyles et al., 2009; Pinho et al., 2006; Reißmann et al., 2010), exert similar functions as the M proteins of *S. pyogenes*. They increase the bacteria's resistance to phagocytosis by binding fibrinogen (Johansson et al., 2004; Nitsche-Schmitz et al., 2007b) and facilitate adhesion to the host tissue, i. e. by binding collagen (Barroso et al., 2009; Nitsche et al., 2006). The presented as well as earlier work (Ben Nasr et al., 1994) identified plasminogen binding M proteins of *S. equisimilis* GCS3, MLC 36 and MLG72 that may contribute to the ability of this streptococcal species to cause severe invasive infections in a similar way as *S. pyogenes* M proteins. Our experiments allow a comparison. Surface plasmon resonance measurements (Fig. 2B) revealed the high affinity of the interaction which is in the same order of magnitude as the interaction of plasminogen with PAM. However, binding and inhibition experiments indicated that GCS3, unlike PAM, MLC36 and MLG72 (Ben Nasr et al., 1994; Wistedt et al., 1998), does not bind to the plasminogen fragment that consists of the kringle domains 1 to 3 (kringle 1-3) (Fig. 3), indicating that their plasminogen binding mechanisms differ. Inhibition experiments with the lysine analogue EACA have shown that lysine binding sites in kringle 1-3 are responsible for the interaction with PAM, MLC36 and MLG72. In case of PAM the kringle 2 domain of plasminogen was identified as the binding site (Wistedt et al., 1998). Although, GCS3 did not bind to kringle 1-3 its interaction with plasminogen could be inhibited by EACA. This suggests the lysine binding sites of kringle domain 4 or 5 (Ho-Tin-Noe et al., 2005; Thewes et

al., 1990; Tulinsky et al., 1988) as the binding motif. Despite this considerable difference in the mode of binding, the function of the interaction appeared to be conserved. Like PAM, GCS3 is not able to activate plasminogen and to generate proteolytic activity, but the bound pro-enzyme can be activated by streptokinase and host plasminogen activators (Fig. 4). The plasminogen/streptokinase complex and the generated plasmin endowed the bacteria with a substantial proteolytic activity as determined in assays with fibrinogen in solution (Fig. 5A) and immobilized on a solid phase (Fig. 5B). Electron microscopic examination demonstrated the plasmin(ogen)-dependent degradation of fibrin (Fig. 6), which suggests that the proteolytic activity may be useful for *S. equisimilis* to escape from entrapment by blood coagulation.

Plasminogen is present not only in human blood plasma but also in human saliva (Moody, 1982) which may be exploited by streptococci. Earlier experiments suggest that *S. pyogenes* may recruit plasminogen with the help of streptococcal enolase in order to enhance adhesion to pharyngeal cells (Pancholi et al., 2003). Our cell culture experiments demonstrate that the plasminogen binding M protein GCS3 immobilizes sufficient amounts of plasminogen on the streptococcal surface and in a mode that increases the adhesion to Detroit 562 human pharyngeal cells (Fig. 7).

Emergence of *S. equisimilis* as an important human pathogen is leading to increased interest in its mechanisms of pathogenesis. Apparently, one of them is the exploitation of plasmin(ogen) for cell adhesion and degradation of host tissue to increase infectivity and bacterial spread. Interference with these processes may support the treatment of severe infections with *S. pyogenes* and *S. equisimilis*, two important human pathogens.

Experimental procedures

Bacterial strains

Streptococcus pyogenes strain A158 and *S. equisimilis* strains C90 , C107, and G35 were isolated from throat swabs. Strains belonged to the *emm*-types emm53.0, stC839, stC36.0 and stC5344, respectively. Streptococci were routinely grown at 37°C without agitation in Todd-Hewitt broth supplemented with 0.5 % yeast extract (THY) or tryptic soy broth. For cultivation of the recombinant *S. gordonii* strain GP1221, erythromycin was added to a final concentration of 3 µg/ml. The strain was kindly provided by Dr. Gianni Pozzi (University of Siena, Italy). *E. coli* strain (HB101), which was used for over expression of recombinant proteins, was grown at 37°C with agitation in Luria Bertani broth with appropriate antibiotic (100 µg/ml ampicillin (Amp)).

Plasma absorption assay

Streptococci from 50 ml overnight cultures were washed with PBS and resuspended to a concentration of 10^9 CFU/ml by adjusting to a transmission at 600 nm of 10%. 1×10^{10} bacteria were pelleted and resuspended in 400 µl PBS with 100 µl human plasma and incubated at 37°C for 1 h. After removal of unbound proteins by washing three times with 1 ml of PBS, bound proteins were eluted with 50 µl 100mM Glycin/HCl, pH = 2 at RT for 15 min. Solid material was removed by centrifugation (5 min, 10,000 × g), before 40 µl of the supernatant was collected and neutralized with 4 µl 1.5 M Tris-HCl, pH = 8.5. This sample was mixed with an equal volume of SDS-PAGE sample buffer (62.5 mM Tris-HCl, 2% (w/v) SDS, 0.001% (w/v) bromophenolblue, 5% (v/v) ß-mercaptoethanol, 10% (v/v) glycerol, pH = 6.8), and subjected to SDS-PAGE using a 12% gel as described by Laemmli (Laemmli, 1970). Following electrophoresis, proteins were visualized by staining with Coomassie stain (0.1% (w/v) Coomassie brilliant blue, 40% (v/v) methanol, 10% (v/v) acetic acid). For

immuno blots, proteins were transferred to nitrocellulose by the method of Towbin (Towbin *et al.*, 1979). Membranes were blocked with 5% (w/v) skim milk powder in TBST (20 mM Tris, 154 mM NaCl, 0.5% (v/v) Tween-20), and probed with polyclonal goat anti-human plasminogen antibodies (1:3000 in TBST). After washing 3 times with TBST, the membrane was probed with a rabbit anti-goat HRP-conjugated secondary antibody (1:5000 in TBST). After washing 3 times with TBST, peroxidase activity was detected by chemoluminescence using 100 mM Tris HCl, 1.25 mM 3-aminopthalhydrazide, 225 μM p-coumaric acid, 0.01% H_2O_2 at a pH = 8.8 in water and chemoluminescence films (GE Healthcare).

^{125}I-plasminogen binding assay

Full-length plasminogen or kringle 1-3 region of plasminogen (Sigma-Aldrich, Germany) were labeled with ^{125}I by the chloramine T method and their binding to whole bacteria was measured as reported elsewhere (McKay *et al.*, 2004). Briefly, 14 ng of ^{125}I-plasminogen was added to 2.5×10^8 bacteria suspended in 250 μl PBST and incubated at room temperature for 45 min. For inhibition experiments 20 μg of unlabeled kringle 1-3 fragment or ε-aminocaproic acid (1 nM to 300 μM) were added to the sample. The cells were washed in PBST and harvested by centrifugation. Supernatant was removed by careful aspiration and pellet-associated radioactivity was measured in an automated Wallac gamma counter (Perkin Elmer, Jugesheim, Germany). Protein binding was expressed as the percentage of total input radioactivity (100,000 cpm). All measurements were carried out in triplicates.

Cloning and expression of recombinant proteins

For the generation of GST–GCS3 or GST–MLC36 proteins, GCS3 *emm* gene and MLC36 *emm* gene fragments were amplified from the chromosomal DNA of the appropriate strains via PCR using the following primers: gcs3Bfor: 5’ CAG AAG TTA AGG GAT CCC ATC CGC GA 3’ (*Bam*HI restriction site); mlc36Bfor: 5’ GAC AGT AGG ATC CAA CGG AGA

AGT TA 3' (*Bam*HI restriction site); and mlc36Srev: 5' CAC CTG TTG AGT CGA CCT GTC TCT TAG TTT 3' (*Sal*I restriction site) for the full length proteins. Standard cloning techniques were used to generate constructs in the pGEX-6P-1 vector system (GE Healthcare, Freiburg, Germany). Proteins were expressed in *E. coil* HB101 and purified under native conditions following the manufacturer's protocol. For production of tag free GCS3 the protein was eluted from the affinity matrix by treatment with PreScission protease (GE Healthcare, Freiburg, Germany) following the manufacturer's protocol.

Construction of *S. gordonii* expression strains

GCS3 was heterologously expressed on the surface of *S. gordonii* GP1221 as described by Talay *et al.* (Talay *et al.*, 2000). The GCS3 *emm* gene was amplified using the primers sgoSrev GCT TCT GCG TCG ACG TTT TCT TGC TCT and gcs3Bfor which is described above.

Dot-blot assay

Recombinant GST tagged proteins GCS3 and MLC36 were dot-blotted onto nitrocellulose (5, 1, and 0.5 µg) before blocking with 5% (w/v) skim milk in PBS for 1 h at RT. The membrane was washed for 5 min in PBS, and probed with 25 µg ^{125}I-plasminogen for 20 min, then washed 3 times for 20 min with PBST. Plasminogen that was bound to the blot was detected by exposing an x-ray film (BioMax MS film, Kodak, Germany).

Surface plasmon resonance measurements

Protein interactions were studied by surface plasmon resonance measurements in a BIAcore 2000 system (BIAcore AB) as described earlier (Nitsche et al., 2006). Human plasminogen (Sigma-Aldrich) was dissolved in 10 mM sodium acetate, pH = 5.0 at a concentration of 40 µg/ml. Injection of 20 µl at a flow rate of 5 µl/min led to immobilization of 600 response

units (RU) of plasminogen. Residual reactive groups were inactivated by a 7 min injection of 1 M ethanolamine, 0.1 M $NaHCO_3$, 0.5 M NaCl, 5 mM EDTA, pH = 8.0. Surface regeneration was achieved by injection of three 20 s pulses of the same ethanolamine solution. Recombinant GCS3 as the analyte was diluted in running buffer (50 mM HEPES, 150 mM sodium chloride, pH = 7.4) at concentrations of 0.5 µM, 1.0 µM, 2.0 µM and 4.0 µM. The interaction was measured at a flow rate of 10 µl/min. The BIAevaluation 3.0 software was used for further analysis of the data. Shown curves represent the difference between the signal of the plasminogen-coupled surface and of a deactivated control surface devoid of protein. They were further corrected by subtraction of the curve that was obtained after injection of buffer alone. Buffer injection led to responses less than 5 RU.

Plasminogen activity assay

Streptococci of 50 ml overnight cultures were washed and adjusted to 10% transmission. 1×10^{10} heat killed bacteria (20 min, 90°C) were incubated with plasma or 100 µg/ml purified plasminogen in 1 ml PBS for 30 min. After washing with PBS, the bacteria were incubated with 200 units streptokinase in 1 ml PBS for 1 h at 37°C, or 100 ng uPA in 1 ml PBS for 2 h at 37°C. Bacteria were washed three times with PBS and pelleted by centrifugation. The pellets were resuspended in 1 ml of a solution that contained the chromogenic substrate S-2251 (80 µM in PBS). After incubation at 37°C for 2 h absorption of the supernatant was measured at a wavelength of 405 nm.

Protein degradation experiments

Degradation of soluble fibrinogen or fibronectin was determined after pre-incubation with plasma as described above. The pre-treated bacteria were incubated with 4 µg fibrinogen or fibronectin for the indicated times at 37°C with or without 25 units of streptokinase. Samples were taken at different time points and boiled with an equal volume of SDS-PAGE buffer

362 before being subjected to SDS-PAGE and Immunoblot analysis. Detection in immunoblots
363 was carried out as described above using rabbit anti-human fibrinogen antibody or rabbit anti-
364 human fibronectin antibody to probe. Degradation of immobilized ^{125}I-fibrinogen was
365 assessed by coating a 96-well microtitre plate with 2.5 µg/ml ^{125}I-fibrinogen overnight. 250 µl
366 of streptococci that had been pre-incubated with 50 µg plasminogen for 30 min at 37°C,
367 washed and resuspended in 1 ml PBS with or without 200 units streptokinase were added to
368 the wells and incubated for the indicated times. At each time point, 25 µl of supernatant of the
369 liquid phase were taken from the wells and radioactivity was measured as described above.

370 ## Degradation of fibrin

371 Fibrin was fibrillized on cover-slips by incubating 1 mg plasminogen depleted human
372 fibrinogen (Calbiochem, Darmstadt, Germany) with 25 U thrombin (from bovine plasma, MP
373 Biomedicals, Ohio, USA) for 12 h at 37°C in 100 µl PBS. SGO GCS3 pretreated with
374 plasminogen and streptokinase as described above, were applied with a dose of 1 x 109 in 100
375 µl PBS containing 10 mM EDTA to the fibrin fibers for 2 h at 37 °C. Fibrin and its
376 degradation by SGO GCS3 was visualized by field emission scanning electron microscopy as
377 described earlier (Bergmann et al., 2005).

378 ## Adherence/Invasion assays

379 The human pharyngeal cell line Detroit 562 (ATCC CLL138) and HEp-2 cells (ATCC CCL-
380 23) was used for adherence and invasion assays with *S. equisimilis* C90 and *S. gordonii* SGO
381 GCS3 and SGO WT. Detroit 562 cells were grown in tissue culture plates containing RPMI
382 1640 medium (Gibco) supplemented with 10% fetal calf serum, L-glutamine (2 mM) and 1%
383 Na-pyruvate. HEp-2 cells were grown in tissue culture plates containing DMEM medium
384 (Gibco) supplemented with 10% fetal calf serum and L-glutamine (2 mM). Streptococcal
385 adhesion to eukaryotic cells was performed essentially as described elsewhere (Talay *et al.*,

2000). For adherence and invasion assay, bacteria were pre-coated with Lys-plasminogen (final 0.74 μg/μl; Sigma-Aldrich, Germany) for 60 min at RT. Washed bacteria were incubated with the cells in the presence of 3.2 units/ml of the trypsin inhibitor aprotinin (Sigma-Aldrich, Germany) to inhibit the protease function of the bound plasmin(ogen).

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Figure Legends

Figure 1: Binding of human plasminogen to streptococci. Streptococci were incubated with human plasma in PBS (+) or PBS alone (-) for 1 h at 37°C. Unbound proteins were removed. Bound proteins were eluted and analyzed by SDS-PAGE (12% gel) (A) and immunoblot probing with polyclonal anti-human plasminogen antibody (B). Potential plasminogen bands in A are indicated by arrow heads. Molecular mass standards in kDa are indicated at the left. (C) Bacteria were incubated with human ^{125}I-plasminogen for 1 h at RT. Bound plasminogen was quantified by measuring the radioactivity associated with the bacteria. Results are expressed as percentage of the total radioactivity added (100,000 cpm). *S. pyogenes* A158 is a PAM positive strain that served as a positive control.

Figure 2: Interaction between human plasminogen and M protein GCS3 of *S. dysgalactiae* ssp. *equisimilis*. (A) The recombinant GST-fusion proteins GST-GCS3, GST-MLC36, GST-M6.1, GST-M3.1 and His_6-GRAB were dot-blotted onto a nitrocellulose membrane in the indicated quantities and probed with ^{125}I-plasminogen. Surface plasmon resonance sensograms of the interaction between tag free M-protein GCS3 in the liquid phase and immobilized plasminogen are depicted in B. Measurements were carried out at the indicated concentrations of GCS3. Injection started at t = 0 s and ended at t = 120 s. Based on the Langmuir model of a 1:1 interaction, an apparent dissociation constant of K_d = 18 nM was determined. (C) The *S. equisimilis* strain that bears GCS3 (C90), wild type *S. gordonii* GP1221 (SGO WT), and the recombinant variant that expresses GCS3 M-protein (SGO GCS3) were incubated with ^{125}I-plasminogen, quantities of bound protein were expressed as the percentage of total radioactivity input (100,000 cpm).

Figure 3: Role of plasminogen kringle domains 1 to 3 in M protein interaction. (A) Binding of ^{125}I-plasminogen to *S. pyogenes* strain A158 (A158) and *S. equisimilis* strain C90

(C90) was measured in absence (*black bars*) and presence (*grey bars*) of the unlabeled plasminogen fragment that comprises kringle domains 1-3 (kringle 1-3). In addition, binding of ^{125}I-kringle 1-3 was determined (*white bars*). (B) binding of ^{125}I-plasminogen to A158 (triangles), C90 (squares), *S. gordonii* strains SGO wt and SGO GCS3 was tested in presence of EACA at different concentrations. The results in A and B are displayed as the percentage of the total radioactivity added (100,000 cpm). Data show mean values ± SD of one representative experiment performed in triplicate. Tukey's post-hoc test was used for statistical analysis. P-values are given in A.

Figure 4: Activation of plasminogen bound to the streptococcal surface by GCS3. *S. equisimilis* strain C90 and the *S. gordonii* strains SGO GCS3 and SGO WT were pre-incubated with human plasma. Unbound proteins were removed before proteolytitc activity against the chromogenic substrate S-2251 was measured in the presence (*black bars*) or absence (*white bars*) of streptokinase (A) or uPA (B). Substrate conversion is expressed as absorbance at 405 nm.

Figure 5: Degradation of fibrinogen by GCS3-bound plasminogen. Following pre-incubation with plasma (A) or plasminogen (B) and removal of unbound protein, *S. equisimilis* strain C90 and the *S. gordonii* strains SGO GCS3 and SGO WT were incubated with fibrinogen for the indicated times at 37°C. (A) Degradation of ^{125}I-fibrinogen immobilized in a microtitre plate was measured for *S. equisimilis* strain C90 (*squares*) and the *S. gordonii* strains SGO GCS3 (*triangles*) and SGO WT (*circles*) in presence (*filled symbols*) and absence (*open symbols*) of streptokinase. Degradation was quantified over time as radioactivity in cpm released into the liquid phase. (B) Samples in which bacteria were co-incubated with fibrinogen in solution were analyzed by immunoblot (12% gel) with antibodies specific for fibrinogen. Negative controls (lanes 2 and 8) were incubated without

559 streptokinase for 4 h. For the experiment depicted in C and D strain C90 was preincubated
560 with human plasminogen where indicated with +. Using bacteria from the same culture their
561 proteolytic effect on fibronectin (C) and fibrinogen (D) in presence + or absence - of
562 streptokinase was investigated. The samples were analyzed by immunoblot (10% gel) with
563 antibodies specific for fibronectin (C) or fibrinogen (D), respectively. Molecular mass
564 standards in kDa are indicated at the left in B, C and D.
565
566 **Figure 6: Electron microscopy of fibrin bundle degradation.** *S. gordonii* strain SGO GCS3
567 was incubated with fibrillated human fibrin in the presence of plasminogen. After addition of
568 streptokinase (A-C) degradation of the fibers was observed discernible by massive formation
569 of thin fibrillar structures. The control sample without streptokinase lacks these signs of fibrin
570 degradation (D). Bars represent 5 µm in A and 1 µm in B-D.
571
572 **Figure 7: Adherence to Detroit 562 cells.** Monolayers of Detroit 562 cells (A and B) or
573 HEp-2 cells (C) were infected with *S. equisimilis* strain C90 (A), the *S. gordonii* strains SGO
574 GCS3 (B and C) and SGO WT (B) in the presence (*black bars*) or absence (*white bars*) of lys-
575 plasminogen. The number of adherent bacteria was determined after 2 h. Adherence is
576 expressed as percentage of the initial inoculum. A value above 100% is due to bacterial
577 growth during incubation. Data show mean values ± SD of one representative experiment
578 performed in triplicate.