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SCM, a novel M-like protein from *Streptococcus canis* binds (Mini)-plasminogen with high affinity and facilitates bacterial transmigration

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Running head: Interaction of *S. canis* with plasminogen

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SYNOPSIS

Streptococcus canis is an important zoonotic pathogen capable of causing serious invasive diseases in domestic animals and humans. In this study we report the binding of human plasminogen to *S. canis* and the recruitment of proteolytically active plasmin on its surface. The binding receptor for plasminogen was identified as a novel M-like protein designated *S. canis* M-like protein (SCM). Surface plasmon resonance analyses, radio-active dot blot analyses and heterologous expression on the surface of *S. gordonii* confirmed the plasminogen-binding capability of SCM. The binding domain was located within the N-terminus of SCM, which specifically bound to the C-terminal part of plasminogen (mini-plasminogen) comprising kringle domain 5 and the catalytic domain. In the presence of urokinase, SCM mediated plasminogen activation on the bacterial surface that was inhibited by serine protease inhibitors and lysine amino acid analogues. Surface-bound plasmin effectively degraded purified fibrinogen as well as fibrin clots, resulting in the dissolution of fibrin thrombi. Electron microscopic illustration and time lapse imaging demonstrated bacterial transmigration through fibrinous thrombi. This study led for the first time to the identification of SCM as novel receptor for (mini)-plasminogen mediating fibrinolytic activity of *S. canis*.

Key words:

Streptococcus canis, M-like protein, mini-plasminogen, transmigration

Abbreviations:

6-AHA, ϵ -amino caproic acid; ECM, extracellular matrix; K(1-3, 1-4, 4), kringle (1-3, 1-4, 4); LB, Luria Bertani; mPLG, mini-plasminogen; PAM, plasminogen-binding group A streptococcal M protein; PLG, plasminogen; SCM, *S. canis* M-like protein; tPA, tissue-type plasminogen activator; TSB, Tryptic-Soy broth; uPA, urokinase-type plasminogen activator

INTRODUCTION

S. canis belongs to group G streptococci and represents a common member of the resident microflora of skin and mucosa of various animals [1]. It is considered as an opportunistic zoonotic pathogen capable of causing serious invasive diseases such as streptococcal toxic shock syndrome, necrotizing fasciitis, septicemia and meningitis in domestic animals [2] as well as humans [3-5]. In spite of the emerging importance of *S. canis* as a health hazard, little is known about its molecular pathogenesis. Many Gram-positive pathogens which can cause invasive diseases in humans exploit the host proteins to invade and disseminate in tissues [6]. The exclusively human pathogenic bacterium, *Streptococcus pyogenes*, uses the host fibrinolytic system and interacts with plasminogen [7]. Plasminogen is

the 92 kDa single-chained pro-enzyme of the broad spectrum serine protease plasmin. It comprises a pre-activation peptide of ~ 8 kDa, five homologous disulfide-bonded triple-loop kringle structures (K1–5; 65 kDa), and a serine-protease domain (25 kDa) [8]. Plasminogen is cleaved by leukocyte elastase activity into mini-plasminogen comprising K5 and the catalytic domain (Val₄₄₂-Asn₇₉₀) [9]. Conversion of the single-chained pro-enzyme to active plasmin is mediated by proteolytic activation via the mammalian plasminogen activators, tissue-type plasminogen activator (tPA), and urokinase (uPA) [10]. Plasmin is involved in intravascular fibrinolysis [11], degradation of extracellular matrix (ECM) material, and is relevant for cell invasion [12]. The capability of bacteria to bind and activate plasminogen to plasmin represents, thereby, a mechanism to invade and disseminate the host. One of the major plasminogen binding components of *S. pyogenes* is the M-like protein PAM which mediates high-affinity binding to K2 [13, 14].

This study describes the identification of a novel M-like protein from *S. canis*, designated SCM that binds plasminogen. The detailed molecular characterization indicated a specific interaction of SCM with the C-terminal part of plasminogen comprising mini-plasminogen. This kind of interaction may represent a novel mechanism for plasminogen interaction different from PAM and other plasminogen binding components of streptococci. Furthermore, the plasminogen activation and the recruitment of plasmin on the bacterial surface of SCM-expressing *S. canis* led to the degradation and dissolution of fibrin thrombi, indicating a contribution of plasminogen-binding to bacterial dissemination within the host.

EXPERIMENTAL

Bacterial strains, media and growth conditions

The strains of β -haemolytic Group A, C, and G streptococci used in this study are listed in table 1. Bacteria were routinely cultivated in tryptic soy broth (TSB) at 37°C without shaking. *Escherichia coli* were grown in Luria Bertani medium. If indicated, antibiotics were added at following concentrations: ampicillin (100 $\mu\text{g}\cdot\text{ml}^{-1}$), kanamycin (25 $\mu\text{g}\cdot\text{ml}^{-1}$).

Proteins and sera

Human plasminogen (Sigma), kringle domains 1-3 (K1-3, Sigma), and angiostatin (K1-4, Hemochrom Diagnostica) were purchased commercially. Blood plasma from pig, goat, cat, and dog was collected at the Veterinary School Hannover; K4 and mini-plasminogen were prepared as previously described [15]. Plasminogen-specific antibodies and horse raddish peroxidase-conjugated anti-goat and anti-mouse secondary antibodies were purchased from Dako. Purified His-tagged SCM was used to raise polyclonal antibodies in BALB/C mice according to standard protocol. Preimmune serum was collected before immunization.

Expression cloning and recombinant DNA techniques

If not stated otherwise, all enzymes were obtained from NEW ENGLAND BIOLABS (NEB). SCM was amplified from the *S. canis* G361 genome using the primer pair MF-1/MF-2 (GGG GGA TTC AAC AGA GTT ACT GAG GCC AGA GC/ CCC GTC GAC TGC TGT GAA GAA TGG GTT GG), incorporating a *Bam*HI and a *Sal*I restriction site, respectively. Subsequent enzymatic restriction and ligation into the *Bam*HI/*Sal*I digested pQE30 vector (Qiagen) resulted in the plasmid pQE30-SCM. The integrity of insert DNA was verified by sequence analysis using ABI Prism dye terminator cycle sequencing (Perkin-Elmer). The N-terminal part of SCM (SCM 25KD) was amplified with the primer pair MF-1/*Sal* fog-S-region (GGG GGA TTC AAC AGA GTT ACT GAG GCC AGA GC/ GCT GTC GAC TTA

TTA TGC TTT GTC GCT TGC TAA TTG TTC) and subcloned as a *Bam*HI/*Sal*I fragment into *pQE30*. Sequence analysis revealed SCM 25KD as the N-terminal part comprising amino acids 37 to 248.

Recombinant proteins were obtained by chromatography under native conditions on Ni-nitrilotriacetic acid resins according to the manufacturer's protocols (Qiagen).

Amplification of the SCM protein from different *S. canis* strains was performed using the primer pair all-canis_fwd/all-canis_rev (TAG CGT TGG AAC AGC ATC AC/ CTG CCT CTG CTT TTG CTT TC).

Circular Dichroism spectroscopy

CD spectra were recorded with a Jasco-J-815 spectrometer using a quartz cell of 1-cm optical path-length, an instrument scanning speed of 100 nm/min and over a wavelength range of 195 nm - 260 nm at 20°C. The reported CD spectrum of each sample represents the average of 10 scans taken at 20°C. PBS was used as a blank. The helical content of the protein was calculated using the Jasco software.

Heterologous expression of SCM protein in *S. gordonii* strains

S. gordonii heterologously expressing SCM on its surface was generated using the mutagenesis system of Oggioni and Pozzi [16]. Full-length SCM was amplified from the *S. canis* G361 genome as described above and cloned into the integration vector *pSMB103*. Transformation of the plasmid in competent *S. gordonii* GP1221 was performed as described by Talay *et al.* [17].

Binding experiments and competitive inhibition assay

Purified plasminogen (Sigma) was radio-labelled with ¹²⁵Iodine by a standard chloramine T method [18], and binding experiments with [¹²⁵I]-plasminogen were performed as described previously [19]. Briefly, 2.5 x 10⁸ bacteria were incubated with 20 nCi of iodinated plasminogen

or iodinated K1-3. Bacteria were sedimented and bacteria-bound radioactivity was measured using a gamma counter (Packard). In competitive inhibition assays, binding to viable streptococci was measured in the presence of increasing molar excesses of glu-plasminogen (Sigma), the lysine amino acid analogue epsilon-amino caproic acid (6-AHA; Merck), or K1-3 (Sigma). For dot blot experiments, purified SCM and SCM 25KD, respectively, were spotted onto a nitrocellulose membrane (Biorad). Blocking was performed in the presence of 5% skimmed milk for 2 h at room temperature. Hybridization with [¹²⁵I]-plasminogen was carried out for 4 h. Binding was detected using X-ray films (Kodak).

Surface plasmon resonance

The association and dissociation reactions of glu-plasminogen (Sigma) and mini-plasminogen to recombinant SCM and its N-terminal derivative, respectively, were analysed in the BIAcore optical biosensor (BIAcore 2000 system) using CM 5 sensor chips. Covalent immobilization of plasminogen and mini-plasminogen was performed by standard amine coupling procedure essentially as described [20]. Additionally, SCM was immobilized on the Chip surface and plasminogen or mini-plasminogen, respectively, was used as analytes. Binding analysis was performed in HBS BIAcore running buffer (10 mM HEPES 150 mM NaCl, 1.4 mM EDTA, 0.05 % Tween-20, pH 7.4) at 20°C using a flow rate of 30 µl·min⁻¹ in all experiments. The affinity surface was regenerated with 10 µl of 20 mM NaOH. Binding was assayed at least in duplicate using independently prepared sensor chips.

Analysis of BIAcore sensogram data

The interaction kinetics were analyzed from raw data of the BIAcore sensograms suitable for analysis using the kinetic models included in the BIAevaluation software version 3.0. The experimental data were fitted globally by using the simple one-step bimolecular association

reaction (1:1 Langmuir kinetic: $A+B \leftrightarrow AB$). For each evaluation, a minimum of four data sets were analyzed.

Plasma absorption, Westernblot and immunoblot analysis

Plasma proteins were eluted from streptococcal surface using 10 mM glycine pH 2.0. After neutralization with 2 N NaOH, proteins were subjected to SDS-PAGE with 12% gels according to the method described by Laemmli [21] and either stained with Coomassie brilliant blue or subsequently transferred to a nitrocellulose membrane (Biorad) using a semi-dry blotting system. In immunoblots, plasminogen binding was detected by incubation of the membrane with monoclonal antibodies directed against human plasminogen (1:300, American Diagnostica), followed by a secondary antibody coupled to HRP. Peroxidase activity was detected by chemoluminescence using 100 mM Tris HCl, 1.25 mM 3-aminophthalhydrazide, 225 μ M p-coumaric acid, 0.01% H_2O_2 at a pH 8.8 in water and exposed to chemoluminescence films (GE Healthcare).

For immunoblot analysis, human plasminogen and its fragments K1-3, K4, K1-4, and mini-plasminogen (mPLG) were spotted onto a nitrocellulose membrane at indicated amounts. Membranes were blocked as described above and incubated with 100 μ g purified SCM overnight at 4°C. Immunoblot analysis with anti-M-protein (1:200) antiserum was performed using HRP-conjugated second antibodies (1:3000) followed by incubation with the substrate solution containing 1 $mg \cdot ml^{-1}$ 4-chloro-1-naphthol and 0.1% H_2O_2 in PBS.

Plasmin activity assay

Activation of plasmin was monitored in 96 microtiter plates. For detection of secreted streptokinase activity, 150 μ l of a 50 ml overnight culture were incubated with the same volume of canine or human plasma, respectively, for 30 min at 37°C. The

chromogenic substrate D-valyl-leucyl-lysine-p-nitroanilide dihydrochloride (S-2251, Fluka) was added to a final concentration of 400 μ M. Proteolytic cleavage was detected photometrically at 405 nm using an ELISA reader (Tecan Sunrise) following time course of 120 min. For detection of cell bound streptokinase activity, *S. canis* bacteria (10^9 cfu $\cdot ml^{-1}$) were preincubated with 40 μ g $\cdot ml^{-1}$ plasminogen in PBS for 15 min at 37°C. After removal of unbound plasminogen by washing, urokinase (500 ng) was added. Plasmin activation was also measured in the presence of 2 U plasmin inhibitor aprotinin or the lysine analogue 6-amino hexanoic acid (6-AHA, Sigma) in concentrations of 2, 40 and 80 μ M. Plasminogen-incubated streptococci without activator and non-treated bacteria were used as controls. For analysis of plasmin activation time of surface-bound *versus* soluble plasminogen, SCM-expressing SGO bacteria were treated essentially as described for radioactive binding assays. Briefly, streptococci were incubated with 55 ng plasminogen for 1 h at room temperature. Then, cells were washed with PBS and 50 ng uPA was added. The amount of soluble plasminogen was calculated from binding experiments as 80% of the iodinated protein. Conversion to plasmin was determined as described above.

Degradation of fibrinogen

S. canis G361 bacteria were preincubated with plasminogen as described, and 5×10^7 cells were incubated with 4 μ g of plasminogen-depleted human fibrinogen (Sigma) following a time series of 5, 10, 15, 20, 30, and 60 min at 37°C. Degradation was monitored in the presence of uPA (500 ng) and the reaction was stopped with 50 μ l of SDS-containing sample buffer. Bacteria were sedimented and samples were separated by SDS-PAGE followed by transfer of proteins to polyvinylidene difluoride membrane (PVDF; Immobilon-P, Millipore) using a semi-dry blotting system. The membranes were blocked as described earlier

prior to incubation with rabbit antiserum directed against human fibrinogen (Dakopatts, 1:2000). Detection of fibrinogen peptides was carried out using HRP-conjugated anti-rabbit antibodies (1:3000) and chemiluminescence as described above.

Degradation of fibrin and transmigration of *S. canis* through fibrin

Fibrin matrix was produced on cover slips and transwell cell culture inserts (polycarbonate membranes with 3.0 μm pore size; Costar) by incubating 100 μl of 50 $\text{mg}\cdot\text{ml}^{-1}$ plasminogen-depleted human fibrinogen (Millipore) in PBS with 2 μl of 1.0 $\text{KU}\cdot\text{ml}^{-1}$ thrombin (from bovine plasma, MP Biomedicals) for 10 h at 37°C. *S. canis* G361 pretreated with plasminogen as described, was applied with a dose of 1×10^7 in 200 μl PBS to the fibrin matrix. Surface-bound plasminogen was activated with uPA (500 ng). Bacterial transmigration from the upper to the lower chamber was quantified by plating serial dilutions of the lower chamber solution on blood agar plates. Experiments were carried out for up to 6 h and samples were plated in intervals of 120 min. Data represent the means \pm SD of one experiment performed in triplicate. Experiments were repeated three times.

Time-lapse microscopy

Fibrin clots were generated in μ -Slides (μ -Slide 8 well, Ibidi, Munich, Germany) as described above. One $\times 10^7$ bacteria were added per well and samples were mounted on an inverted microscope (Axio Observer.Z1) equipped with a 25 \times /0.8 NA LCI Plan-Neofluar objective driven by AxioVision Software 4.7 (Zeiss, Jena, Germany). Time-lapse imaging was performed at 37°C and frames were taken every two minutes. Video frames were processed for contrast and brightness using Fiji/ImageJ.

Electron microscopy

Samples were fixed in 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer (0.1 M

cacodylate, 0.01 M CaCl_2 , 0.01 M MgCl_2 , 0.09 M sucrose, pH 6.9) for 1 h on ice and washed with TE-buffer (20 mM TRIS, 1 mM EDTA, pH 7.0) before 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 CO_2 (CPD 30, Bal-Tec, Liechtenstein). Dried samples were covered with a gold film by sputter coating (SCD 500, Bal-Tec, Liechtenstein) before examination in a field emission scanning electron microscope Zeiss DSM 982 Gemini (Zeiss, Germany) using the Everhart Thornley SE detector and the SE-inlens detector in a 50:50 ratio with an acceleration voltage of 5 kV. Images were recorded onto a MO-disk, contrast and brightness was adjusted applying Adobe Photoshop CS3.

Computational analysis

Prediction of Gram-positive signal sequences was carried out using the SignalP program (<http://www.cbs.dtu.dk/services/SignalP>).

Prediction of fibrillar structure was performed *in silico* using the coiled-coil program (<http://www.ch.embnet.org/software/COILS>).

RESULTS

Plasminogen binding to Group C and G streptococci

During the course of wound infection, streptococci encounter different components from host blood and tissue. Recruitment of human plasma components to bacterial *S. canis* isolates G1, G2, G14, G361 and *S. equisimilis* C90 has been analysed after plasma incubation. Coomassie stain detected the presence of a 90 kDa protein after elution of surface-bound plasma proteins from G361, C90, and to a lesser extent, G1 (Fig. 1A, black arrow). Immunoblot analysis with antibodies directed against plasminogen identified the respective protein as human plasminogen (Fig. 1B). In addition, *S.*

canis isolate G361 can also recruit plasminogen from plasma derived from different mammalian species including pig, goat, cat, and dog (Fig. 1C, black arrow). In radioactive interaction studies binding of iodinated human plasminogen, was detected for *S. canis* isolates G1, G8, G13, G15, and G361 ranging from 16% binding for strain G8 up to 60% for *S. canis* G361 (Fig. 1D). Comparative plasminogen binding activity was determined for PAM-expressing *S. pyogenes* A158 (data not shown), whereas only marginal plasminogen binding has been observed for the isolates G2, G14, and G17 (Fig. 1D). These results demonstrate a specific interaction of zoonotic *S. canis* isolates with human plasminogen and a non-species specific recruitment of plasminogen from plasma on the *S. canis* cell surface.

Identification of SCM as a M-like protein of *S. canis* (SCM) mediating plasminogen binding

M- and M-like proteins of human group A, C and G streptococci have been identified as plasminogen receptors [22]. Based on a DNA sequence, that has been supposed to encode a M-like protein of *S. canis* (accession number: FJ594772) and an ongoing genome sequencing project, the respective gene was amplified from the genome of strain G361 and termed SCM. The amino acid sequence of the purified His-tagged protein elucidates a N-terminal signal sequence with a predicted cleavage site between alanine and glutamic acid (VKA-EH) and a LPxTG motif for sortase-mediated covalent cell wall attachment at the C-terminus (Fig. 2A). Using the prediction program COILS a probability for coiled-coil formation of more than 90% was calculated for amino acids 109 to 360 of full length SCM. In further studies, CD spectroscopy revealed a typical curve progression for alpha-helical proteins with two minima at 208 and 222 nm, respectively. The helical content was estimated as 72.4%. Furthermore, a 222:208 nm ratio of 1.13 was calculated (Fig. 2B). Since this ratio is ≥ 1 for

coiled-coil proteins [23, 24], these data suggest a dimeric form of SCM under physiological conditions. In addition, a typical feature of coiled-coil proteins is a repeating heptad of the generalized sequence a-b-c-d-e-f-g, whereas positions a and d are occupied by hydrophobic residues [25]. An alignment with other M- and M-like proteins elucidated the presence of the hydrophobic amino acids in the positions a and d also within the SCM sequence (Fig. 2C). Furthermore, sequence alterations occur as deletion of seven amino acids supporting the coiled-coil structure of SCM.

In order to detect *scm* in the genome of different *S. canis* isolates, a specific PCR with oligonucleotides based on the N-terminal signal sequence and the conserved C-terminal LPxTG motif has been established (Fig. 2D). Sequence analysis revealed that the *scm* fragment is not ubiquitously present in all *S. canis* isolates (Fig. 2D). The *scm* gene could be detected in the genomes of strains G1, G8, G13, G15 and G361. Within these strains, presence of *scm* correlates with a high plasminogen binding capacity (Fig. 1D). In contrast, no amplification of *scm* was observed for the *S. canis* strains G2, G14 and G17 which correlates with only a weak plasminogen binding capacity (Fig. 2D). A total number of 19 strains have been tested and all strains showing strong plasminogen binding were also positive for *scm* (Fig. 2D). These results indicated that SCM is necessary for plasminogen binding in *S. canis*.

Biochemical analyses of plasminogen binding to SCM

SCM was heterologously expressed in the non-plasminogen binding *S. gordonii*. In contrast to the wild type strain (SGO WT), the SCM expressing *S. gordonii* strain (SGO SCM) showed strong binding to iodinated human plasminogen (Fig. 3A). Results of dot blot overlay analyses confirmed the recombinant purified SCM as plasminogen-binding protein of *S. canis*. Furthermore, the plasminogen binding domain of SCM could be narrowed

down to the first 214 amino acids of the mature protein representing SCM 25KD (Fig. 3B). Surface plasmon resonance analysis was performed by using plasminogen as immobilized ligand and purified SCM and SCM 25KD as analytes in a series of concentrations (0.125 μ M - 4 μ M). Sensogram data of SCM binding to immobilized plasminogen revealed a specific and concentration-dependent binding interaction (Fig. 3C). Evaluation of the binding data determined dissociation constants of 2.69×10^{-8} M for the plasminogen interaction with SCM (Fig. 3C) and 2.07×10^{-8} M for the N-terminal part of SCM (Fig. 3D). Dissociation constants within the same range (5.10×10^{-7} M) could also be confirmed in kinetic analysis with SCM as immobilized ligand and plasminogen as analyte in solution (Fig. 3E). A summary of the BIAcore results representing the association rate constant (k_a), the dissociation rate constant (k_d), the affinity constant (K_D), and the chi square values (χ^2) were depicted in Table 2.

Characterization of SCM-binding domain in human plasminogen

Kringle domains of plasminogen are well known binding sites for a variety of bacterial plasminogen receptors containing exposed lysine residues [26, 27]. In order to characterize the region of interaction between SCM and the plasminogen molecule to detail, different inhibition studies were performed in radioactive binding experiments. A dose-dependent inhibition of plasminogen binding to the surface of *S. canis* G361 was observed in the presence of increasing amounts of 6-AHA (Fig. 4A). Addition of 300 mM 6-AHA led to a residual binding capacity of 25.4%. A dose-dependent inhibition of plasminogen binding from 66.5% with 62.5 nM inhibitor to 19.2% with 2 μ M of non-labelled plasminogen underlined the specificity of the interaction (Fig. 4B). In contrast, no difference in plasminogen binding capacity was observed in the presence of 2 μ M non-labelled K1-3 (Fig. 4B). PAM expressing *S. pyogenes* strain A158 bound to K1-3 with a

capacity of 58.0% (Fig. 4D), which was inhibited by the addition of 2 μ M non-labelled plasminogen and 2 μ M K1-3 (Fig. 4C). These results were supported by the fact that neither *S. canis* strain G361 (7.6%) nor the SCM expressing *S. gordonii* strain SGO SCM (2.8%) were able to bind to iodinated K1-3 (Fig. 4D). For mapping the binding site on plasminogen, dot blot experiments using different fragments of the entire plasminogen molecule and recombinant SCM was performed. Fig. 4E showed a specific, dose-dependent binding signal of SCM only to full length plasminogen and to the C-terminal elastase-digested product mini-plasminogen, whereas no interaction could be detected using the fragments K1-3, K1-4, and K4, respectively. Surface plasmon resonance analyses of binding kinetics with immobilised mini-plasminogen and SCM as analyte revealed a high dissociation constant of 1.38×10^{-8} M which is within the same high affinity range as determined for the kinetics between SCM and plasminogen (Fig. 4F, Table 2).

Activation of plasminogen and recruitment of plasmin on *S. canis* cell surface

Activation of plasminogen to proteolytic active plasmin was determined by photometrical measurement of the plasmin specific chromogenic substrate S-2251 (Fig. 5A). No plasmin activity was detected in the supernatant of the zoonotic isolate G361 with both, human or canine plasma (Fig. 5A, B). In contrast, the *S. equisimilis* strain C90 recruited plasminogen from human plasma (shown in Fig. 1B) and converted human plasminogen but not canine-plasma derived plasminogen into plasmin (Fig. 5A, B). These results indicate that no endogenous plasminogen activator like streptokinase is expressed in *S. canis*, and also emphasize the species-specific activity of streptokinase derived from *S. equisimilis* [28]. Interestingly, human plasminogen bound to the surface of *S. canis* G361 was activated in a time dependent manner in the presence of the

eukaryotic activator urokinase (Fig. 5C), whereas no plasmin activity was detected in the absence of plasminogen or of plasminogen activators (Fig. 5C). The serine protease inhibitor aprotinin inhibits plasminogen activation on the bacterial surface, indicating the serine protease specificity of plasmin-mediated substrate conversion (Fig. 5C). Plasmin activity was also significantly reduced in the presence of 2, 40, and 80 mM 6-AHA, in a dose-dependent manner (Fig. 5D). In addition to the results obtained from binding analyses, the inhibitory activity of 6-AHA for plasminogen activation confirmed, that the lysine binding sites within plasminogen kringle domains are involved in interaction of SCM with plasminogen. Interestingly, plasminogen bound to SCM-expressing SGO underwent a faster conversion into plasmin by urokinase as compared to soluble plasminogen (Fig. 5E). These results indicate that binding of plasminogen to SCM on bacterial surface accelerates the rate of plasmin formation.

Binding of SCM protein to plasminogen promotes degradation of fibrinogen and fibrin and transmigration of streptococci through microthrombi

Fibrinogen is the major component of fibrin clots generated in response to vessel injuries and inflammatory processes. Immunoblot analysis of fibrinogen degradation detected a time-dependent degradation of purified human fibrinogen by plasminogen-coated *S. canis* bacteria in the presence of urokinase (Fig. 6A). Degradation products of fibrinogen fragments were produced after 5 min of incubation with bacteria and fragmentation was enhanced following a time series up to 60 min. In contrast, no degradation was detected by plasminogen-coated *S. canis* bacteria without any activator (Fig. 6A lane 2). In order to analyse fibrinolytic activity by plasminogen-coated bacteria, fibrin clots were generated on glass cover slips by thrombin incubation of plasminogen-depleted fibrinogen. Photographic

visualization of the fibrin clots demonstrated that the white and turbid microthrombi were totally dissolved into a clear solution by plasminogen-coated *S. canis* G361 in the presence of urokinase within 4 h of incubation (Fig. 6B). No dissolution of the fibrin clots could be observed after incubation with bacteria or plasminogen-coated bacteria without activator (Fig. 6B), indicating that the fibrin thrombi represent a tight but protease-sensitive barrier. Plasmin-mediated transmigration activity of *S. canis* bacteria through fibrin clots generated on membranes of transwell cell culture inserts was determined by plating of diluted aliquots from the lower well (Fig. 6B, table). Transmigration of $3.7 (\pm 0.7) \times 10^6$ cfu·ml⁻¹ *S. canis* bacteria was detected after 4 h and $8.3 (\pm 0.8) \times 10^6$ cfu·ml⁻¹ bacteria after 6 h of incubation of thrombi with plasmin-coated bacteria. Dissolution of fibrin bundles by plasmin-coated *S. canis* bacteria was also visualized by time lapse microscopy. The time-dependent fibrin degradation is shown in Fig. 6C illustrating the dynamics of plasmin-mediated fibrin degradation.

Electron microscopic visualization of fibrin degradation by *S. canis* and SCM-expressing *S. gordonii*

Electron microscopic studies illustrated the dissolution of the fibrin bundles of *S. canis* G361 (Fig. 7A-H). Fibrin fibres of a generated fibrinogen thrombus were microscopically visualized as ordered, cable-like structures of up to 0.35 µm in diameter (Fig. 7A, B). Without plasmin-mediated fibrinogen degradation, the cell chains of *S. canis* bacteria showed strong adherence to the fibrin surface layer (Fig. 7C) and also to the non-dissolved fibrin fibrils (Fig. 7D, E). The fibrin bundle structure remained unchanged after incubation with plasminogen-coated bacteria in the absence of a plasminogen activator (Fig. 7E). Fibrin fibres were substantially degraded after incubation of thrombi with plasminogen-pretreated bacteria in the presence of urokinase (Fig. 7F-H). Higher

magnifications visualized, that the bacteria-bound proteolytic activity resulted in an enhanced dissolution of the fibrin matrix forming a halo of dissolved fibrin around the bacteria and led the bacteria sink into the underlying fibrin layer (Fig. 7G and H). Plasmin-mediated fibrin degradation was also analysed by electron microscopy after incubation of the microthrombi with *S. gordonii* bacteria expressing SCM (Fig. 7I-P). No fibrin degradation was detected by *S. gordonii* bacteria (Fig. 7I) or plasminogen-preincubated *S. gordonii* (Fig. 7J) which lack SCM on its surface (SGO WT). Moreover, no dissolution of the fibrin bundles was observed by plasminogen preincubated *S. gordonii* (SGO WT) bacteria in the presence of the plasminogen activator urokinase (Fig. 7K, L). Similar to *S. canis*, *S. gordonii* bacteria expressing SCM on the surface (SGO SCM) show no fibrin degradation activity but strong adherence to the fibrin matrix (Fig. 7M, N). Moreover, in conjunction to *S. canis*, dissolution of the ordered fibrin structures was only detected by plasmin-coated *S. gordonii*-expressing SCM (Fig. 7O and P). In conclusion, the degradation analyses indicate that plasmin-mediated dissolution of soluble fibrinogen and fibrinous thrombi represents a key function of the *S. canis* M-like protein for streptococcal transmigration and dissolution of the fibrin matrix.

DISCUSSION

S. canis is well known as an emerging infective agent responsible for a variety of invasive diseases in domestic animals [2] as well as humans [3-5]. Despite its zoonotic potential, the knowledge about virulence mechanisms required for establishing a *S. canis* infection remained mostly elusive. The subversion of host-derived proteolytic activity for invasive and degradative dissemination within the host tissue belongs to one of the most important pathogenicity mechanisms described for pathogenic bacteria so far [6]. Several bacterial plasminogen binding proteins have been identified up to now, but the pathophysiological

role of plasminogen binding to zoonotic streptococci like *S. canis* has not been investigated in detail. The presented study revealed the presence of a novel plasminogen receptor (SCM) on the surface of *S. canis*. Detailed biochemical analysis characterized SCM as a high-affinity ligand of the elastase-digested plasminogen fragment mini-plasminogen. Despite the lack of endogenous streptokinase activity, surface-bound plasminogen is convertible to plasmin by host-derived plasminogen activators, which enables *S. canis* to degrade ECM matrix molecules and dissolve aggregated fibrin thrombi. The use of plasmin might therefore represent an important virulence trait for establishing a *S. canis* infection.

S. canis is mostly isolated from animals, in particular, from dogs, cats, and cows [1]. A close genetic relationship and a similar clinical phenotype to other group A, C, and G streptococci, lead to inclusion of *S. canis* in the pyogenic group of streptococci [29]. It has been described for the species *S. pyogenes* (group A streptococci, GAS) and *S. dysgalactiae* sub. *equisimilis* (group C and G streptococci, GCGS) that their ability to bind and activate plasminogen has an outstanding role in pathogenicity [30, 31]. Receptors for plasminogen binding on the streptococcal surface are M- and M-like proteins like PAM, GCS3, and MLG72 [13, 31]. M proteins form fibrillar structures on the surface of streptococci which have been characterized extensively as important virulence factors in the pathogenesis of GAS and GCGS infections [32, 33]. Usually, *emm* genes are detectable by specific oligonucleotides. *S. canis* is characterized as *emm*-negative in the established PCR-test described by the Centre of Disease Control (CDC) for *emm* typing. One exception is published by Ahmad and colleagues [34]. Very recently, Yang and coworkers identified a protein of *S. canis* (SPASc) showing similarity to the protective antigen SpaZ of *S. equi* sub. *zooepidemicus*, SPA of *S. pyogenes*, and SeM, the M-like protein of *Streptococcus equi* [35]. An orthologue of SPASc was recombinantly

expressed as a His-tag fusion. CD spectroscopy demonstrated an alpha-helical structure and a high probability for dimerization under physiological conditions. The formation of a coiled-coil structure is supported by the fact that an alignment of the amino acid sequence with known M- and M-like proteins revealed the generalized heptad sequence characteristic for alpha-helical coiled-coil proteins. In summary, structural properties, binding of ECM proteins and sequence homologies characterize SCM as an M-like protein of *S. canis*.

Dot blot overlay assays showed a concentration-dependent binding of SCM to iodinated plasminogen. The quality of SCM mediated plasminogen binding was supported by the determination of a dissociation constant within the nanomolar range by surface plasmon resonance studies. Furthermore, strong plasminogen binding activity of heterologously SCM-expressing *S. gordonii* bacteria also confirmed the high relevance of SCM for plasminogen interaction of *S. canis*.

The PAM-binding site has been identified within the kringle domain 2 of plasminogen [36, 37]. Kringle domains have been shown to participate in high affinity binding interactions with streptokinase and also mediate interaction of plasminogen with plasminogen activators, plasmin substrates, fibrin, and cell surface receptors [38, 39]. In contrast to PAM, binding analysis revealed that SCM shows no interaction with the first four kringle domains of plasminogen. Moreover, to the best of our knowledge, SCM is the first bacterial plasminogen binding protein interacting in lysine dependent manner with the C-terminal part of plasminogen, termed mini-plasminogen. Surface plasmon resonance studies confirmed the interaction of SCM with mini-plasminogen reaching kD values within nanomolar range. This kind of interaction has only been suggested for the plasminogen interaction with *H. pylori* [40], *Trypanosoma cruzi* epimastigotes, and streptococci from bovine origin [41, 42]. Several reports describe an interaction of plasminogen binding proteins with the lysine

binding sites present in the N-terminal kringle domains of plasminogen. Since we demonstrate an interaction of SCM with the C-terminal part of plasminogen, composed of kringle domain 5 and the catalytic domain, the different interaction sites may also contribute to a different mechanism of host-pathogen interaction. Detection of mini-plasminogen was associated with low levels of plasminogen and high levels of elastase-al protease inhibitor complex in patients suffering from septicemia [43, 44] and is also able to produce clot lysis after activation with tPA in plasminogen-depleted plasma [9]. Therefore, interaction of pathogenic *S. canis* with mini-plasminogen may enable the streptococci to recruit plasmin activity after binding of plasminogen or mini-plasminogen present under different pathophysiological conditions. The inhibition of plasminogen binding by the lysine analogue 6-AHA is often interpreted as identification of lysine binding sites of kringle domains as major interaction sites [13, 45, 46]. Plasminogen binding of SCM as well as conversion of plasminogen to proteolytic active plasmin on the *S. canis* surface was inhibited in concentration-dependent manner by 6-AHA. These results would support an interaction of SCM with the lysine binding site present in kringle domain 5 of mini-plasminogen. In contrast to SCM, group A streptococcal PAM has been shown to bind to the K2 of plasminogen, which exhibits a low affinity lysine binding site [36]. X-ray analysis of the PAM-kringle-2 protein complex revealed that a "pseudo-lysine" like binding is formed in PAM by positively and negatively charged amino acids within the plasminogen binding region of PAM [37]. This kind of pseudo-lysine interaction has also been proposed for binding of lysine analogues to K1 and K5 [37]. The detailed characterization of specific amino acids involved in interaction of SCM with mini-plasminogen is currently in progress and will elucidate the role of lysine residues in binding of SCM to mini-plasminogen.

Immobilization of plasminogen on physiological fibrin surfaces or on cell surface of bacteria has been shown to accelerate conversion into proteolytic active plasmin by eukaryotic activators like tissue-type activator or urokinase type plasminogen activator, respectively [47]. Plasmin activation measurements confirmed an increase in activation of plasminogen bound to SCM-expressing *S. gordonii* bacteria in contrast to significantly slower plasmin activation in the presence of soluble plasminogen. Accelerated activation was also shown for plasminogen which was bound to surface displayed eukaryotic enolase [26]. In contrast to GAS and GCGS, *S. canis* is not expressing an endogenous plasminogen activator [31]. Therefore, a host-derived plasminogen activator is required for activation of cell surface bound plasminogen in an aprotinin-sensitive manner. Blood coagulation resulting in fibrinogen-enriched thrombi has been suggested to capture invasive pathogenic microorganisms in order to prevent spreading of bacteria into bloodstream and into deeper tissue sites [36, 48]. Electron microscopic illustration and time-lapse visualization clearly demonstrate the proteolytic degradation activity recruited on the surface of *S. canis* or SCM expressing *S. gordonii* bacteria. Generated fibrin thrombi were completely dissolved by plasmin-enclosed bacteria. Therefore, bacteria-bound proteolytic activity enables the bacterial release from the coagulative capture within blood thrombi and presents an effective evasion mechanism for invasive pathogenic bacteria. Moreover, it has been shown, that bacterial-bound plasmin activity is associated with pathological phenotype. In patients with Gram-negative septicemia, high levels of fibrin degradation products have been detected [49] and also, interaction of Group A streptococcal streptokinase with host fibrinolytic system facilitates bacterial access to the vasculature [50]. Interestingly, neonatal septicemia and

meningitis, which is a clinical feature of *S. canis* as well as *E. coli* infections, has also been associated with tPA-catalysed activation of plasminogen bound to *E. coli* fimbriae [2, 3, 51, 52].

The recruitment of plasminogen to the surface of the invasive zoonotic pathogen *S. canis* might, therefore, represent an important virulence mechanism leading to effective evasion of proteolytic active bacteria from thrombus capture within vasculature of the host and to bacterial dissemination into deeper tissue sites. The elucidation of the interaction mechanism between SCM and mini-plasminogen offers new insights into understanding interaction of M-like proteins with the human fibrinolytic system and enables the development of new intervention therapies.

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FIGURE LEGENDS

Fig. 1. Plasminogen recruitment to *S. canis* and *S. equisimilis* after incubation with human plasma or purified radiolabelled plasminogen. A, Coomassie stain of plasma proteins eluted from streptococcal surface after plasma incubation. Arrow indicates the 90 kDa plasminogen protein. B, Immunoblot analysis with plasminogen-specific antibodies after incubation of different *S. canis* (G1, G2, G14, G361) and *S. equisimilis* strains (C90) with human plasma. C, Coomassie stain of plasminogen recruitment to the human *S. canis* isolate G361 after incubation with plasma derived from pig (swine), goat (caprine), cat (feline), dog (canine), and human. D, Binding of 125 I-labelled plasminogen to streptococci. Plasminogen binding was performed in triplicates and was calculated in percentage of employed iodinated plasminogen.

Fig. 2. Identification of plasminogen binding protein as M-like protein of *S. canis*. A, Amino acid sequence of *S. canis* M like protein SCM. Signal sequence (VKA-EH, in standard font), LPxTG-motif (written in italic letters), and the last amino acids of the 25 kDa N-terminal part of SCM (SCM 25KD, underlined). B, Circular dichroism (CD) spectrum showing molar ellipticity versus wavelength for native SCM. The value for 222:208 nm ratio is depicted. C, Alignment of the SCM with selected alpha-helical, coiled-coil proteins, including SpaSc of *S. canis* (ACM47242.1), SpaZ of *S. equi* sub. *zooepidemicus* (ACG63105.1), Spa of *S. pyogenes* (AAL98520.1), SzM of *S. equi* sub. *zooepidemicus* (CAW97903.1), Mrp4 of *S. pyogenes* (AAB33261.1), M18.1 of *S. pyogenes* (AAB03086.1), PAM of *S. pyogenes* (CAA80222.1), and M6 of *S. pyogenes*. The generalized heptad a-b-c-d-e-f-g sequence is depicted above the alignment; respective amino acids are indicated in grey. D, Illustration of PCR analysis of different *S. canis* isolates with oligonucleotides specific for *S. canis scm* gene. Positiv (+) and negativ (-) plasminogen binding of the respective strains is depicted below.

Fig. 3. Biochemical characterization of plasminogen binding of *S. canis* isolates and SCM protein. A, Plasminogen binding analysis of *S. gordonii* heterologously expressing SCM (SGO-SCM) was analysed with iodinated plasminogen. B, Dot blot overlay of recombinant *S. canis* M-like protein SCM and its N-terminal fragment SCM 25KD. SCM and SCM 25KD were immobilized in serial dilutions on nitrocellulose (0.25 μ g, 0.125 μ g, 0.063 μ g and 0.031 μ g) followed by incubation with iodinated human plasminogen. C-E, Sensograms of surface plasmon resonance kinetics of plasminogen binding to SCM (C) and to the N-terminal fragment of SCM 25KD (D). Plasminogen was immobilized on Biacore CM5 Sensorchips and both, SCM and SCM 25KD were used as analytes. E, Binding kinetic analysis after immobilization of SCM full length and with plasminogen as analyte.

Fig. 4. Binding analysis and inhibition studies of plasminogen and different plasminogen derivatives to different streptococci. A, Inhibition of lysine-dependent plasminogen binding to *S. canis* G361 in the presence of 0.001 mM, 0.01 mM, 0.1 mM, 10 mM, 100 mM, 1000 mM of lysine analogue 6-AHA. B, C, Inhibition of plasminogen binding of *S. canis* G361 (B) and *S. pyogenes* isolate A158 (C) by non-labelled plasminogen and non-labelled K1-3, respectively. Inhibitors were employed in concentrations of 0.5 μ M, 1.0 μ M, 1.5 μ M and 2.0 μ M. D, Binding analysis of iodinated human K1-3 to *S. canis*, *S. gordonii* and *S. pyogenes*. E, Dot blot analysis of SCM binding to plasminogen and plasminogen derivatives: K1-3, angiostatin (representing K1-4), K4, and mini-plasminogen (representing K5 and the catalytic domain). Plasminogen and its fragments were immobilized on nitrocellulose in an amount of 3.0 μ g, 1.5 μ g, 0.75 μ g, 0.38 μ g and 0.19 μ g. Binding of SCM was detected with SCM-specific antibodies and HRP-conjugated secondary antibodies.

Unspecific cross-reactivity of antibodies was not detected (data not shown). F, Kinetic analysis of SCM binding to immobilized mini-plasminogen using surface plasmon resonance technique.

Fig. 5. Plasmin activation mechanism of *S. canis* strain G361. A, B, Determination of secreted plasmin activity by measurement of chromogenic plasmin substrat S-2251 at 405 nm in a time series including 0 min, 30 min, 90 min and 120 min after incubation of *S. canis* G1, G2, G361 and *S. equisimilis* C90 with human plasma (A) and canine plasma (B). C, Plasmin activity was measured after incubation of *S. canis* bacteria with purified human plasminogen in the presence of urokinase. Plasmin activity was determined over a time period up to 120 min and also in the presence of the serine protease inhibitor aprotinin. D, Dose-dependent inhibition of plasmin activation by the addition of 2.0 mM, 40 mM and 80 mM of the lysine analogue 6-AHA. E, Time dependent conversion of plasminogen bound to surface of SCM-expressing SGO and soluble plasminogen. No plasmin activation was detected without plasminogen or urokinase, respectively (data not shown).

Fig. 6. Plasmin-mediated degradation of fibrinogen and fibrin matrices by *S. canis* G361. A, Immunoblot analysis of degradation of human fibrinogen by plasminogen encotated *S. canis* G361 bacteria. Degradation was analysed in the presence of urokinase after indicated time points (0 min, 5 min, 10 min, 20 min, 30 min, and 60 min) with fibrinogen-specific antibodies. Fibrinogen was not degraded after incubation of plasminogen-coated *S. canis* for 0 min and for 60 min with fibrinogen without activator (Ctrl 0, Ctrl 60). B, Illustration of plasminogen-mediated degradation of fibrin generated on a porous transwell membrane by *S. canis* G361 in the presence or absence of urokinase. Plasmin-mediated transmigration of *S. canis* 361 through fibrin clots was quantified by determination of bacterial cfu·ml⁻¹ after different incubation periods (0 h, 2 h, 4 h, 6 h) by plating of serial dilutions from the lower well. C, Time-lapse imaging of fibrin degradation by plasmin-encotated *S. canis* G361 bacteria. *S. canis* G361 bacteria (arrows) are visible within the dissolved fibrin solution.

Fig. 7. Visualization of fibrin degradation by plasmin-coated *S. canis* G361 and *S. gordonii* bacteria analysed by field emission scanning electron microscopy (FESEM). A, B, Microscopic images illustrate a dense network of microthrombi formed by bundled fibrin fibres (control). C-E, Bacterial attachment to the fibrin matrix was shown by non-treated *S. canis* G361 (C, D) as well as by plasminogen-preincubated bacteria (E, PLG). F-H, The fibrin bundles were degraded by proteolytic active *S. canis* bacteria in the presence of urokinase (PLG, uPA). I, J, Only minor bacterial attachment to fibrin thrombi was detected by *S. gordonii* lacking the SCM protein on the surface (I, SGO WT, white arrows) and plasminogen-preincubated SGO WT (J; SGO WT PLG, white arrows). K-M, No degradation of fibrin bundles was observed with plasmin-encotated *S. gordonii* (K, L; PLG, uPA) or SCM-expressing *S. gordonii* (SGO-SCM) without plasminogen incubation (M; PLG). N, Plasminogen preincubation was not mediating microthrombi dissolution by SGO-SCM. O, P, Remarkable degradation of fibrin bundles was only detected by *S. gordonii* expressing the SCM protein on its surface (SGO-SCM) supplemented with plasminogen and urokinase (PLG, uPA).

TABLE 1

Bacterial strains

<u>Isolate</u>	<u>Strains</u>	<u>Host</u>
G1	<i>S. canis</i>	dog
G2	<i>S. canis</i>	dog
G8	<i>S. canis</i>	dog
G13	<i>S. canis</i>	dog
G14	<i>S. canis</i>	dog
G15	<i>S. canis</i>	dog
G17	<i>S. canis</i>	dog
Sc1 4074-03 ^a	<i>S. canis</i>	human
Sc2 6381-02 ^a	<i>S. canis</i>	human
Sc3 8881-03 ^a	<i>S. canis</i>	human
Sc4 5426-05 ^a	<i>S. canis</i>	human
G361 ^b	<i>S. canis</i>	dog
39126/96 ^c	<i>S. canis</i>	dog
2572 ^c	<i>S. canis</i>	dog
2424/96 ^c	<i>S. canis</i>	dog
1022/96 ^c	<i>S. canis</i>	dog
2424 ^c	<i>S. canis</i>	dog
566714 ^c	<i>S. canis</i>	dog
734/95 ^c	<i>S. canis</i>	dog
C90	<i>S. equi</i> sub. <i>equisimilis</i> (GCS3)	human
A158	<i>S. pyogenes</i> (PAM)	human

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^b M. van der Linden, National Reference Centre for Streptococci, Aachen, Germany

^c J.F. Prescott, Department of Pathobiology, University of Guelph, Ont., Canada

TABLE 2

Kinetic parameters of Biacore Sensogram

<u>ligand</u>	<u>analyte</u>	<u>k_a</u> <u>(1/Ms)</u>	<u>k_d</u> <u>(1/s)</u>	<u>kD</u> <u>(M)</u>	<u>χ²</u>
PLG	SCM	5.76e ³	1.55e ⁻⁴	2.69e ⁻⁸	20
PLG	SCM25KD	1.10e ⁴	2.28e ⁻⁴	2.07e ⁻⁸	4
mPLG	SCM	9.63e ³	1.33e ⁻⁴	1.38e ⁻⁸	16
SCM	PLG	3.11e ³	1.59e ⁻³	5.10e ⁻⁷	27.7

Figure 1

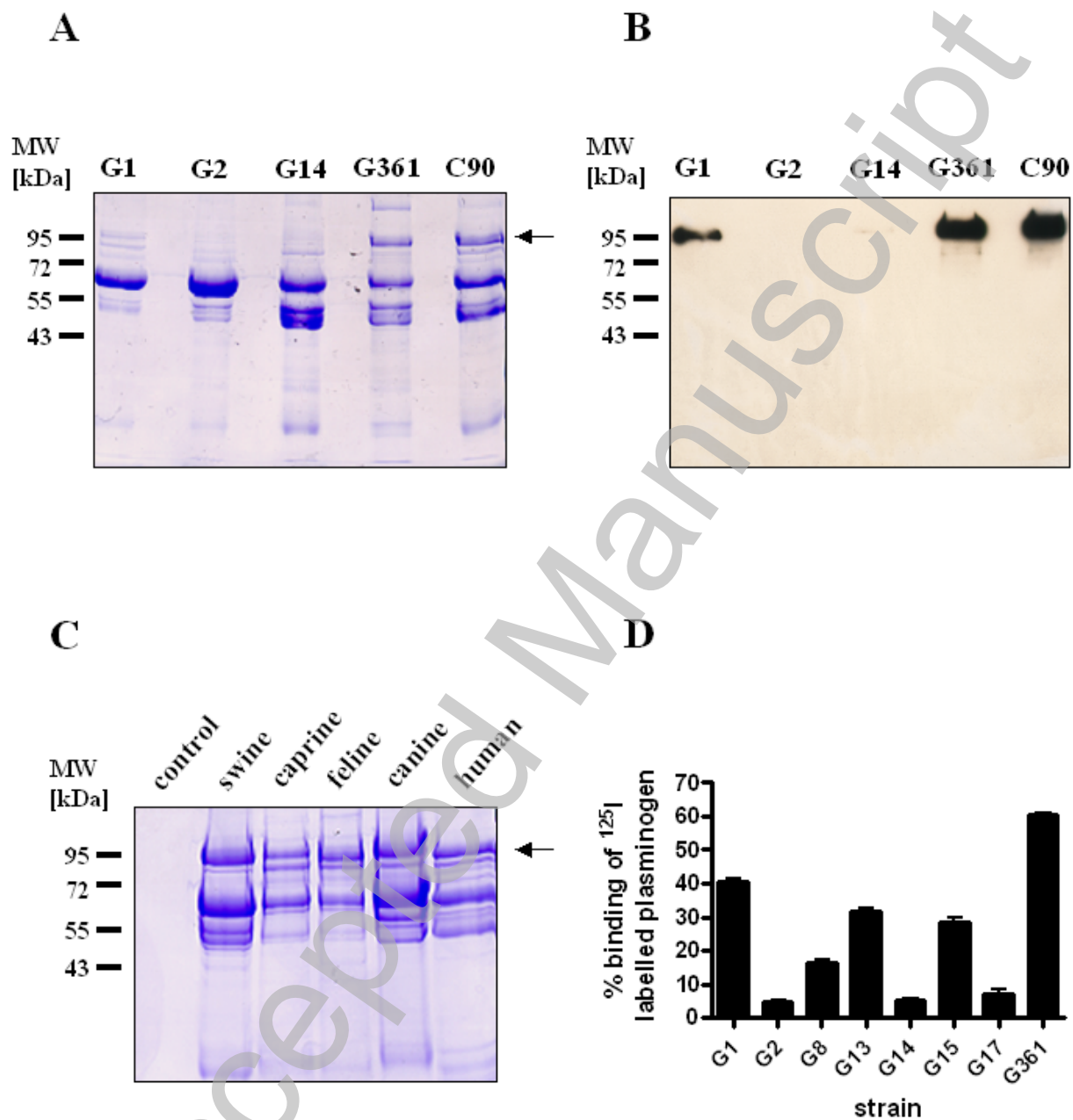


Figure 2

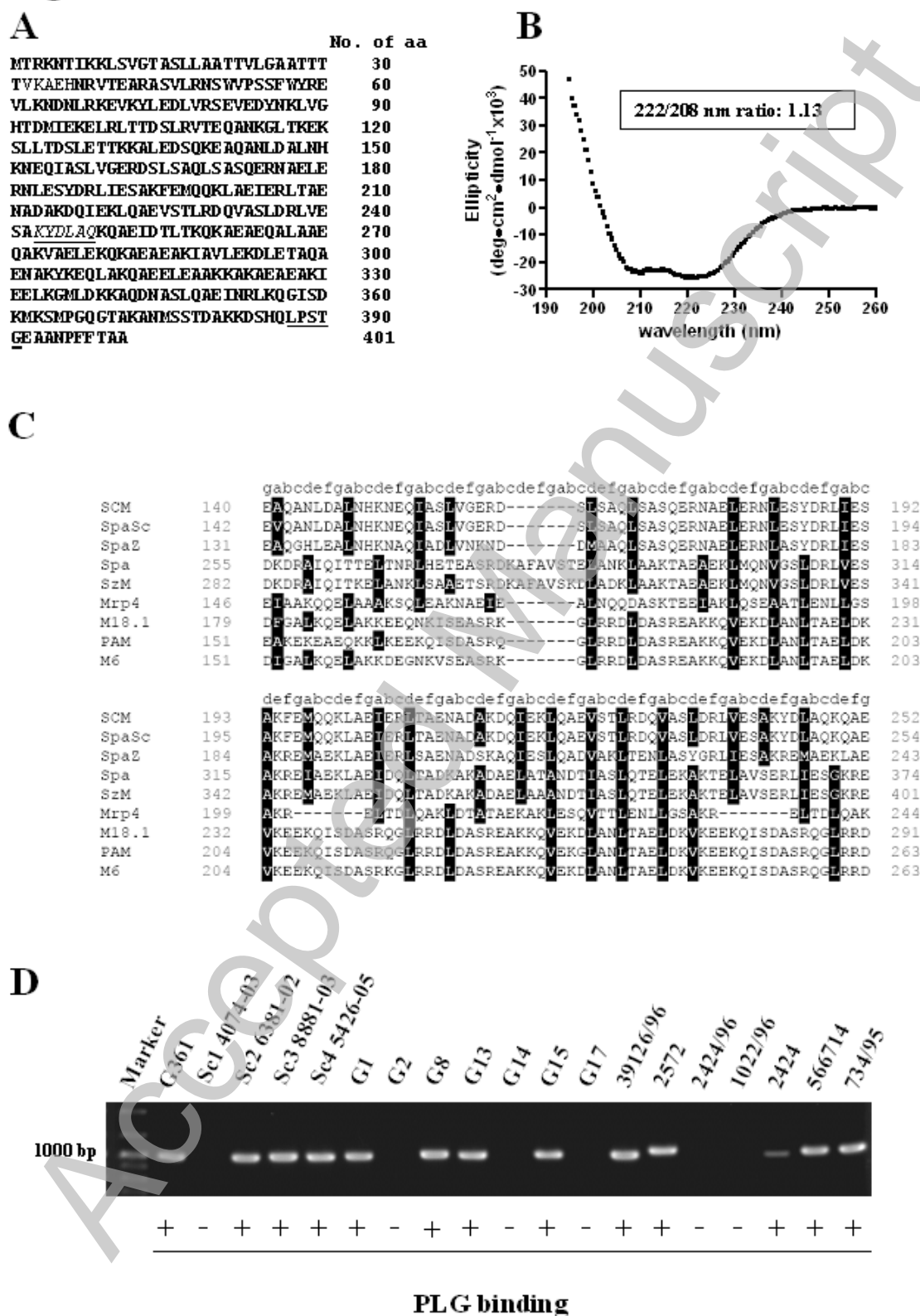
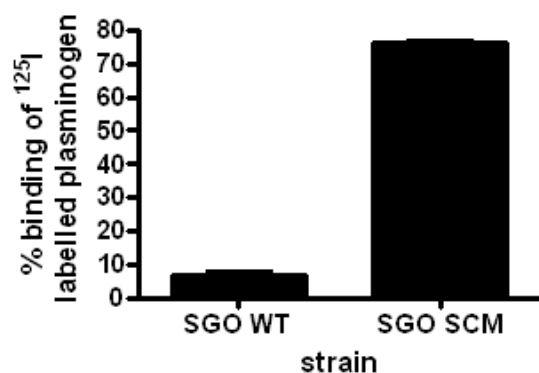
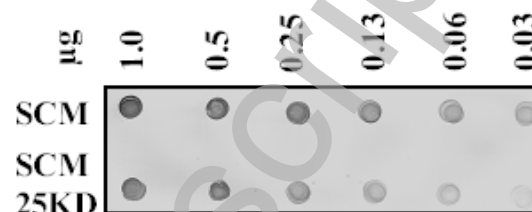


Figure 3

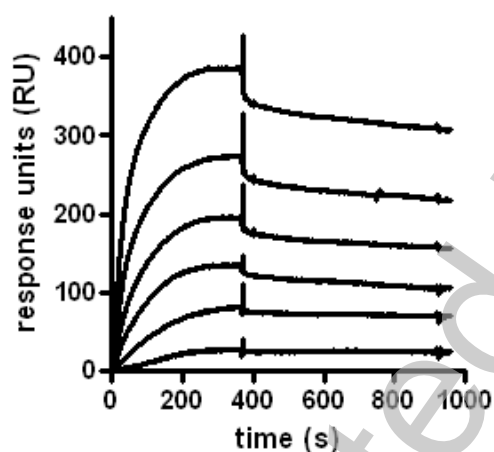
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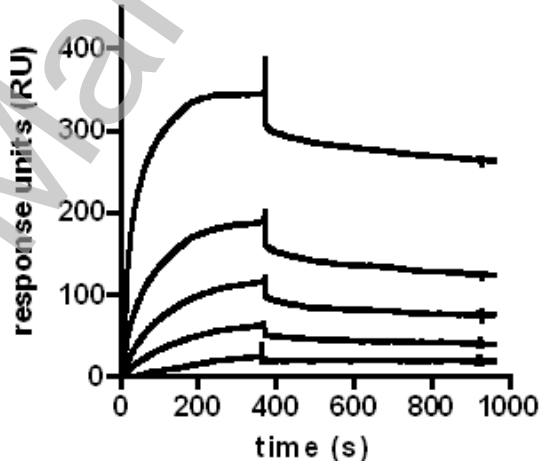
B



C



D



E

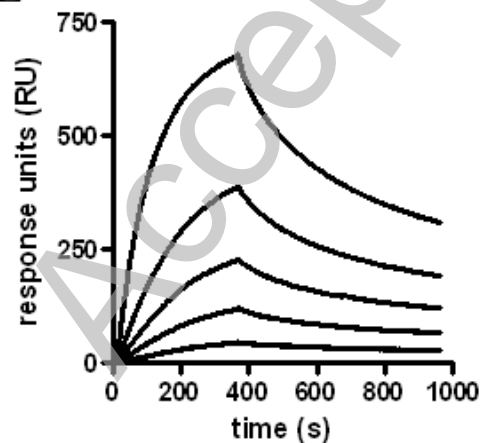


Figure 4

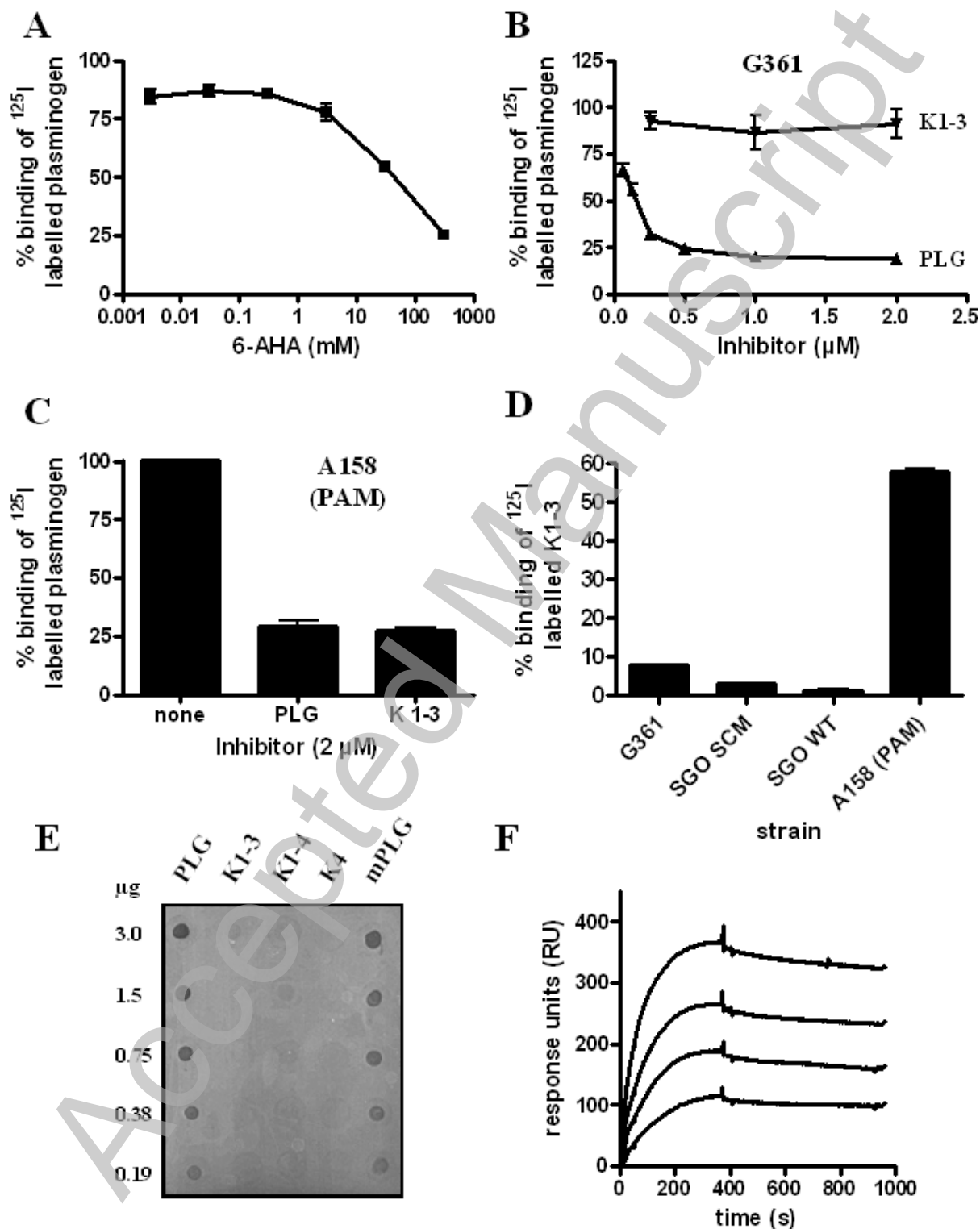


Figure 5

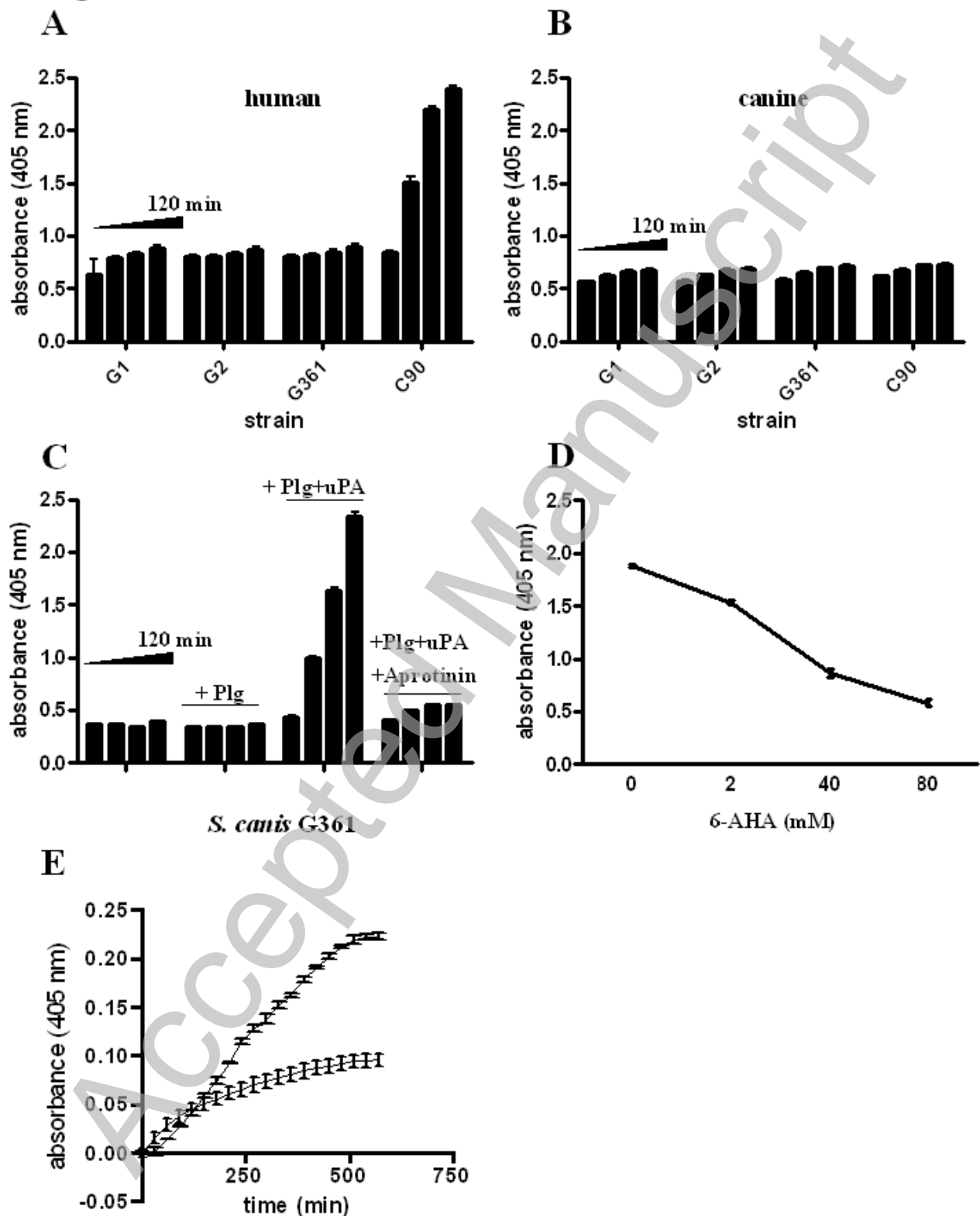
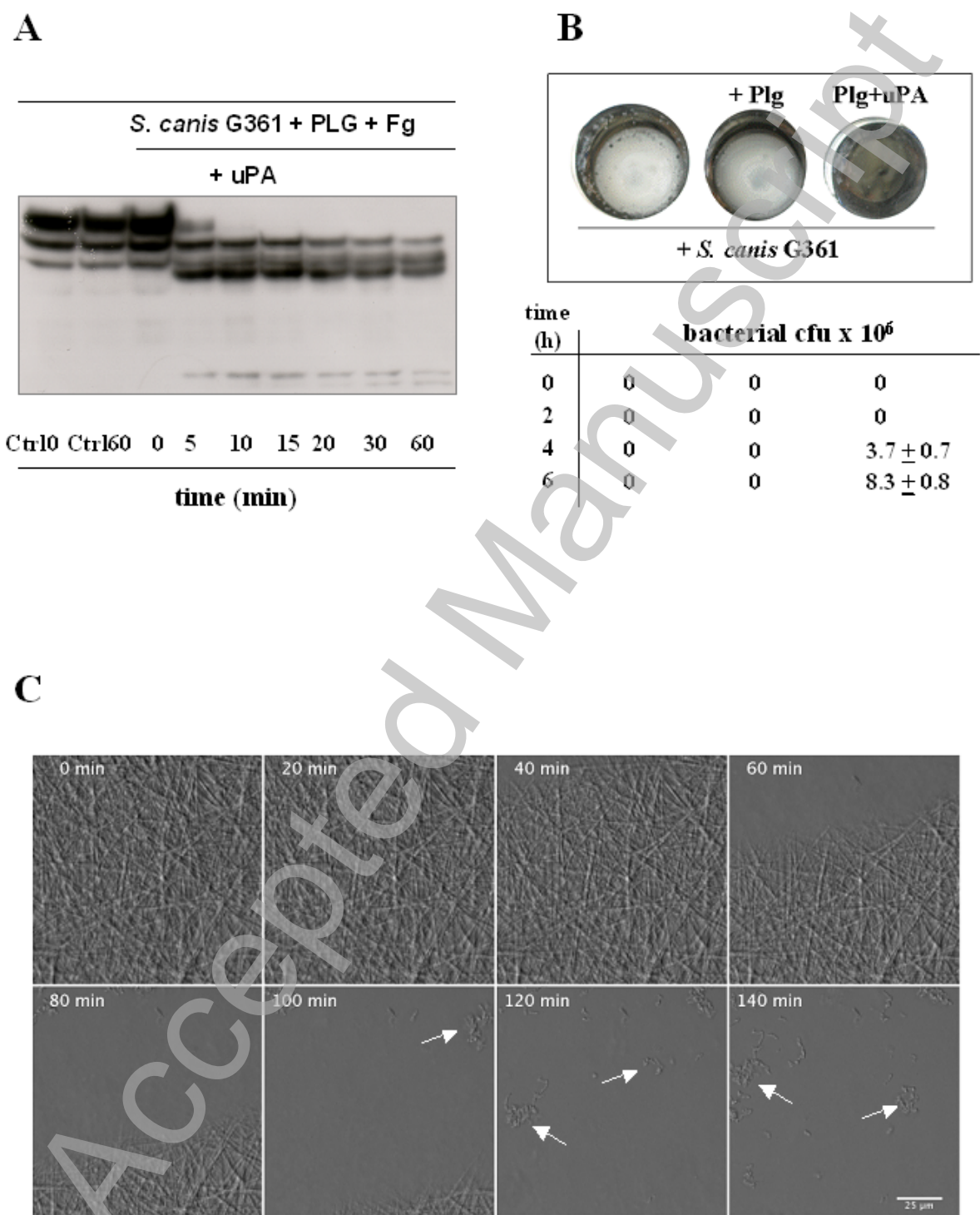


Figure 6



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Figure 7

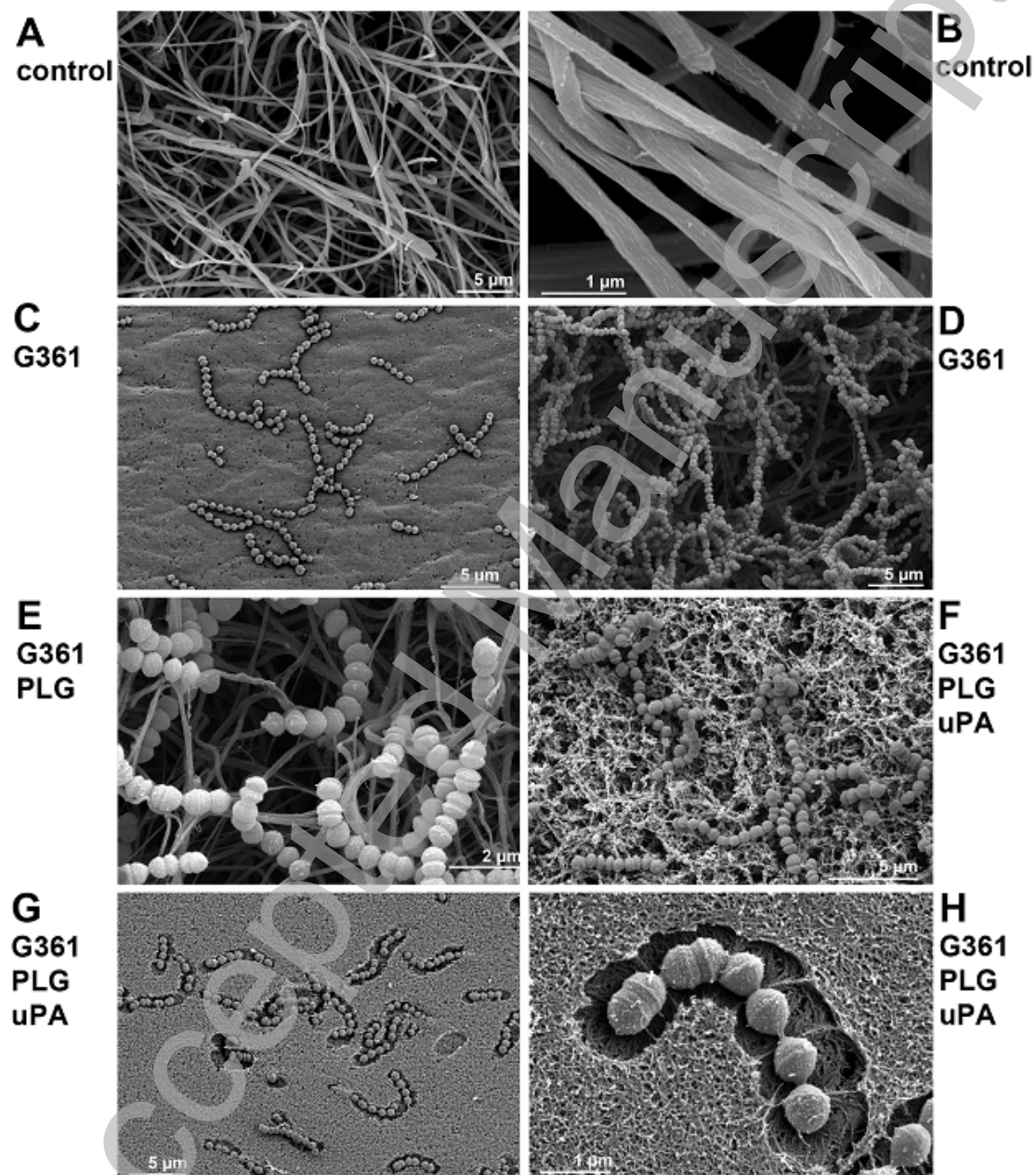
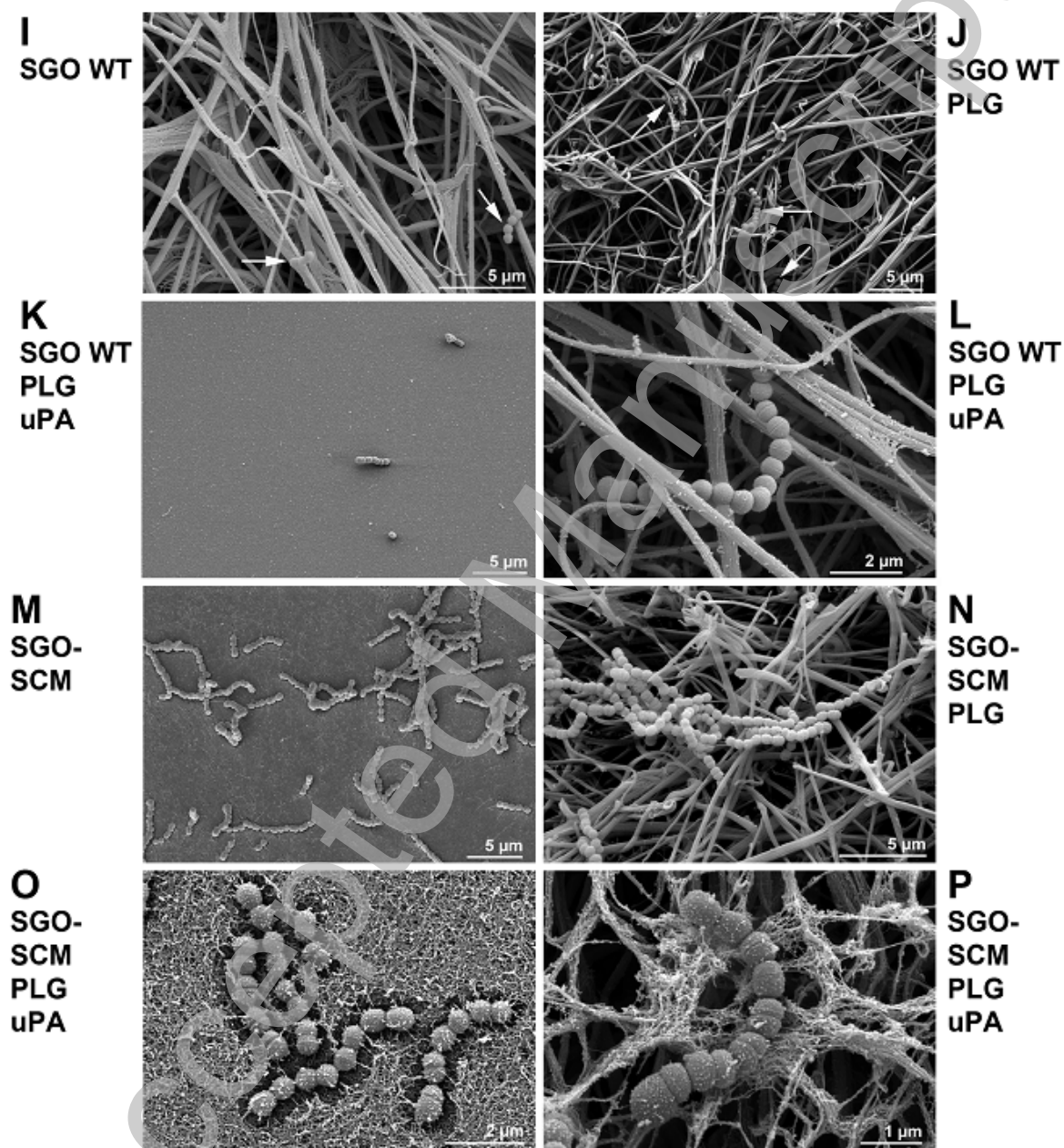


Figure 7



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