Protein FOG – a streptococcal inhibitor of neutrophil function

Helena M. Johansson, Matthias Mörgelin and Inga-Maria Frick

Correspondence

Inga-Maria Frick

inga-maria.frick@medkem.lu.se

Department of Cell and Molecular Biology, Section for Clinical and Experimental Infectious Medicine, BMC, B14, Lund University, S-221 84 Lund, Sweden

Several strains of group G streptococci (GGS) form aggregates when grown *in vitro*. Aggregating strains interact with fibrinogen, and this study reports the isolation of a novel self-associating and fibrinogen-binding protein of GGS, denoted protein FOG. Sequencing of the *fog* gene revealed structural similarity with M proteins of both GGS and group A streptococci (GAS). Analogous to GAS, GGS were found to multiply in human blood. All strains of GGS express protein G, a protein known to interact with the constant region of immunoglobulin G and albumin. Surprisingly, a clinical isolate expressing protein G, but lacking protein FOG, was killed in human whole blood; however, the addition of intact soluble protein FOG restored the ability of the bacteria to survive and multiply in human blood. This is believed to be the first report of a soluble M-like protein salvaging an M-negative strain from being killed. The antibactericidal property of protein FOG is dependent on its fibrinogen-binding activity. Thus, in plasma, FOG precipitates fibrinogen, and when added to whole blood, protein FOG triggers the formation of visible aggregates comprising fibrinogen and neutrophils that are disabled in their killing of the bacteria. Moreover, the results emphasize the importance of an intact FOG molecule, as presented on the bacterial surface, for full protective effect.

Received 22 April 2004

Revised 6 August 2004

Accepted 9 September 2004

INTRODUCTION

Human isolates of group C and G streptococci (GCS and GGS) are generally regarded as commensals in the throat, skin and gastrointestinal tract, but they are also associated with infections of clinical importance. Diseases caused by these bacteria are similar to those caused by group A streptococci (GAS), including pharyngitis and impetigo, to more severe conditions such as necrotizing fasciitis (for references, see Oster & Bisno, 2000). In addition, GCS and GGS are animal pathogens, causing diseases like bovine mastitis and equine strangles (Calvinho *et al.*, 1998; Timoney, 1993). GCS and GGS are closely related; genetic analysis based on household genes failed to separate them into two distinct groups (Kalia *et al.*, 2001). During the last decade, several studies have shown an increase in the number of human skin infections and cases of bacteraemia caused by GCS and GGS (Lewthwaite *et al.*, 2002; Oster & Bisno, 2000; Sylvetsky *et al.*, 2002). Pharyngeal carriage of these bacteria has also been suggested as a conceivable cause of acute rheumatic fever (Haidan *et al.*, 2000).

Abbreviations: α_2 -M, α_2 -macroglobulin; FOG, fibrinogen-binding protein of G streptococci; GAS, group A streptococci; GCS, group C streptococci; GGS, group G streptococci; GST, glutathione S-transferase; HBP, heparin-binding protein; IgGfc, constant region of immunoglobulin G.

The GenBank/EMBL/DBJ accession number for the sequence reported in this paper is AY600861.

The best-characterized surface protein produced by GCS and GGS is protein G, which has affinity for the constant region of immunoglobulin G (IgGfc) and albumin (Björck *et al.*, 1987; Björck & Kronvall, 1984; Reis *et al.*, 1984). Protein G also interacts with α_2 -macroglobulin (α_2 -M) and high-molecular-weight (H-) kininogen (Sjöbring *et al.*, 1989). The role of protein G in virulence is not known, but it has been suggested that the binding of host proteins may aid in evasion of host defence mechanisms. Protein G also acts mitogenically on peripheral blood lymphocytes (Otten & Boyle, 1991). It has also been proposed that protein G could function as an environmental sensor ensuring an appropriately adapted gene expression (Cleary & Retnoningrum, 1994). The protein-G-related MIG protein of *Streptococcus dysgalactiae*, an organism which causes bovine mastitis, was recently shown to prevent phagocytosis by bovine neutrophils, most likely through an interaction with IgG or α_2 -M (Song *et al.*, 2001); however, it is not known whether protein G expressed on human isolates of GCS and GGS has a similar function. In addition to protein G, a number of strains of GCS and GGS express M or M-like proteins that are similar in structure and function to those described for GAS (Campo *et al.*, 1995; Collins *et al.*, 1992; Schnitzler *et al.*, 1995).

M proteins are cell wall attached, elongated molecules forming α -helical coiled-coil dimers. Their N-terminal parts are highly variable, whereas the C-terminal regions are more

conserved. M proteins have affinity for numerous plasma proteins, such as fibrinogen, albumin, IgG and complement factors (for references, see Fischetti, 1989). The M protein is traditionally regarded as a major virulence factor, primarily through its ability to promote bacterial survival in human blood. Specific interactions with fibrinogen and regulatory proteins of complement have been reported as important mechanisms by which M protein of GAS exerts its anti-phagocytic effect (Horstmann *et al.*, 1988; Perez-Casal *et al.*, 1995; Ringdahl *et al.*, 2000; Thern *et al.*, 1995; Whitnack & Beachey, 1982). Possible proposed mechanisms are the circumvention of opsonization by C3 at the bacterial surface, or the hindering of binding to granulocytes (Podbielski *et al.*, 1996). Although M proteins of GCS and GGS have also been reported to confer resistance of these bacteria to phagocytosis, it is not known whether these bacteria utilize mechanisms identical to those described for GAS to achieve survival in human blood.

In the present work, the contribution of protein G to survival of GGS in human blood was investigated. Surprisingly, a clinical isolate expressing protein G was found to be rapidly killed. Instead, the experiments performed identified a self-associating M-like protein, denoted FOG for fibrinogen binding protein of G streptococci, as crucial for bacterial survival.

METHODS

Bacterial strains, growth conditions and sedimentation analysis. GGS were clinical isolates collected at the Department of Clinical Microbiology, Lund University Hospital, Sweden. Bacterial strains were cultured on blood agar plates from which single colonies were inoculated into Todd-Hewitt (TH) broth (Difco). When relevant, plating of bacteria was performed using TH with 2% agar (THA; Difco). BMJ71 is a GAS mutant strain lacking M and M-like proteins, which are regulated by *mga* (Kihlberg *et al.*, 1995). Bacteria were grown at 37 °C in a 5% CO₂-enriched environment. To analyse sedimentation, bacterial cultures were grown in 10 ml TH overnight, resuspended through gentle agitation, and then allowed to settle at room temperature. By measuring the OD₆₂₀ in the upper half of the tubes at various time points, the degree of sedimentation was determined. Bacteria treated with cyanogen bromide (CNBr) were washed and resuspended in PBS [0.12 M NaCl, 0.03 M phosphate (KH₂PO₄/Na₂HPO₄), pH 7.4] before sedimentation was analysed.

CNBr extraction and papain digestion. CNBr chemically cleaves proteins on the carboxyl side of methionine residues. Bacterial surface proteins were extracted as described by Otten & Boyle (1991). In brief, bacterial cells were collected by centrifugation at 1400 g for 10 min, and resuspended in 2 ml PBS (g bacteria)⁻¹. An equal volume of CNBr solution (30 mg per ml of 0.2 M HCl) was added, and the sample was rotated overnight at room temperature, followed by centrifugation at 12 900 g for 15 min. The supernatant was filter-sterilized to remove remaining bacteria, dialysed against 0.1 M HCl for removal of excess CNBr, and, finally, the pH was raised to 7.5 by the addition of 1 M Tris. Released material was then subjected to SDS-PAGE and Western blotting. G148 bacteria were digested with papain, as described by Björck & Kronvall (1984), and analysed for binding of IgG.

SDS-PAGE, ligand blotting and N-terminal amino acid sequencing. SDS-PAGE was performed using the buffer system described by Laemmli (1970). Separated proteins were stained with Coomassie brilliant blue, or transferred onto PVDF membranes (Amersham Biosciences) using the Bio-Rad TransBlot SemiDry Transfer Cell System. Membranes were washed with blocking buffer (PBS containing 0.25% Tween 20, 0.25% gelatin) four times for 20 min, and then incubated with radiolabelled protein for 3 h at room temperature, or overnight at 4 °C. The membranes were subsequently washed four times with blocking buffer. Bound ligand was measured using the Fuji Imaging System, after an exposure of 3 h minimum. For detection of protein G in CNBr-released material, membranes were blocked in PBS containing 0.05% Tween 20 (PBST) and 5% dry milk powder (blocking buffer), incubated with antibodies against protein G (see next section; 1:1000) in blocking buffer for 30 min at 37 °C, washed with PBST, and incubated with horseradish-peroxidase-conjugated protein A (1:5000; Sigma) for 30 min at 37 °C. The membranes were washed, and bound antibodies were detected by the chemiluminescence method (Nesbitt & Horton, 1992). Proteins were also directly applied onto PVDF membranes using a Milliblot-D System. Membranes were incubated with radiolabelled probe as described above. Samples subjected to N-terminal amino acid sequencing were separated by 10% SDS-PAGE, and transferred onto a PVDF membrane as described by Matsudaira (1987). Membranes were stained with 0.1% Coomassie blue R-250 in 50% methanol, bands of interest were excised, and sequence analysis was performed at Eurosequence (Mediatech Center, Groningen, The Netherlands).

Reagents, labelling and binding assays. Human serum albumin (HSA), human fibrinogen and polyclonal human IgG were purchased from Sigma. Human factor H was kindly provided by Dr L. Truedsson, Lund University, Sweden. Protein PAB from *Fingoldia magna* was purified as described by de Chateau & Björck (1994), and human α_2 -M was kindly provided by Dr H.-P. Müller, Lund University, Sweden. GAS M1 protein was purified as described by Åkesson *et al.* (1994). Recombinant protein G containing the IgGFC-binding regions was obtained from Sigma, while protein G containing both the IgGFC- and the albumin-binding regions was purified as described by Björck *et al.* (1987). Antiserum against the N-terminal albumin-binding region of protein G was raised in rabbits. HSA was labelled with ¹²⁵I using the Bolton and Hunter reagent (Amersham). Fibrinogen, IgG and factor H were radio-labelled using the Chloramine T method as described by Greenwood *et al.* (1963), and purified protein FOG fragments were labelled using Iodobeads (Pierce). Overnight cultures, and bacteria subjected to CNBr extraction or papain digestion, were harvested by centrifugation at 1400 g for 10 min, and washed with PBST. ¹²⁵I-labelled proteins were bound to bacterial cells, as described by Björck & Kronvall (1984).

Bactericidal assay, analysis of blood aggregates and blood exchange experiments. Bacteria were grown to early mid-exponential phase (OD₆₂₀ 0.15) and diluted to approximately 1.5 × 10³ cells ml⁻¹ in TH. A 100 µl bacterial suspension was then added to 1 ml human heparinized whole blood or 1 ml human plasma, and incubated with gentle rotation at 37 °C. Aliquots were taken at different time points, mixed with 2.5 ml 0.5% agar, plated onto THA, and incubated at 37 °C overnight. The number of c.f.u. was counted, and the multiplication factor was calculated by dividing the number of c.f.u. by the initial number of c.f.u. In protection assays, 100 µl G148 bacterial suspension was mixed with soluble proteins, prior to incubation with blood. Samples were taken and plated as above. For cocultivation experiments, G41 was heat inactivated at 80 °C for 10 min, and then 2.5 × 10⁴ cells were mixed with 100 µl G148 (1.5 × 10³ cells ml⁻¹), and the mixture was added to 1 ml whole blood. Samples were taken and plated directly on THA.

From the 5 h sample, single colonies were further cultivated in TH for binding analysis and PCR. Heparinized blood was used in all experiments. After 1 h incubation of whole blood and protein FOG, when aggregates were seen, the blood was removed by pipetting, and the aggregates were carefully washed four times in isotonic PBS (0.1 M NaCl, 1.7 mM KH_2PO_4 , 4.9 mM Na_2HPO_4 , pH 7.2). A 1 ml volume of fresh blood from the same donor was then added together with a fresh aliquot of diluted G148 bacteria. Samples were incubated at 37 °C, and bacterial survival was monitored as described above. For analyses by light microscopy, the aggregates were resuspended in 100 µl isotonic PBS, diluted 1:15, and applied onto glass slides by means of cytospin, using 300 r.p.m. for 4 min. Specimens were fixed in 2% paraformaldehyde on ice for 30 min in a humidified chamber, washed twice with cold PBS, stained with haematoxylin and eosin, and analysed by light microscopy or subjected to immunostaining. For immunostaining, samples were incubated with rabbit anti-human fibrinogen (1:3000; Dako) for 1 h at 37 °C, washed extensively with PBST, and then incubated with Alexa-Fluor-488-conjugated goat anti-rabbit Fab'2-fragments as secondary antibody (1:2000; Molecular Probes). After washing with PBST, samples were analysed using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled CCD camera, using a Plan Apochromat ×100 objective and a high numerical aperture oil-condenser.

Electron microscopy. Human polymorphonuclear neutrophils were isolated from heparinized blood using Polymorphprep (Nycomed Pharma), according to the manufacturer's instructions. For scanning electron microscopy, the cells were resuspended in RPMI (Difco), and diluted to a final concentration of 1×10^7 cells ml^{-1} . An aliquot (1×10^6 cells) was incubated with 5 µg protein FOG, or fragments 1-C or 1-B, at 37 °C for 30 min. Aliquots (30 µl) were then applied onto poly-L-lysine-coated coverslips, and subsequently fixed in 2.5% glutaraldehyde in 0.15 M sodium cacodylate, pH 7.4, (cacodylate buffer) for 30 min at room temperature. For analyses of G41 and G148 bacteria, 100 µl overnight culture, containing 2×10^7 c.f.u., was applied onto poly-L-lysine-coated coverslips. After 30 min incubation, the samples were fixed as described above. Specimens were washed with cacodylate buffer, and dehydrated with an ascending ethanol series from 50% (v/v) to absolute ethanol (10 min per step). The specimens were then subjected to critical-point drying in carbon dioxide, with absolute ethanol as intermediate solvent, mounted on aluminium holders, sputtered with 50 nm palladium/gold, and examined in a JEOL JSM-350 scanning electron microscope.

For transmission electron microscopy, bacterial cells were pelleted, fixed for 1 h at room temperature, and then overnight at 4 °C in 2.5% glutaraldehyde in cacodylate buffer. Samples were washed with cacodylate buffer, postfixed for 1 h at room temperature in 1% osmium tetroxide in cacodylate buffer, dehydrated in a graded series of ethanol, and then embedded in Epon 812 using acetone as an intermediate solvent. Specimens were sectioned into 50-nm-thick ultrathin sections with a diamond knife on an LKB ultramicrotome. The ultrathin sections were stained with uranyl acetate and lead citrate. Specimens were observed in a JEOL JEM 1230 electron microscope operated at 80 kV accelerating voltage, and images were recorded with a Gatan Multiscan 791 CCD camera.

Sequencing, cloning procedures, PCR and computational analysis. Genomic DNA was isolated according to Pitcher *et al.* (1989), with modifications described by Rasmussen *et al.* (1999). Primers were based on the signal sequence (5'-AGAAAATTAATAAAGGTACTGCATC-3') of an M-like protein of GGS (Schnitzler *et al.*, 1995), and on a conserved area (5'-TGCCATAACAGCAAGGGC-3') in *emm* genes of GCS, GGS and GAS, located C-terminally of the cell wall anchor motif LPXTG. PCR was performed using 200 ng DNA, 25 pmol of each primer, 0.2 mM dNTP mix

(Amersham Pharmacia Biotech), 2 mM MgCl_2 , $1 \times$ PCR buffer, 5 U *Taq* polymerase (all from Sigma), and sterile H_2O to a final volume of 100 µl. Primers were from DNA Technologies, and all reactions were performed with an Eppendorf Mastercycler Personal. Amplification was initiated at 94 °C for 10 min, and terminated at 72 °C for 10 min. Thirty cycles of amplification were run, each cycle consisting of 94 °C for 1 min, 56 °C for 1.5 min, and 72 °C for 1.5 min. PCR products were analysed by agarose (1%) gel electrophoresis, and purified by using the High Pure PCR product purification kit (Roche). For sequencing of the *fog* gene, reactions were carried out by primer walking, using BigDye sequencer version 3.0 (Applied Biosystems). Each sequencing reaction consisted of 2 µl BigDye terminator, 40 ng PCR-product, 5 pmol primer, and sterile H_2O to a final volume of 10 µl. Sequencing PCR was performed in accordance with the manufacturer's protocol. Samples were precipitated with 29 µl 95% ethanol and 1 µl 3 M NaAc, pH 5.2, and subsequently centrifuged at 16 100 g for 30 min at room temperature. Samples were vacuum centrifuged, and the sequencing reactions were performed on an ABI 3100 at the BM-unit, Lund University, Sweden. New primers were based on the sequences obtained. Sequences were aligned using MacVector version 7.0, with a minimum overlap of 100 bp, and each base pair was sequenced at least twice with independently purified templates. Computational prediction of signal sequence cleavage was performed using the web-based program SignalP V1.1 (<http://www.cbs.dtu.dk/services/SignalP/>). Prediction of dimerization and coil formation was done using services available at <http://www.ch.embnet.org> and <http://multicoil.lcs.mit.edu/cgi-bin/multicoil>.

Fragments of protein FOG were cloned and expressed in *Escherichia coli* using the GST Gene Fusion System (Amersham Biosciences). The mature protein FOG (aa 1–557) was amplified by PCR using the 5' primer 5'-GCGGATCCGCGGAGAATACATACGATAGATGG-3', containing a *Bam*HI site, and the 3' reverse primer 5'-GCTGAAT-TCTTATTAACCTGTTGATGGTAAGTCTCTT-3', containing an *Eco*RI site. For the generation of fragment 1-B (aa 1–278), the 3' reverse primer 5'-GTTGAATTCCTTATTAAGCTGTTAGACTGTCAACAATGCC-3', containing an *Eco*RI site, and for fragment 1-C (aa 1–493), the 3' reverse primer 5'-GATGAATTCCTTATTAGTTAAGTTTTCAAGAGCAGCTAATTT-3', containing an *Eco*RI site, were used in combination with the 5' primer used for the mature protein FOG. After digestion with the indicated restriction enzymes (New England Biolabs), the fragments were cloned into the pGEX-6P-1 vector. Following standard ligation (Invitrogen), the plasmid was transformed into competent *E. coli* JM109 cells. Transformants were grown on Luria–Bertani agar containing 100 µg ampicillin ml^{-1} , and screened for the correct fragment insert. Plasmid DNA was then isolated, and transformed into competent *E. coli* BL21. Induction and procedures of purification followed the instructions of the manufacturer.

PCR amplification of the protein G gene was performed using the 5' primer 5'-TTGGTCGACTGATGATAGGAGATTATTG-3', containing a *Sall* restriction site, and the 3' reverse primer 5'-CGGGGATCCCATATTGAAAAGGCTCAATG-3', containing a *Bam*HI site. Template DNA was prepared by boiling G41 or G148, grown on THA overnight, in sterile water for 5 min. Following centrifugation at 10 000 g, the supernatant was recovered and used as a template.

Surface plasmon resonance spectroscopy and flow cytometry analyses. The association and dissociation rate constants for the interactions between protein FOG and fibrinogen, HSA or IgG were determined by surface plasmon resonance spectroscopy, using a Biacore-X system. Protein FOG, HSA and fibrinogen were immobilized on research-grade CM5 sensor chips in 10 mM sodium acetate at pH 3.5, using the amine coupling kit supplied by the manufacturer. Analyses were performed in PBST at 25 °C and at a flow rate of 10 µl min^{-1} . Analyte (35 µl) was applied in serial

dilutions starting at a concentration of 1 mg ml⁻¹. Surfaces were regenerated with 35 µl 0.1 M glycine/HCl, pH 2.0, at a flow rate of 10 µl min⁻¹. The kinetic data were analysed by the BIAEVALUATION 2.2 program (Biacore).

Complement deposition on the cell surface of G41 and G148 was analysed by measuring the amount of C3 deposition, as described by Kotarsky *et al.* (2001). For detection of fibrinogen on the neutrophil surface, purified neutrophils (1 × 10⁶) were incubated for 30 min at 37 °C with rabbit anti-human fibrinogen diluted 1:3000 (Dako) in MEM (Life Technologies), washed twice with PBS, and subsequently incubated for another 15 min in darkness at room temperature with Alexa-Fluor-488-conjugated Fab'2-fragments from goat (1:800) (Molecular Probes) directed towards rabbit IgG. Neutrophils were washed twice in PBS for 5 min, resuspended, and analysed by flow cytometry using a FACS-Calibur flow cytometer (Becton-Dickinson) equipped with a 15 mW argon laser tuned at 488 nm. The FL1 fluorescence channel (λ_{em} 530 nm) was used to record the emitted fluorescence of Alexa 488.

Heparin-binding protein (HBP) release and precipitation experiments. Varying amounts of protein FOG, or fragments thereof, were incubated for 30 min at 37 °C in 1 ml human whole blood diluted 1:10 in Dulbecco's PBS (Difco). For maximum release of HBP, 5% Triton X-100 was used, and for background level, one tube was put directly on ice, and one was included in the series incubated at 37 °C. Following incubation, samples were centrifuged at 10 400 g for 15 s, and the resulting supernatants were recovered. HBP was quantified by means of ELISA as described by Tapper *et al.* (2002). Precipitation of protein FOG in PBS containing 10% plasma or fibrinogen (300 µg ml⁻¹) was performed as described by Herwald *et al.* (2004). Fibrinogen-deficient human plasma was purchased from Enzyme Research Laboratories.

RESULTS

Survival of GGS correlates with bacterial aggregation and fibrinogen binding

Isolates of GGS are known to express protein G, a protein that interacts with IgGfC and albumin. To analyse the contribution of protein G to bacterial survival in human whole blood, a number of clinical isolates were tested for growth in a bactericidal assay. There was an interexperimental variation in multiplication rate, but the relative outcome of each experiment was clear. All strains tested, except G148, multiplied as shown in Table 1. To confirm the expression of protein G on the bacterial surface, binding experiments with human IgG were performed. These data demonstrated that all strains listed in Table 1 bound between 75 and 80% of added radiolabelled IgG. Treatment of G148 bacteria with papain, an enzyme known to release protein G from the bacterial surface (Björck & Kronvall, 1984), reduced the binding of IgG to background levels (2%). Thus, the results of the bactericidal assay indicated that protein G did not protect GGS from phagocytosis. During growth of GGS in liquid medium, we observed that most strains formed aggregates. When the sedimentation rate for the isolates in Table 1 was determined by measuring OD₆₂₀ of the cultures over time, all strains except G148 sedimented within 30 min (Fig. 1a, lower panel), demonstrating that protein G was not responsible for bacterial aggregation. Instead, we hypothesized

Table 1. Multiplication factors of ten GGS strains

Bacteria were incubated in whole blood at 37 °C, and samples were taken after 3 and 5 h. The multiplication factor represents the increase in c.f.u. at the time points of incubation. The experiment was performed four times. Data from a representative experiment are shown.

Incubation time (h)	Strain									
	G6	G11	G26	G36	G41	G42	G43	G46	G55	G148
3	7	43	14	81	6	6	42	15	44	0
5	463	754	4200	7356	106	537	1479	1668	149	0

that M- or M-like proteins expressed on the bacterial surface are involved in both aggregation and survival in human blood. M proteins are known to interact with fibrinogen (Kantor, 1965); therefore, we analysed the strains for binding of radiolabelled fibrinogen, and apart from the non-aggregating G148, all strains bound the radiolabelled probe (Fig. 1a).

The G41 and G148 strains were selected for further analysis of fibrinogen binding and aggregation. Electron microscopy analysis of the bacterial cultures demonstrated the presence of large aggregates of G41, while almost no aggregates could be seen in cultures of G148 (Fig. 1b, upper panels). Also, self-associating hair-like structures protruding from the surface were seen in G41, but not in G148 (Fig. 1b, lower panels). After treatment with CNBr, aggregation of G41 was abolished, as demonstrated by a sedimentation rate of the bacterial suspension that was similar to the sedimentation of G148 bacteria (Fig. 1c). Moreover, the fibrinogen-binding capacity of G41 was greatly reduced and both G41 and G148 no longer bound radiolabelled IgG (data not shown). Solubilized peptides were separated by SDS-PAGE, and, as shown in Fig. 1(d), Stain, material from G41 gave rise to several bands, whereas only one major band was obtained from G148. In ligand blotting, three bands from G41, with the apparent molecular mass values of 66, 64 and 32 kDa, showed affinity for radiolabelled fibrinogen, while the G148 band did not react with the probe (Fig. 1d, blot). This band represents a fragment of protein G, as demonstrated by using antibodies against protein G as a probe (not shown). The fibrinogen-binding bands were subjected to N-terminal amino acid sequencing. Identical sequences were obtained from the 64 and 32 kDa bands, suggesting that these represent fragments of the same protein (Fig. 1d). The N-terminal amino acid sequence of the 66 kDa fragment (AENTYDRWKAQT) was found to be identical in the first eight positions to a partial sequence of an M-like protein from a GGS strain (Schnitzler *et al.*, 1995), while no homology was found to the N-terminal sequence of the 64 kDa fragment (DELQKLKDFSKQ). We therefore decided to sequence this fibrinogen-binding protein of GGS, denoted protein FOG.

Fig. 1. Analysis of fibrinogen binding and aggregation. (a) The binding of 125 I-labelled fibrinogen to GGS was measured at a concentration of 2×10^9 bacteria ml^{-1} . Bars represent mean values $\pm$ SEM from three experiments. The GGS isolates were also analysed for aggregation. Bacteria grown at 37 °C overnight in TH were resuspended and left to settle at room temperature. The sedimentation rate was obtained by measuring the OD₆₂₀ in the upper half of the tubes: +, aggregation; –, no aggregation. (b) Electron micrographs showing G41 (left) and G148 (right) from overnight cultures in TH. Upper panels, scanning electron micrographs; bar, 10 μm . Lower panels, transmission electron micrographs; bar, 5 μm . (c) G41 and G148 were incubated with PBS or CNBr. After digestion, bacteria were washed and analysed for sedimentation: ◆, G41-PBS; ■, G41-CNBr; ▲, G148-PBS; ×, G148-CNBr. (d) CNBr-released material from G41 and G148 was subjected to SDS-PAGE analysis. One gel was stained with Coomassie blue (Stain) and one was blotted onto a PVDF membrane and probed with 125 I-labelled fibrinogen (Blot). Material corresponding to the three bands indicated in the G41 lane were subjected to N-terminal amino acid sequencing. Molecular markers (kDa) are shown to the left.

Sequencing of the gene encoding protein FOG

In order to sequence the *fog* gene, primers were constructed based on a region in the signal sequence of an M-like protein from GGS (Schnitzler *et al.*, 1995), and on a region in the homologous C-terminal membrane-spanning part of M proteins from GCS, GGS and GAS, which was found in database searches (Altschul *et al.*, 1990). Using these

primers, a PCR product of 1.8 kb was produced, with chromosomal DNA from G41 used as a template. This PCR product was then used as a template in sequencing reactions (see Methods). Analysis of the protein sequence deduced from the nucleotide sequence (GenBank/EMBL accession number AY600861) demonstrated that the N-terminal amino acid sequence obtained from the 66 kDa protein band (see Fig. 1d) was consistent with the N-terminal of the mature protein FOG. This was also in agreement with a putative cleavage of the signal sequence predicted by the web-based server SignalP V1.1. Protein FOG is structurally similar to other M-like proteins of GAS, GCS and GGS species, and it has a signal sequence typical of surface proteins from Gram-positive bacteria. Hence, we follow the structural nomenclature used by Åkesson *et al.* (1994). A schematic representation of protein FOG is shown in Fig. 2(a). The A domain contains two short repeated sequences (aa 84–88 and 108–112), and is followed by two identical B domains (B1, aa 133–172; B2, aa 193–232). The S region is 108 aa long, and is followed by three highly homologous repeats (C1–C3, aa 340–368, 375–406 and 424–448, respectively). C1 and C2 are 100 % identical on the amino acid level, and they are encoded by nucleotides that are identical to a level of 92 %. C3, being the shortest of the three repeats, shows 100 % identity on the amino acid level, and 93 % identity on the genetic level. The D domain shows sequence identity with M proteins of class I (Bessen & Fischetti, 1992), and it includes an LPXTG anchor motif. The size of the fibrinogen-binding fragments generated by CNBr cleavage corresponded to the positions of the methionine residues 38, 284 and 546 (Fig. 2a). Computer-aided analysis showed that protein FOG has a high probability of dimerization and α -helical coil formation. Amino acids 227–245, 336–355, 371–389 and 406–424 of protein FOG show similarity to a region of other M and M-like proteins described to be responsible for self-association (Frick *et al.*, 2000). Analogous to the M-like protein FAI of GCS (Talay *et al.*, 1996), there are unique segments in protein FOG, such as the B-repeats, showing no homology to M proteins or any other protein available in the database.

Binding properties of protein FOG

The mature protein FOG was recombinantly expressed in *E. coli* using the glutathione S-transferase (GST) fusion system (Fig. 2a). As mentioned above, all strains of GGS interact with IgGfC and albumin through protein G, but slot-binding experiments revealed that protein FOG also has affinity for these plasma proteins (Fig. 2b). As shown in Fig. 2(b), protein FOG has no affinity for human α_2 -M, a protein that interacts with protein G (Sjöbring *et al.*, 1989). DNA fragments corresponding to the A–C3 region (fragment 1-C) and the A–B region (fragment 1-B) of protein FOG were also expressed in fusion with GST to produce these peptides in *E. coli* (Fig. 2a). There was no difference in binding activity to fibrinogen, indicating that the N-terminal A–B part represents the binding region

Fig. 2. Binding of protein FOG to plasma proteins. (a) A schematic representation of protein FOG and its domains is shown. The three fibrinogen-binding fragments obtained with CNBr-digestion (see Fig. 1d) are depicted above the protein FOG molecule. The positions of the methionine residues are indicated with an M in the schematic figure of protein FOG, and the position of the LPXTG motif is shown. The recombinantly expressed fragments 1-C and 1-B of protein FOG are also shown. Numbers in the figure refer to amino acid residue positions, where 1 is the first residue of the mature molecule. (b) Different amounts of human fibrinogen (Fib), HSA, polyclonal human IgG and human α_2 -M were applied to PVDF membranes. The membranes were incubated with 125 I-labelled protein FOG, and fragments 1-C and 1-B. Bound ligand was detected using the Fuji Imaging System.

(Fig. 2a). Like the mature protein FOG, fragments 1-C and 1-B blocked the binding of radiolabelled fibrinogen to G41 bacteria, further emphasizing the A–B region on protein FOG as the fibrinogen-binding site (data not shown). The binding site for IgG was located to the C-terminal part of protein FOG, as fragment 1-B did not show any affinity for this molecule. Only the mature protein FOG bound serum albumin in the slot-binding assay (Fig. 2b), but surface plasmon resonance spectroscopy demonstrated that fragment 1-C also interacted with albumin, although the affinity was of a lower magnitude compared to the mature protein FOG (not shown). These results indicated that albumin binding resides in the C-terminal region of protein FOG, although it is possible that overlapping modules are needed for a full binding of HSA, as described for protein FAI of GCS (Talay *et al.*, 1996). The affinity constants for the interactions between protein FOG and albumin, fibrinogen and IgG were determined to 1.4×10^7 , 2.6×10^6 and 1.1×10^7 M $^{-1}$, respectively, by plasmon resonance spectroscopy. There was no binding of the GST tag to any of these ligands.

Survival of GGS in human blood is mediated by protein FOG

As shown above, the G41 strain survives and multiplies 100-fold in human whole blood, while the G148 strain, which lacks protein FOG, is killed (Table 1). In contrast, both strains multiply equally well in human plasma (not shown). To confirm the absence of the *fog* gene in G148 bacteria, PCR was performed with the primers used for the sequencing of *fog*, and chromosomal DNA from G148 as the template. No product was obtained with these primers or others used in primer walking the *fog* sequence, confirming that the G148 strain lacks the *fog* gene (data not

shown). A PCR product corresponding to the 1.8 kbp product was generated in another 30 clinical isolates of GGS, all of which bound fibrinogen (not shown).

To analyse the contribution of protein FOG to the survival of G41 bacteria in blood, the G148 strain was used in a series of experiments. Soluble protein FOG, and fragments 1-C or 1-B, at a concentration of 5 μ g ml $^{-1}$, were added to G148, and growth in human whole blood was monitored for a period of 5 h. The amount of protein FOG added corresponds well with the amount that is secreted into the growth medium of G41 bacteria (2.5–10 μ g ml $^{-1}$) in late-exponential growth phase. As shown in Table 2, the addition of FOG resulted in a 500-fold multiplication

Table 2. Salvage experiments

Multiplication factors of strains G148 and BMJ71 when salvaged by the addition of 5 μ g soluble protein FOG, fragments thereof, or M1. Experiments were carried out using whole blood. Representative data of at least three experiments using blood from three different donors are shown.

	Incubation time (h)		
	1.5	3	5
G148 + PBS	1	0	0
G148 + GST	0	0	0
G148 + FOG	11	224	564
G148 + FOG1-C	3	1	1
G148 + FOG1-B	1	0	0
BMJ71 + FOG	5	34	137
G148 + M1	12	39	183

of G148. FOG also mediated survival of BMJ71, an *mga* mutant of the AP1 strain of GAS (Kihlberg *et al.*, 1995) lacking M protein. The M1 protein of the wild-type strain AP1 was also found to protect G148 from being killed (Table 2). Addition of fragment 1-C gave an intermediate response, resulting in a maintained but not increased number of c.f.u. In contrast, the addition of fragment 1-B, the GST-tag alone or the buffer control failed to restore growth (Table 2). Neither protein PAB, an albumin-binding surface protein of *F. magna* (de Château & Björk, 1994) nor protein G could protect G148 bacteria from phagocytosis (data not shown).

Next, we tested the ability of G41 to support growth of the G148 strain in human blood. Cocultivation of the two strains resulted in an increase in multiplication factor of 1.5 as compared to G41 alone. The number of FOG molecules on the surface of the G41 bacterium has been estimated to be 17 000 by using Scatchard analysis (Kronvall *et al.*, 1970). Thus, about 25 000 c.f.u. would equal 5 µg soluble FOG. In order to identify growing G148 bacteria in such experiments, heat-inactivated G41 bacteria were used. There is no difference in binding activity to viable or heat-inactivated G41 regarding any of the known ligands. Cocultivation for 5 h resulted in a 160-fold multiplication of G148. To ensure that the colonies represented G148, binding studies with fibrinogen and IgG were performed. This was further confirmed with PCR analyses, since the protein G gene differs in size between GGS strains (Sjöbring *et al.*, 1991). Taken together, these results suggest that protein FOG mediates survival and multiplication of GGS in whole blood.

Protein FOG promotes survival of GGS through aggregation of neutrophils

Survival of GAS in human blood has been attributed to M protein binding of fibrinogen, as well as proteins regulating complement activity (Horstmann *et al.*, 1988; Perez-Casal *et al.*, 1995; Ringdahl *et al.*, 2000; Thern *et al.*, 1995; Whitnack & Beachey, 1982). The ability of protein FOG to interact with factor H was tested in the slot-binding assay. As shown in Fig. 3(a), radiolabelled factor H bound to protein FOG, suggesting that this interaction might be of some benefit for FOG-expressing bacteria. The binding was retained in fragment 1-C, but not fragment 1-B. However, protein G was also found to interact with factor H (Fig. 3b), and no difference in complement deposition on the surfaces of G41 and G148 by the alternative pathway was detected in flow cytometry analyses (not shown).

Despite having fibrinogen-binding capacity, fragment 1-B, corresponding to the N-terminus of protein FOG, failed to protect G148 from being killed (Table 2). Recently it was demonstrated that M protein of GAS forms complexes with fibrinogen in human plasma that cause release of HBP from human neutrophils, thereby inducing vascular leakage (Herwald *et al.*, 2004). This information raised the question of whether formation of such complexes could explain the

Fig. 3. Analysis of factor H binding. Various amounts of (a) protein FOG, and fragments 1-C and 1-B of protein FOG, and (b) CNBr-digested material from G148 (CNBr G148), and recombinantly expressed protein G containing both the albumin- and IgGfC-binding regions (Rec. prot G), were applied to PVDF membranes and probed with ¹²⁵I-labelled factor H. Bound ligand was detected using the Fuji Imaging System.

capacity of FOG-expressing bacteria to survive in human blood. We therefore investigated the ability of the different FOG fragments to precipitate fibrinogen. Table 3 shows that protein FOG, at concentrations equal to those used in the bactericidal assay, formed precipitates in human plasma and in a fibrinogen solution. Precipitates were also formed in plasma with fragments 1-C and 1-B, although the degree of precipitation was of a lower magnitude compared to that seen with the full-length protein (Table 3). In contrast, even at high concentrations of the fragments, the degree of fibrinogen precipitation in buffer was close to background levels (Table 3). The GST tag alone caused no precipitation of fibrinogen (not shown). To emphasize the importance of fibrinogen binding, a recombinant FOG fragment lacking the N-terminal B-repeats would have been useful as a negative control. However, we did not succeed in making

Table 3. FOG-induced precipitation of fibrinogen

FOG-mediated precipitation in 10% plasma (top 3 rows), in fibrinogen solution (300 µg ml⁻¹) (middle 3 rows), and in 10% plasma that was fibrinogen deficient (bottom row). Values (±SEM) represent the percentage of total radioactivity of the respective fragments (*) that were recovered in the precipitate.

	Amount of respective fragment added (pmol):			
	0	10	30	100
FOG*	6.7 ± 1.5	17 ± 10.3	30.2 ± 15.1	46.1 ± 9.7
FOG1-C*	7.8 ± 1.3	10.0 ± 2.7	18.9 ± 2.8	27.0 ± 6.2
FOG1-B*	13.3 ± 5.0	12.9 ± 3.6	22.6 ± 4.6	26.5 ± 6.2
FOG*	26 ± 3.7	29 ± 1.2	44 ± 5.0	44 ± 6.9
FOG1-C*	19 ± 1.5	23 ± 2.8	23 ± 4.0	26 ± 4.6
FOG1-B*	22 ± 5.6	24 ± 4.1	26 ± 6.0	26 ± 3.5
FOG*	4.5 ± 0.9	4.1 ± 0.5	3.1 ± 1.1	3.9 ± 0.9

such a construct, which was traced to an undefined property of the construct itself. Instead, when protein FOG was incubated with fibrinogen-deficient plasma, no precipitation occurred (Table 3), a result that excludes formation of complexes between protein FOG and other plasma proteins. Furthermore, when added to human whole blood, FOG triggered a release of HBP that was 40% of the total HBP content released by lysing neutrophils with Triton X-100. Fragments 1-C and 1-B, at equimolar amounts (30 pmol), or even at a molar excess compared to the intact molecule, failed to cause any release of HBP (data not shown), as compared to background levels obtained from unstimulated cells. These results suggest that the intact protein FOG molecule is required for optimal formation of complexes with fibrinogen.

When added to heparinized whole blood, protein FOG and fragment 1-C, but not fragment 1-B and GST, gave rise to macroscopically visible aggregates on the walls of the test tubes after 1 h. Such aggregates were also seen with G41. When G148 was added to depleted blood, i.e. blood from which precipitates were removed, it multiplied to a similar rate as when it was added to blood preincubated with protein FOG (Fig. 4a). In contrast, when aggregates formed with protein FOG were added to fresh blood, together with G148, bacterial killing was restored, suggesting that the addition of fresh unaffected neutrophils abolished the effect of FOG (Fig. 4a). The partial killing seen with the addition of purified neutrophils (1×10^6) to depleted blood most likely depends on a lower amount of neutrophils as compared to the addition of whole blood. Light microscopy analysis demonstrated that the aggregates contained large clusters of neutrophils (Fig. 4b). The presence of fibrinogen in the clusters was confirmed by fluorescence microscopy (Fig. 4c).

Next, purified neutrophils were incubated with protein FOG, or with fragment 1-C or 1-B, for 30 min at 37 °C, followed by electron microscopy analyses. An extensive clumping of the neutrophils was seen with protein FOG, and although less pronounced, fragment 1-C also caused clumping. The appearance of neutrophils incubated with fragment 1-B was similar to the PBS control (Fig. 5). The surface of purified neutrophils was coated with fibrinogen, as determined by the use of antibodies in flow cytometry analyses, suggesting that the clumping caused with protein FOG was a result of fibrinogen binding (not shown). In summary, for efficient aggregation of neutrophils and full protection of bacterial killing, an intact protein FOG is required, although the binding of fibrinogen resides in the N-terminal part of the molecule.

DISCUSSION

Like GAS, in the absence of opsonic antibodies, isolates of GGS readily survive and multiply in human whole blood. However, in contrast to GAS, this important characteristic has not been extensively studied for GGS. The best-characterized surface protein of GGS is protein G (Björck

Fig. 4. Protein FOG inactivates neutrophils in whole blood. (a) Protein FOG (5 µg) was incubated with 1 ml heparinized human whole blood for 1 h at 37 °C. Visible aggregates were formed and the multiplication of G148 was determined as described in Methods. ▲, Blood incubated with protein FOG for 1 h prior to addition of G148; ●, G148 added to blood depleted by FOG, i.e. precipitates were removed; ○, depleted blood to which purified neutrophils and G148 were added; ■, fresh blood was added together with G148 to aggregates preformed with protein FOG. (b) The aggregates formed by protein FOG in whole blood were carefully washed, resuspended in PBS, and applied onto glass slides by cytospin. Neutrophils were stained with haematoxylin and eosin, and analysed by light microscopy. Bar, 10 µm. (c) The presence of fibrinogen in the aggregates (left) was demonstrated by fluorescence microscopy (right) using rabbit anti-human fibrinogen, followed by Alexa-Fluor-488-conjugated goat anti-rabbit Fab'2-fragments. Bar, 10 µm.

& Kronvall, 1984). Protein G has affinity for IgGFc, and the observation that the Fc-binding regions on protein G have evolved convergently suggests that the interaction has functional significance (Frick *et al.*, 1992). For example,

Fig. 5. Protein FOG mediates clumping of neutrophils via fibrinogen present on neutrophils. Purified neutrophils were pre-incubated with 5 µg FOG (b), fragments 1-C (c) and 1-B (d), respectively for 30 min at 37 °C in RPMI. Control cells (a) were treated with PBS. Cells were subsequently applied onto a poly-L-lysine-coated coverslip, and analysed by scanning electron microscopy. Bar, 25 µm.

when the surface of GGS is covered with IgG it may be able to evade immune recognition by the host. However, despite expressing protein G, the G148 strain, which lacks protein FOG, was rapidly killed in human blood (Table 1), excluding non-immune binding of IgG or an interaction with α_2 -M as a protective mechanism for GGS. Neither does albumin binding to GGS contribute to survival, further accentuated through experiments in which the albumin-binding protein PAB was unable to rescue G148 from phagocytosis. Instead, we find that in the presence of soluble protein FOG, G148 is able to survive and multiply. To our knowledge, this is the first example where addition of a soluble M-like protein exerts an antiphagocytic effect in whole blood, thereby rescuing a bacterial strain that is normally killed in this environment.

Protein FOG is a self-associating surface protein of GGS. Self-association among GAS has been ascribed to M proteins (Frick *et al.*, 2000); however, other bacterial species, such as *Staphylococcus aureus*, *Mycobacterium tuberculosis* and *Bordetella pertussis*, also show similar self-associating characteristics (McDevitt *et al.*, 1994; Menozzi *et al.*, 1994, 1996). Bacterial aggregation may be of benefit for the bacteria, since larger particles are less at risk of being engulfed by host phagocytes. Also, aggregation may mediate a firmer attachment to host cells, facilitating invasion, and supporting a sustained infection. Protein FOG contains several regions that show high similarity to the AHP sequence involved in self-association of GAS M proteins (Frick *et al.*, 2000). These regions are located in the middle and C-terminal parts of protein FOG, which might help to explain the antiphagocytic effect of the intact FOG molecule.

A multitude of studies have demonstrated that M protein interactions with regulating proteins of complement, as well as with fibrinogen, play important roles in the survival of GAS in human blood. Factor H was shown early on to bind to the C-repeated region of M proteins, suggesting that an activation of the alternative pathway of complement would thus be inhibited (Horstmann *et al.*, 1988). Protein FOG interacts with factor H in a similar mode, but the fact that protein G also has affinity for factor H, and still has no protective effect, further excludes factor H-binding as an important mechanism for GGS survival in blood. This is in agreement with Kotarsky *et al.* (2001), who demonstrated that resistance to phagocytosis of GAS was independent of factor H.

Fibrinogen binding to M-protein-expressing GAS has for some strains been suggested to modulate bacteria–phagocyte interactions, resulting in inefficient killing of bacteria (Ringdahl *et al.*, 2000; Whitnack & Beachey, 1982). The mechanism is unclear, but the finding that proteins M1 and M5 both contribute to streptococcal survival, although they interact with different regions of fibrinogen (Ringdahl *et al.*, 2000), underlines the importance of fibrinogen binding. Neutrophils interact with human fibrinogen through the CD18 family of integrins, where the N-terminal part of the α chain of fibrinogen binds to CD11c/CD18 (Loike *et al.*, 1991). This has consequences such as cell spreading, respiratory burst and degranulation, which all are relevant in the bacteria–host interplay. Furthermore, the C-terminal part of the γ chain has been reported to bind to CD11b/CD18 (Wright *et al.*, 1988). In addition, both receptors mediate attachment of unopsonized bacteria to neutrophils (Ross *et al.*, 1992). Thus, it is conceivable that, by binding fibrinogen, M and M-like proteins, such as protein FOG, could interfere with receptor attachment to activated C3 and C4 on the bacterial surface. Recently, it was also demonstrated that M protein–fibrinogen complexes aggregate and activate neutrophils by cross-linking of the CD18 integrins (Herwald *et al.*, 2004). Activation resulted in release of HBP and ‘frustrated phagocytes’, which, as in our case, seem to be unable to function.

Although protein FOG is structurally related to GAS M proteins, differences in terms of function appear. The binding site for fibrinogen on protein FOG is located in the N-terminal region, but, surprisingly, only the mature molecule exerted a protective effect on killing of non-protein FOG-expressing bacteria (Table 2). Moreover, to achieve an efficient precipitation of fibrinogen and neutrophil clumping, a FOG fragment equivalent to that presented on the bacterial surface is needed. This is in contrast to the GAS M1 protein, in which a fibrinogen-binding N-terminal fragment still forms active complexes with fibrinogen (Herwald *et al.*, 2004). The affinity constant for the interaction between M1 and fibrinogen is 2.5×10^8 (Ringdahl *et al.*, 2000), which is about 100-fold more than the affinity between protein FOG and fibrinogen. Thus, it

is tempting to speculate that, due to lower affinity, the complex formation between protein FOG and fibrinogen depends on a conformationally stable α -helical dimer. In this context, it is notable that the C-terminal parts of M proteins, which are well conserved within members of this family, were found to adopt a more stable folded structure than the N-terminal regions (Nilson *et al.*, 1995). This was later found to be the case also with the fibrinogen-binding protein (FgBp) of *Streptococcus equi* subsp. *equi*, in which the C-terminal part contributes to thermal stability of the molecule (Meehan *et al.*, 2002). The same group also showed that neither A nor B repeats are important for binding of FgBp to fibrinogen (Meehan *et al.*, 2000). These domains, however, seem important for conformation and multimerization. The results of Meehan and co-workers support our findings that emphasize the difference between having the ability to bind fibrinogen (fragment 1-B) and actually reaching the effect of the binding, i.e. inhibition of neutrophil function. Our results clearly show that an intact protein FOG molecule is a structural prerequisite for the functions investigated in this study, and from which the bacterium may benefit.

Although GGS most often cause skin or mucosal infections, and are less frequently found in blood, neutrophils will be recruited to the site of infection. A possible cross-linking of the CD18 integrins, triggered by protein FOG, would result in HBP release and exhausted neutrophils. This could contribute to streptococcal survival, and, in addition, the HBP release will cause vascular leakage, providing the bacteria with nutrients and a route of bacterial dissemination. During cultivation of FOG-expressing strains, the protein is found in the growth medium at late-exponential growth phase. Most likely, protein FOG is also released from the bacterial surface *in vivo*; thereby an inhibition of neutrophil function could take place at sites distant from the bacterial surface. In GAS, M proteins are cleaved from the bacterial surface by the bacterial cysteine protease SpeB (Berge & Björck, 1995). Such enzymic activity has not yet been reported for GGS, but the possibility of enzymic surface protein release cannot be ruled out. Streptococcal resistance to neutrophilic killing is a complex mechanism. Here we demonstrate that an M-like molecule of GGS, protein FOG, triggers aggregation of neutrophils in human whole blood, at least in part through an interaction with fibrinogen. However, other properties of protein FOG might affect the function of neutrophils in later stages of encounter. Studies to further understand the molecular interactions between M-like proteins and phagocytic cells are of great interest and importance.

ACKNOWLEDGEMENTS

We are indebted to Dr Heiko Herwald for helpful suggestions, and to Ulla Johannesson for technical assistance. This work was supported by the Swedish Research Council (project 7480), the Foundations of Crafoord, Bergvall and Österlund, the Royal Physiographic Society and Hansa Medical AB.

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