Streptococcal Inhibitor of Complement Inhibits Two Additional Components of the Mucosal Innate Immune System: Secretory Leukocyte Proteinase Inhibitor and Lysozyme

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Streptococcal inhibitor of complement (SIC) is a 31-kDa extracellular protein of a few, very virulent, strains of *Streptococcus pyogenes* (particularly M1 strains). It is secreted in large quantities (about 5 mg/liter) and inhibits complement lysis by blocking the membrane insertion site on C5b67. We describe investigations into the interaction of SIC with three further major components of the innate immune system found in airway surface liquid, namely, secretory leukocyte proteinase inhibitor (SLPI), lysozyme, and lactoferrin. Enzyme-linked immunosorbent assays showed that SIC binds to SLPI and to both human and hen egg lysozyme (HEL) but not to lactoferrin. Studies using ¹²⁵I-labeled proteins showed that SIC binds approximately two molecules of SLPI and four molecules of lysozyme. SLPI binding shows little temperature dependence and a small positive enthalpy, suggesting that the binding is largely hydrophobic. By contrast, lysozyme binding shows strong temperature dependence and a substantial negative enthalpy, suggesting that the binding is largely ionic. Lysozyme is precipitated from solution by SIC. Further studies examined the ability of SIC to block the biological activities of SLPI and lysozyme. An M1 strain of group A streptococci was killed by SLPI, and the antibacterial activity of this protein was inhibited by SIC. SIC did not inhibit the antiproteinase activity of SLPI, implying that there is specific inhibition of the antibacterial domain. The antibacterial and enzymatic activities of lysozyme were also inhibited by SIC. SIC is the first biological inhibitor of the antibacterial action of SLPI to be described and may prove to be an important tool for investigating this activity in vivo. Inhibition of the antibacterial actions of SLPI and lysozyme would be advantageous to *S. pyogenes* in establishing colonization on mucosal surfaces, and we propose that this is the principal function of SIC.

In the last decade there has been a global marked increase in the number of severe infections caused by *Streptococcus pyogenes* (group A streptococci [GAS]) (9). Many of these infections are associated with certain particularly virulent strains, such as M1 strains, which account for a large proportion of the cases of invasive infections and streptococcal toxic shock syndrome. However, the majority of GAS infections are at superficial sites, giving rise to pharyngitis or skin infections.

In 1996 Åkesson and coworkers (1) identified a novel protein secreted by an M1 strain of GAS, which they termed streptococcal inhibitor of complement (SIC) because of its ability to inhibit complement lysis. SIC is a 31-kDa extracellular protein produced by a few very virulent strains of GAS, and a number of observations support the proposition that it is an important virulence factor. For example, the *sic* gene is extremely polymorphic and to date nearly 300 variants have been found (17). A high level of polymorphism is usually a characteristic of genes encoding exported molecules which interact with the host environment (22). This extreme variability is unprecedented among bacterial genes studied so far and implies that the gene is under strong selective pressure, probably to evade the host antibody response, since SIC has also been shown to be highly immunogenic (16). In addition, a recent

study by Lukomski and coworkers (20), who used an M1 isogenic mutant strain in which the *sic* gene was inactivated, showed that mice inoculated intranasally with the wild-type parental strain had a significantly higher incidence of throat colonization within 4 days postinfection than those inoculated with the *sic* knockout mutant strain. The authors concluded that SIC promotes the early stages of infection in some as-yet-uncharacterized way.

The original study of Åkesson and coworkers (1) indicated that purified SIC could block complement lysis in vitro assays. We have subsequently shown that SIC inhibits lysis by preventing the uptake of C5b67 complexes onto cell membranes, presumably by blocking the membrane insertion site transiently expressed on C7 (11). However, it is unlikely that inhibition of the complement membrane attack complex (MAC) is really the primary role of SIC in vivo, since GAS are already resistant to lysis by the MAC as their membranes are protected by a thick cell wall and, during exponential growth, a capsule as well. Therefore, we decided to look for interactions between SIC and other components of the innate host defense mechanisms, particularly those found in the throat in airway surface liquid (ASL).

ASL contains a number of antimicrobial agents, produced largely by the submucosal glands and epithelial cells, which are the first line of defense against bacteria. These include lysozyme, lactoferrin, and secretory leukocyte proteinase inhibitor (SLPI), which are the most abundant antimicrobial agents and form the subjects of our study, together with elafin, secre-

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tory phospholipase A2, anionic peptides, cathelicidin LL-37, and defensins (28). Lysozyme acts by cleaving the glycosidic bonds of *N*-acetylmuramic acid, damaging the bacterial cell wall, and ultimately killing the organism by lysis (18). Lactoferrin is primarily bacteriostatic by chelating iron and making it inaccessible to invading organisms, but a direct antibacterial action which is thought to be restricted to a domain near the N terminus and distinct from the iron binding domain has been reported (4). The antimicrobial mode of action of SLPI is as yet unknown (15). Antimicrobial factors found in ASL also occur in other mucosal secretions such as tears, saliva, urine, and breast milk, where mucosal surfaces are constantly exposed to low levels of bacteria.

Although GAS have been reported not to be directly susceptible to the effects of either lysozyme or lactoferrin (2, 12), a recent electron microscopy study found that lactoferrin was bound to the surfaces of clinical isolates of *S. pyogenes* (26). The potential action of SLPI against GAS has not hitherto been investigated. SLPI is a dual-function molecule comprising two similar domains, one of which contains the enzyme inhibitory activity (10) and the other of which contains the antimicrobial activity (15). We speculated that SIC might enhance the infectivity of M1 GAS by interfering with the activities of some of these innate defense proteins and here report the results of our studies on the interactions between SIC and lysozyme, SLPI, and lactoferrin.

MATERIALS AND METHODS

Bacterial strains. *S. pyogenes* type M1 strain NCTC 8198 (ATCC 12344) was obtained from the National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom. The sequence of its *sic* gene is identical to that originally published (GenBank accession no. X92968) (1, 11). The bacteria were grown in Todd-Hewitt broth–0.2% yeast extract (THBY) (Oxoid Ltd., Basingstoke, Hants., United Kingdom) and maintained short-term on selective Columbia agar–5% horse blood agar plates (HBA) (containing oxolinic acid at 5 µg/ml and colistin sulfate at 10 µg/ml; Sigma-Aldrich Co. Ltd., Gillingham, United Kingdom). To select for maximum expression of M protein and other virulence factors, the bacteria were passaged three times in fresh human plasma as described previously (3). *Streptococcus suis* type 2 strain P117, originally isolated from a pig with meningitis, was kindly provided by Andrew Allen, Bacterial Infection Group, Centre for Veterinary Science, University of Cambridge. The bacteria were grown in Todd-Hewitt broth and maintained on selective HBA plates as described above.

Purification of SIC. SIC was purified from 1-liter overnight-culture supernatants of strain M1 GAS, to which 10 mM EDTA had been added, as follows. A SIC-containing fraction was precipitated from the culture supernatant with 30% saturated solution of ammonium sulfate at room temperature for 1 h, the pH was adjusted to 5.5 with HCl, and the precipitate was spun down at $22,000 \times g$ for 30 min at room temperature. The SIC precipitate was recovered from the sides of the tube by dissolving it in 50 mM phosphate–10 mM EDTA (pH 7) (PE), and this solution was made by adding solid ammonium sulfate to a final concentration of 0.4 M and applied to a 20-ml butyl Sepharose hydrophobic interaction chromatography (HIC) column (Amersham Pharmacia Biotech, Little Chalfont, Bucks., United Kingdom) equilibrated in the same buffer. The column was washed with PE–0.4 M ammonium sulfate, and the SIC was eluted with a 7-column-volume decreasing ammonium sulfate gradient from 0.4 down to 0 M in PE. SIC eluted as a clean prominent peak in the latter part of the gradient. We found that concentrating the fractions by ultrafiltration caused substantial loss of protein. Therefore, as a means of concentrating, the SIC was reprecipitated with 50% ammonium sulfate at room temperature and, to avoid any loss of protein, precipitation was carried out in the centrifuge tubes, because a significant proportion of the SIC precipitate sticks to the wall of any vessel used. The precipitate was spun down as before, redissolved in a minimal volume of phosphate-buffered saline (PBS), and dialyzed into PBS. Aliquots were stored at -70°C . Typically, 5 mg of SIC can be purified from 1 liter of culture supernatant by this procedure.

ELISA. Enzyme-linked immunosorbent assay (ELISA) plates (Microtiter; Greiner Bio-One, Stroud, Glos., United Kingdom) were coated with either hen egg lysozyme (HEL), human lysozyme, lactoferrin from human milk (Sigma Aldrich Co. Ltd.), or recombinant human SLPI (rhSLPI) (R & D Systems Ltd., Abingdon, Oxford, United Kingdom) at 2 µg/ml in triplicate in 50 mM carbonate-bicarbonate buffer, pH 9.6 (or with coating buffer only as a control), and the plate was blocked with PBS–1% gelatin (VWR International Ltd., Lutterworth, United Kingdom). SIC at 10 µg/ml was added, and bound protein was detected with an in-house rabbit anti-SIC (immunoglobulin [Ig] fraction) (11) at 2 µg/ml followed by alkaline phosphatase-conjugated goat anti-rabbit Ig (Sigma Aldrich Co. Ltd.) diluted 1/1,500. All dilutions were in PBS–0.1% gelatin–0.05% Tween 20.

In other ELISAs, plates were coated with SIC at 1 µg/ml as described above and blocked with PBS–1% bovine serum albumin (BSA) or PBS–2.5% dried skim milk. Human lysozyme or lactoferrin was added at 5 µg/ml or HEL was added at 10 µg/ml, and bound protein was detected with sheep anti-human lysozyme (IgG fraction; CN Biosciences UK Ltd., Nottingham, United Kingdom) at 35 µg of total protein per ml, rabbit anti-human lactoferrin (Ig fraction; DAKO Ltd., Ely, United Kingdom) at 2.5 µg/ml, or rabbit anti-HEL (Ig fraction; Abcam Ltd., Cambridge, United Kingdom) at 5 µg/ml and then with alkaline phosphatase-conjugated monoclonal anti-sheep or -goat Ig (Sigma Aldrich Co. Ltd.) diluted 1/1,500 or alkaline phosphatase-conjugated anti-rabbit Ig. All dilutions for the human lysozyme plate were in PBS–0.1% BSA–0.05% Tween 20, and those for the lactoferrin or HEL plate were in PBS–0.5% dried skim milk–0.05% Tween 20. In a similar ELISA, rhSLPI at 5 µg/ml was added to the SIC-coated plate and detected with goat anti-rhSLPI, the IgG fraction, at 1 µg/ml (R & D Systems Ltd.) and then with alkaline phosphatase-conjugated monoclonal anti-goat Ig as described above, with blocking and dilutions being in PBS–0.1% gelatin–0.05% Tween 20.

In all ELISAs, coating was at 4°C overnight, subsequent incubations were for 1 h at 37°C , and the final reagent was the Sigma Fast pNPP substrate-buffer system (Sigma Aldrich Co. Ltd.). One hundred-microliter volumes of all reagents were used throughout, except in the blocking step, for which reagents were at 200 µl. Absorbance was read at 405 nm (reference wavelength, 490 nm) in a model 3550 microplate reader (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts., United Kingdom). All ELISAs were performed at least three times with triplicate wells.

Iodination of proteins. Five hundred micrograms of HEL, 100 µg of rhSLPI, and 100 µg of lactoferrin were radiolabeled by the iodogen method (14) with 1 mCi of ^{125}I each (Amersham Pharmacia Biotech). Free iodine was removed by gel filtration on a PD10 column (Amersham Pharmacia Biotech). Specific activities of the labeled proteins were on the order of 10^6 cpm/µg.

Interactions of SIC with SLPI, lysozyme, and lactoferrin binding studies. Double dilutions of rhSLPI or lactoferrin from 200 µg/ml (17.5 and 2.6 µM, respectively) at pH 7.3 in PBS–10 mM sodium azide–1 mg of BSA per ml, containing iodinated ligand to approximately 1.3×10^4 cpm/µg, were added to SIC at 200 µg/ml (6.45 µM) and incubated overnight at 4, 22, or 37°C . Serial dilutions of HEL from 500 µg/ml (35 µM) containing the same proportion of labeled protein were combined with SIC at 100 µg/ml (3.22 µM) and incubated as described above. In all experiments complexes were precipitated with 1.5 M ammonium sulfate and centrifuged. The pellets and supernatants were counted in an LKB Wallac (Turku, Finland) 1277 Gamma Master automatic gamma counter. Results were analyzed by fitting data to the hyperbolic binding curve using nonlinear regression in order to obtain the K_d and number of ligand molecules bound. To make sure that we used only points where equilibrium had been reached, we made a preliminary plot of percentages of ligand bound versus amounts of ligand offered. All points where the percentage bound fell as ligand concentration decreased were rejected. Thermodynamic properties of the reactions were derived from the dissociation constants obtained at the three temperatures. Data were processed using the SPSS 10 statistical data analysis program (SPSS Inc., Chicago, Ill.). All binding experiments were performed at least three times. Due to the high cost of rhSLPI, the SLPI experiments were performed in duplicate, while the HEL and lactoferrin experiments were performed in triplicate.

Other binding studies. To investigate whether any breakdown of either protein occurred during incubation of SIC with lysozyme or SLPI, 5 µg of SIC was incubated overnight at 4°C with 5 µg of human lysozyme, HEL, or SLPI in 20-µl volumes. Samples (5 µg) of the individual proteins incubated alone served as controls. Reducing Laemmli loading buffer (20 µl) was added, and the samples were boiled for 3 min, subjected to sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS–12.5% PAGE), and stained with Coomassie blue.

Antibacterial assays. (i) Titration of the effect of rhSLPI on M1 GAS. The effect of rhSLPI on the viability of the M1 (SIC-positive) strain of GAS was investigated by a method adapted from the work of Wiedow et al. (30). Overnight cultures of M1 GAS were grown at 37°C in THBY and used to inoculate fresh broth at a 1/100 dilution. Bacteria were grown to mid-log phase, spun down, washed twice in 10 mM sodium phosphate buffer (pH 7.2), and resuspended to a concentration of 2×10^5 /ml in phosphate buffer–1% THBY–2 mg of BSA/ml. Aliquots (15 µl) of bacteria were added to equal volumes of double dilutions of rhSLPI from 40 µM in phosphate buffer–THBY (or phosphate buffer–THBY only), in duplicate, giving final concentrations of rhSLPI from 20 µM, with BSA at 1 mg/ml and bacteria at 10^5 /ml, and incubated for 2 h at 37°C. Bacteria were then plated, and percent survival was calculated as described below. This experiment was performed twice in duplicate under the above conditions exactly and on more than six other occasions under a variety of slightly different conditions, all of which gave the same result.

(ii) Determination of the effect of SIC and rhSLPI on M1 GAS. M1 GAS were grown and prepared as described above and resuspended to a concentration of 2.5×10^5 /ml in phosphate buffer–1% THBY. Doubling dilutions of SIC from 20 µM were preincubated with 20 µM rhSLPI (both in phosphate buffer–1% THBY as described above) in a total volume of 15 µl (in duplicate) for 2 h at 37°C. Twelve microliters of M1 GAS at 2.5×10^5 /ml was added together with BSA to 1 mg/ml and incubated for a further 2 h at 37°C. Final overall concentrations were from 10 µM for SIC, 10 µM for rhSLPI, and 10^5 /ml for bacteria. Controls were bacteria in phosphate buffer–1% THBY, with dilutions of SIC from 10 µM or with 10 µM rhSLPI alone and with all suspensions containing BSA at 1 mg/ml. This experiment was performed twice in duplicate under the above conditions exactly and on more than six other occasions under a variety of slightly different conditions, all of which gave the same result.

(iii) Determination of the effect of HEL and SIC on *S. suis*. Overnight cultures of *S. suis* were grown at 37°C in THB, prepared as described above, and resuspended to a concentration of 2.5×10^9 /ml in phosphate buffer–1% THB. Doubling dilutions of SIC from 17.5 µM were combined with 17.5 µM HEL (both in phosphate buffer–THB) in a total volume of 15 µl and incubated overnight at 4°C. Twelve microliters of *S. suis* at 2.5×10^9 /ml was added together with BSA to 1 mg/ml, and the mixture was incubated for a further 15 min at 37°C. Final overall concentrations were from 8.5 µM for SIC, 8.5 µM for HEL, and 10^9 /ml for bacteria. Controls were bacteria in phosphate buffer–1% THB, dilutions of SIC from 8.5 µM, or 8.5 µM HEL alone, all of which contained BSA at 1 mg/ml.

In all antibacterial assays, 10-fold dilutions of the reaction mixtures were made in 100-µl volumes of phosphate buffer–THBY or –THB, plated on selective HBA plates, and incubated overnight at 37°C. The numbers of colonies were counted, and the results were plotted as percentages of bacteria that survived relative to the number of bacteria incubated in buffer only that survived $\pm$ the standard errors of the means (SEM). This experiment was performed twice in duplicate under the above conditions exactly and on more than four other occasions under a variety of slightly different conditions, all of which gave similar results.

HNE inhibition assays. (i) Titration of HNE activity and its inhibition by rhSLPI. Double dilutions of human neutrophil elastase (HNE) (CN Biosciences UK Ltd.) from 500 nM were made up in 0.1 M Tris–0.5 M NaCl, pH 7.5 (assay buffer). Eighteen microliters of each dilution was added to 100 µl of substrate solution [0.2 mM *N*-(methoxysuccinyl)-Ala-Ala-Pro-Val 4-nitroanilide; Sigma-Aldrich Co. Ltd.] in a flat-bottomed microtiter plate (Greiner Bio-One Ltd.) and incubated at room temperature for up to 1 h, and successive readings of optical density at 405 nm (OD_{405}) were taken. The optimum concentration of HNE for use in this system was 250 nM (7.37 µg/ml). Therefore, 10 µl of HNE at 14.75 µg/ml (500 nM) was added to 10-µl aliquots of double dilutions of rhSLPI from 11.7 µg/ml (1 µM), both of which were in Tris–NaCl assay buffer (experiment performed in duplicate), and incubated at 37°C for 15 min (final concentrations of 250 nM HNE and SLPI from 500 nM). Elastase activity was then measured as described above. A concentration of 500 nM SLPI was required to give complete inhibition of substrate digestion.

(ii) Effect of SIC on the inhibition of HNE activity by rhSLPI. Twenty microliters of SIC at 2.2 mg/ml (71 µM) was incubated with an equal volume of SLPI at 17.55 µg/ml (1.5 µM) or 20 µl of SLPI was incubated with an equal volume of HNE at 22.2 µg/ml (750 nM) for 15 min at 37°C. Twenty microliters of HNE or assay buffer was then added to the SIC and SLPI or 20 µl of SIC was added to the SLPI and HNE and incubated for a further 15 min at 37°C. Controls were mixtures of SLPI, HNE, and BSA at 2.5 mg/ml; SIC and HNE; HNE and BSA; SIC alone; SLPI and BSA; BSA alone; and buffer alone. All reaction mixtures were made up in duplicate. BSA was included in all the reaction mixtures not containing SIC as we found that merely having extra protein in the system greatly enhanced the function of HNE, and the substitution of BSA avoided this problem. Triplicate 18-µl aliquots of the duplicate reaction mixtures were then trans-

ferred to a microtiter plate, 100 µl of substrate solution was added, and the OD_{405} was monitored. This experiment was performed four times in triplicate.

In a similar experiment, 6-µl aliquots of SIC at 350 µg/ml (11.3 µM) were combined with equal volumes of SLPI at 133 µg/ml (11.3 µM) or 6 µl of SLPI was combined with an equal volume of HNE at 3.33 µg/ml (0.113 µM) and incubated for 30 min at 37°C. Six microliters of HNE or buffer was then added to the SIC and SLPI mixture or 6 µl of SIC was added to the SLPI and HNE mixture, and the mixtures were incubated for a further 15 min at 37°C. Controls were a mixture of SLPI and HNE, a mixture of SIC and HNE, and SIC, SLPI, or HNE alone. Reducing Laemmli loading buffer (18 µl) was added, and the samples were boiled for 3 min, run on an SDS–12.5% PAGE gel, and stained with Coomassie blue.

Effect of SIC on the catalytic activity of lysozyme. Sequential dilutions of 3 parts SIC (from 0.5 mg/ml, 16.1 µM) and 1 part diluent were combined with HEL or human lysozyme at a constant 0.25 mg/ml (17.5 or 14.7 µM, respectively) in 15-µl volumes (both in 50 mM sodium phosphate buffer, pH 7) in duplicate and incubated overnight at 4°C. Sequential dilutions of HEL or human lysozyme alone from 0.5 mg/ml (3 parts lysozyme to 1 part diluent) were also made up and incubated as described above to provide a standard curve. Five-microliter portions of each set of reaction mixtures together with a set of standard-curve dilutions were added to 3-mm-diameter wells of duplicate agarose lysoplates containing *Micrococcus lysodeikticus* cell walls (catalog no. M3770; Sigma-Aldrich Co. Ltd.) at 0.5 mg/ml, according to the method described previously (7). The plates were incubated at room temperature for approximately 4 h, and the diameters of the zones of bacterial cell wall clearance were measured with a magnifying graticule with divisions of 0.1 mm. To confirm that SIC itself did not digest *M. lysodeikticus* cell walls, a comparable series of dilutions of SIC were investigated and no digestion was observed. The experiment with HEL was performed numerous times in duplicate, and that with human lysozyme was performed three times in duplicate. In another experiment to investigate the effects of temperature on the system, dilutions of SIC from 16.1 µM (3 parts SIC to 1 part diluent) were combined with 17.5 µM HEL, incubated overnight at 4 or 37°C, as were standard-curve dilutions, and assayed as described above. This experiment was performed three times in duplicate.

Effect of SLPI on the interaction between SIC and lysozyme. In order to ascertain whether there was any competition between SLPI and lysozyme for binding sites on SIC, double dilutions of SLPI from 28.5 µM were combined with 5 µM SIC and 10 µM HEL in 50 mM sodium phosphate buffer (in duplicate) in 15-µl volumes and incubated overnight at 4°C. Controls were 10 µM HEL, 5 µM SIC, 10 µM HEL plus 5 µM SIC, and buffer only, together with dilutions of HEL for a standard curve as described above. Samples (5 µl) of each reaction mix were added to duplicate lysoplates as described above, and zones of clearance were measured. This experiment was performed four times in duplicate. In order to confirm that SLPI itself did not digest *M. lysodeikticus* cell walls, a comparable series of dilutions of SLPI were investigated and no digestion was observed.

In all the above-described lysoplate assays, results were expressed as percentages of lysozyme activity remaining relative to the level of activity of the control (lysozyme alone).

Precipitation of HEL by SIC. Initial inhibition studies showed that when SIC and HEL were combined, a visible precipitate formed almost instantaneously. In order to investigate this further, double dilutions of SIC from 2 mg/ml (64 µM) were combined with HEL at 0.5 mg/ml (35 µM) in 100-µl volumes (in duplicate) and incubated overnight at 4°C. The precipitate was spun down and assayed by the method of Lowry et al. (19). Standard curves were constructed for SIC and HEL separately, and the mean of these curves was plotted to approximately represent a pellet comprising a mixture of the two proteins. This experiment was performed three times in duplicate.

Affinity purification of SIC on a lysozyme column. HEL was coupled to cyanogen bromide-activated Sepharose CL4B (14) at 8 mg/ml, and an 8-ml column was equilibrated in 50 mM Tris–10 mM EDTA, pH 7.5 (TE). One liter of M1 GAS overnight-culture supernatant was precipitated with 30% saturated ammonium sulfate at room temperature, adjusted to pH 5.5, resuspended in 30 ml of TE, dialyzed, and loaded onto the column at 4°C. The column was washed with TE, and the SIC eluted with a 20-column-volume steep sodium chloride gradient from 0 to 2 M.

RESULTS

SIC binds to rhSLPI, human lysozyme, and HEL but not to lactoferrin as determined by ELISA. SIC bound strongly to plates coated with rhSLPI (Fig. 1a). Similarly, rhSLPI bound strongly to plates coated with SIC (data not shown). SIC also

FIG. 1. Binding of SIC to secretory leukocyte proteinase inhibitor, HEL, and human lysozyme by ELISA. In each panel, bar 1 is coating protein plus ligand plus first and second antibodies; bar 2 is coating protein plus first and second antibodies; bar 3 is coating protein plus second antibody; and bar 4 is coating protein plus ligand plus second antibody. Results are expressed as net ODs after deduction of background values from matching uncoated wells $\pm$ SEM. Where no error bars are shown, the SEM is too small to be seen. (a) Coating protein, secretory leukocyte proteinase inhibitor; ligand, SIC; (b) coating protein, HEL; ligand, SIC; (c) coating protein, SIC; ligand, human lysozyme.

bound to plates coated with HEL (Fig. 1b) but only weakly to plates coated with human lysozyme (data not shown). However, human lysozyme bound to plates coated with SIC (Fig. 1c). No binding was observed between SIC and lactoferrin in either ELISA orientation.

Under physiological conditions SIC binds to rhSLPI at higher affinity than to HEL and not at all to lactoferrin. Equilibrium binding studies were performed using a hyperbolic-curve fit in order to characterize further the interactions of SIC with SLPI and HEL, and the thermodynamic properties were calculated. The results are shown in Tables 1 and 2. No binding was observed between SIC and lactoferrin. At 37°C the affinity of SLPI is substantially higher than that of lysozyme, with K_d s of 19.7 and 85.4, respectively, while at 4°C they are much the same. This is because the SIC-SLPI interaction shows little temperature dependence while the SIC-lysozyme interaction is highly temperature dependent. Approximately two molecules of SLPI bind to SIC, while approximately four molecules of lysozyme bind to SIC. The SLPI-SIC interaction shows a small positive enthalpy of 6.4 kJ/mol, while the lysozyme-SIC interaction shows a negative enthalpy of -34.6 kJ/mol. Similarly, the SLPI-SIC interaction shows a positive entropy of 0.111

kJ/mol/K while the lysozyme-SIC interaction shows a negative entropy of -0.035 kJ/mol/K.

SLPI kills the M1 strain of GAS and is inhibited by SIC. In order to investigate whether the M1 strain of GAS was susceptible to killing by SLPI, doubling dilutions of rhSLPI from 20 μ M were incubated with the bacteria at 10^5 /ml. The results show that rhSLPI kills M1 GAS at concentrations similar to those published for other organisms and that virtually complete killing of the bacteria occurred at about 20 μ M rhSLPI (Fig. 2a).

In order to investigate the effects of SIC upon the antibacterial properties of rhSLPI, double dilutions of SIC from 10 μ M were combined with 10 μ M rhSLPI and then incubated with M1 GAS at 10^5 /ml. SIC totally inhibited the killing of M1 GAS by 10 μ M rhSLPI at an optimum concentration of 5 μ M SIC, i.e., at a SIC/SLPI molar ratio of 1:2 (Fig. 2b). BSA was added throughout, as initial experiments showed that the addition of SIC alone caused enhanced growth compared to that of bacteria in phosphate buffer alone. This was shown to be an effect of protein concentration in the reaction mixture, the same effect being produced by the addition of comparable amounts of BSA, human serum albumin, or gelatin. The addition of BSA at 1 mg/ml throughout eliminated the problem.

SIC has no effect on HNE inhibition by rhSLPI. As the two functionally distinct domains of SLPI are structurally very similar, the potential effect of SIC upon elastase inhibition by

TABLE 1. Binding characteristics of SIC with SLPI and HEL^a

Protein	Temp (°C)	Mean $K_d \pm$ SEM	Mean no. of molecules bound $\pm$ SEM
SLPI	4	26.1 ± 4.1	1.6 ± 0.1
	22	19.3 ± 3.4	1.6 ± 0.1
	37	19.7 ± 3	1.9 ± 0.3
HEL	4	18.9 ± 3	4.2 ± 0.2
	22	74.4 ± 18.5	3.3 ± 0.4
	37	85.4 ± 2.5	3.4 ± 0.3

^a Values are means of results of three assays performed in duplicate (SLPI) or triplicate (HEL).

TABLE 2. Physical chemical data for interactions of SIC with SLPI and HEL^a

Protein	Enthalpy (ΔH) (kJ/mol)	Entropy (ΔS) (kJ/mol/K)	Free energy (ΔG) (kJ/mol)
SLPI	6.4 ± 2.3	0.111 ± 0.008	-26.4 ± 0.4
HEL	-34.6 ± 3	-0.035 ± 0.009	-24.2 ± 0.3

^a Values are calculated from data in Table 1 and are means $\pm$ SEM.

FIG. 2. SLPI kills the M1 strain of GAS, and killing is inhibited by SIC. (a) Doubling dilutions of SLPI from 20 μM were combined with bacteria at $10^5/\text{ml}$ together with BSA at 1 mg/ml and incubated for 2 h at 37°C. Tenfold dilutions of the bacteria were plated. Results are from two experiments performed in duplicate. (b) Doubling dilutions of SIC from 40 μM were incubated with 40 μM SLPI for 2 h at 37°C. Bacteria at $2.5 \times 10^5/\text{ml}$ were added together with BSA to 1 mg/ml, incubated for a further 2 h at 37°C, and processed as described above. Final concentrations were $10^5/\text{ml}$ for bacteria, from 10 μM for SIC, and 10 μM for SLPI. Controls were bacteria in 10 μM SLPI, buffer, or dilutions of SIC. Results are from a typical experiment performed in duplicate. In both panels, results are expressed as percentages of survival relative to the survival of bacteria incubated in buffer only $\pm$ SEM.

rhSLPI was also investigated. Results showed that although SIC inhibited the antibacterial properties of SLPI as described above, it had no effect on the inhibition of elastase by SLPI. Figure 3a shows that preincubation of SLPI with SIC followed by the addition of HNE, or preincubation of SLPI with HNE followed by the addition of SIC, had no effect on the ability of SLPI to inhibit HNE activity. The second experiment, in which the reaction mixtures were analyzed by SDS-PAGE, essentially

made the same point but also demonstrated that HNE digests SIC in the absence of SLPI; no digestion of SIC by HNE was observed in the presence of SLPI, irrespective of the order in which the reagents were combined (Fig. 3b). These experiments also showed that there is no competition for binding sites on SLPI between SIC and HNE.

SIC inhibits killing of *S. suis* by HEL. In order to investigate the effects of SIC upon lysozyme function, SIC at different concentrations was preincubated with HEL and added to *S. suis* at $10^9/\text{ml}$. SIC partially inhibited killing of *S. suis* by HEL at an optimum SIC/HEL molar ratio of between 1:2 and 1:3 (Fig. 4). Even at the optimum molar ratio, only about 10% of the bacteria survived.

SIC inhibits the catalytic activity of lysozyme. Investigation of the catalytic activity of human lysozyme and HEL in the presence of SIC measured by digestion of bacterial cell walls showed that the maximum inhibition of HEL activity occurred at a SIC/HEL molar ratio of about 1:3 after overnight incubation at 4°C. With less SIC, the amount of inhibition diminished very sharply. The highest level of inhibition obtained was approximately 75%. Addition of more SIC does not increase the level of inhibition. A similar inhibition curve was obtained with human lysozyme, where the maximum inhibition of activity also occurred at a SIC/lysozyme molar ratio of approximately 1:3. The highest level of inhibition seen was never more than about 50% (Fig. 5a). However, after overnight incubation at 37°C, no inhibition of HEL activity was observed (Fig. 5b).

SLPI competes with lysozyme for binding sites on SIC. In order to investigate whether there was competition between SLPI and HEL for binding to SIC, dilutions of SLPI were added to a mixture of SIC and HEL and the level of inhibition of HEL activity was assayed on lysoplates. SLPI at the highest concentration used completely negated the inhibition of HEL by SIC and restored HEL activity to 100%. SLPI competed with HEL for binding to SIC in a dose-dependent manner. No digestion was caused by SLPI alone. The results are shown in Fig. 6.

SIC does not break down SLPI or lysozyme. Incubation of SIC with SLPI, human lysozyme, or HEL does not result in the breakdown of any of the proteins. Coomassie blue-stained SDS-PAGE gels of the three protein combinations, and of individual proteins postincubation, showed no breakdown products (Fig. 7). It should be noted that SIC runs with an apparent molecular mass of around 45 kDa, although its actual molecular mass is 31 kDa, and that the slightly lower-molecular-mass band in the SIC tracks is a spontaneous breakdown product in which 33 amino acids have been lost from the N terminus (11).

SIC precipitates HEL at 4°C. SIC precipitates HEL from solution at 4°C at an optimum HEL/SIC molar ratio of between 2.2 and 4.4:1, at which ratios greater than 80% of the total protein in the system is precipitated. The results are shown in Table 3.

Affinity purification of SIC on a lysozyme column. SIC protein from bacterial culture supernatant bound to HEL linked to a column of Sepharose CL4B and eluted at approximately 0.7 M sodium chloride in a single peak, as shown by SDS-PAGE. A typical elution profile is shown in Fig. 8. The eluted SIC was of a purity similar to that eluted from the HIC column as described in Materials and Methods. This HEL-Sepharose

FIG. 3. SIC has no effect on HNE inhibition by SLPI. (a) Twenty-microliter aliquots of 71 μ M SIC were incubated with equal volumes of 1.5 μ M SLPI or 20 μ l of SLPI was incubated with an equal volume of 750 nM HNE for 15 min at 37°C. Twenty microliters of HNE or assay buffer was then added to the SIC and SLPI, and 20 μ l of SIC was added to the SLPI and HNE; both mixtures were incubated for a further 15 min at 37°C. Triplicate 18- μ l aliquots of duplicate reaction mixtures were transferred to a microtiter plate, 100 μ l of the substrate solution was added, and the OD₄₀₅ was monitored. Bar 1, SIC plus SLPI; bar 2, SIC plus SLPI and then HNE; bar 3, SLPI plus HNE and then SIC; bar 4, SLPI plus HNE; bar 5, SIC plus HNE; bar 6, HNE plus BSA; bar 7, SIC; bar 8, SLPI plus BSA; bar 9, BSA; bar 10, buffer. Results are the means of results of three experiments performed in duplicate $\pm$ SEM. Error bars are too small to be seen. (b) Six-microliter aliquots of SIC and SLPI or of SLPI and HNE were incubated together for 30 min at 37°C. Six microliters of HNE or buffer was then added to the SIC and SLPI or 6 μ l of SIC was added to the SLPI and HNE and incubated a further 15 min, as were controls. The samples were then run on an SDS-12.5% PAGE gel and stained with Coomassie blue. Molecular mass markers appear at both sides. Lane 1, SIC plus SLPI; lane 2, SIC plus SLPI and then HNE; lane 3, SLPI plus HNE and then SIC; lane 4, SLPI plus HNE; lane 5, SIC plus HNE; lane 6, SIC; lane 7, SLPI; lane 8, HNE (the quantity of HNE loaded was too small to be shown by Coomassie blue staining).

affinity procedure is now used as a final purification step after SIC is separated on the HIC column. SIC-containing fractions are collected from the HIC column, pooled, and dialyzed into TE as described above, run on an 8-ml lysozyme-Sepharose column, and eluted with a shallow 15-column-volume sodium

FIG. 4. Inhibition of lysozyme killing of *S. suis* by SIC. Double dilutions of SIC from 17 μ M were incubated with 17 μ M HEL overnight at 4°C. Equal volumes of *S. suis* at 2.5×10^9 bacteria/ml were added together with BSA to 1 mg/ml and incubated for 15 min at 37°C. Final concentrations were 10^9 /ml for bacteria, from 8.5 μ M for SIC, and 8.5 μ M for HEL. Controls were bacteria in 8.5 μ M HEL, dilutions of SIC, or buffer. Tenfold dilutions of the bacteria were plated. Results are expressed as percentages of surviving bacteria relative to the survival of bacteria incubated in buffer only $\pm$ SEM.

chloride gradient from 0 to 1 M. SIC then elutes at around 0.3 M sodium chloride. SIC-containing fractions are then pooled and precipitated as described above.

DISCUSSION

As shown by ELISA and binding studies, SIC binds strongly to rhSLPI, less strongly to human lysozyme and HEL, and not at all to lactoferrin. As determined by hyperbolic-curve fit, the interaction of SIC with SLPI at 37°C is of higher affinity than that of SIC with HEL, which is consistent with the results of ELISAs and the functional inhibition studies. Again, no binding was observed between SIC and lactoferrin. The positive enthalpy (endothermic reaction) and higher entropy imply that the interaction between SIC and SLPI is primarily hydrophobic, and the negative enthalpy (exothermic reaction) and lower entropy imply that the interaction between SIC and lysozyme is primarily ionic. These implications are confirmed by the facts that the K_d for HEL-SIC varies considerably with temperature but that the K_d for SLPI-SIC is largely independent of temperature. The number of SLPI molecules bound per mole of SIC is 1.6 to 1.9, probably therefore really 2, while the number of HEL molecules bound is 3.3 to 4.2 and could be either 3 or 4, both of which figures are in agreement with the results of functional and precipitation studies. The relatively weak binding of lysozyme and SIC in ELISAs can be explained by the variation in K_d with temperature, as the ELISAs are normally performed at 37°C, when the K_d is actually at its highest. The relatively strong interaction between SIC and HEL at 4°C forms a convenient method for purifying SIC on a HEL affinity column.

SIC does not appear to have any enzymatic activity against

FIG. 5. Inhibition of the catalytic activities of human lysozyme and HEL by SIC. (a) Three in four dilutions of SIC from 16.1 μM were combined with 14.7 μM human lysozyme or 17.5 μM HEL and incubated overnight at 4°C. (b) Three in four dilutions of SIC from 16.1 μM were combined with 17.5 μM HEL and incubated overnight at 4 or 37°C. Residual lysozyme activity was assayed by the lysoplate method. Data are from three experiments performed in duplicate and are expressed as percentages of lysozyme activity remaining relative to the activity of the control (lysozyme alone) $\pm$ SEM.

SLPI or lysozyme, as incubation of SIC with these proteins does not cause any breakdown, so the inhibition of activity is presumably due to binding and blocking of the antimicrobial site of SLPI and the antimicrobial or catalytic site of lysozyme.

We have shown that recombinant SLPI kills the M1 strain of GAS at a concentration comparable to that shown for other microorganisms (15) and that SIC completely inhibits the antibacterial function of rhSLPI at a SIC/SLPI molar ratio of around 1:2. SIC has no effect on the ability of rhSLPI to inhibit HNE. Similar experiments performed using trypsin gave identical results (data not shown). This implies that the interaction is either specifically with the antimicrobial domain of SLPI or

FIG. 6. Competition between SLPI and HEL for binding to SIC. Double dilutions of SLPI from 28.5 μM were incubated with 10 μM HEL and 5 μM SIC. HEL activity released (and hence the degree of competition by SLPI for binding to SIC) was assayed by the lysoplate method. Results are the results of four experiments performed in duplicate and are expressed as percentages of lysozyme activity relative to the level of activity of the control (10 μM lysozyme alone) $\pm$ SEM.

that any interaction of SIC with the enzyme inhibitory domain does not inhibit its function.

SIC inhibited the ability of both human lysozyme and HEL to digest *M. lysodeikticus* cell walls, with 75% inhibition being achieved with HEL and 50% inhibition being achieved with human lysozyme after incubation together at 4°C overnight, in both cases with an optimal SIC/lysozyme molar ratio of 1:3. However, no inhibition of HEL activity occurred after incubation with SIC at 37°C. SIC inhibited the bactericidal activity of HEL against *S. suis*, but at best only about 10% of the bacteria survived. *S. suis* was chosen for these experiments because it is so exquisitely sensitive to lysozyme that the amount of HEL that is freely in solution at any one time is enough to kill nearly 90% of the bacteria, even at $10^9/\text{ml}$, and after only a 15-min incubation at 37°C. This is in marked contrast to the results for

FIG. 7. SIC does not break down SLPI, human lysozyme, or HEL. Five micrograms of SIC was incubated overnight at 4°C with 5 μg of human lysozyme, HEL, or SLPI or 5 μg of the individual proteins alone as controls. The samples were run on an SDS-12.5% PAGE gel and stained with Coomassie blue. Molecular mass markers are at both sides. Tracks: 1, SIC; 2, SIC plus SLPI; 3, SLPI; 4, SIC plus human lysozyme; 5, human lysozyme; 6, SIC plus HEL; 7, HEL; 8, SIC.

TABLE 3. Precipitation of HEL by SIC at 4°C

Sample	µg of SIC offered/ 50 µg of HEL	HEL/ SIC molar ratio	Amt of protein in pellet (µg) ^a	% of total amt of protein precipitated ^a
1	200	0.5:1	78.6 ± 4.8	31.4 ± 1.9
2	100	1:1	83.9 ± 6	55.9 ± 4
3	50	2.2:1	84 ± 5.7	84 ± 5.7
4	25	4.4:1	64.6 ± 4.8	86.1 ± 6.4
5	12.5	8.8:1	35.6 ± 2.8	57 ± 4.5
6	6.3	17.5:1	15.1 ± 1.3	27 ± 2.3

^a Data are the means of results of three experiments performed in duplicate ± SEM.

M1 GAS and SIC-SLPI, with which 100% protection is afforded by the addition of SIC, presumably because the binding between SLPI and SIC is much stronger at 37°C and GAS are less sensitive to SLPI than *S. suis* is to HEL. These observations are in agreement with the physicochemical data above.

SLPI neutralized the inhibition of HEL activity by SIC in a dose-dependent manner, indicating that there is competition for binding sites on SIC. This result implies that the binding sites are either identical or near each other. In view of the different binding mechanisms of SLPI and HEL for SIC, it is unlikely that the binding sites are the same even though they are in the same region and that the interference is caused by steric hindrance.

The most common site of infection by GAS is the respiratory tract, and ASL contains a number of antimicrobial substances, the major components being lysozyme, lactoferrin, and SLPI. The interaction with lysozyme, though interesting, does not

explain in itself the function of SIC since GAS cell walls are largely resistant to direct breakdown by lysozyme (12). However, inhibition of lysozyme activity in vivo may still be advantageous to the bacteria for a number of reasons. It has recently been shown in vitro with purified components that some of the antimicrobial substances in ASL act synergistically in killing a variety of bacteria; examples are combinations of lysozyme and lactoferrin, lysozyme and SLPI, and lactoferrin and SLPI (25). The triple combination of lysozyme-lactoferrin-SLPI showed even greater synergy. Supporting evidence for synergy in vivo was shown in another study (7) which showed that the antimicrobial activity of boiled nasal secretions could not be restored by the addition of lysozyme or lactoferrin alone, suggesting that the interaction of multiple factors was required.

Since SLPI and lysozyme kill many other organisms found on mucous surfaces, the protection afforded by SIC may also facilitate increased growth of other bacteria when GAS are present. A recent study showed a high incidence of mixed infection with a variety of aerobes and anaerobes, including β-lactamase-producing organisms, on tonsils and adenoids removed from children with a history of recurrent group A beta-hemolytic streptococcal adenotonsillitis and with an average of nine different isolates per specimen (6). It would, of course, be particularly advantageous to GAS to enhance the survival of β-lactamase-producing organisms as they would afford some protection against penicillin treatment. In an earlier study using a mouse model, mutual enhancement of growth was observed between several combinations of different bacteria, including GAS (5), implying a synergistic role for factors yet to be determined.

Secretory leukocyte proteinase inhibitor is a low-molecular-mass nonglycosylated protein (11.7 kDa) originally identified as a serine proteinase inhibitor. It has a boomerang-shaped secondary structure composed of two highly homologous domains (13, 15), with both domains containing four cysteines arranged in a pattern characteristic of a group of proteins known as the four-disulfide core family of proteins (23). The carboxy-terminal domain contains the antiprotease active site (10), and the amino-terminal domain contains the antibacterial activity (15). The antimicrobial mode of action is still unknown. A protein of similar structure and function, eNAP-2, is produced by equine neutrophils (8), and the smaller elastase inhibitor, elafin (or SKALP), which has 42% sequence homology with SLPI, has also recently been shown to have antibacterial properties against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (24). SLPI has also been reported to have antifungal properties (27) and is known to be a potent inhibitor of human immunodeficiency virus type 1 (21). The mode of action for this property is thought to be by binding to, and blocking, a host cell-associated receptor molecule on macrophages. A recent study (29) using a *Saccharomyces cerevisiae* two-hybrid screen of a peripheral blood leukocyte cDNA library showed that the SLPI-binding protein is the human scramblase protein, which is an integral membrane protein involved in the movement of membrane phospholipids and which is present in a wide range of cells and tissues (31).

It appears probable that the ability to inhibit SLPI's antibacterial activity and, to a lesser extent, that of lysozyme is the property of SIC that accounts for its activity as a virulence factor, both by the direct protection of GAS themselves and

FIG. 8. Affinity purification of SIC on a lysozyme-Sepharose column. HEL was coupled to cyanogen bromide-activated Sepharose CL4B to make an 8-ml affinity column and equilibrated with TE (pH 7.5). A 30% ammonium sulfate precipitate from a 1-liter M1 GAS overnight-culture supernatant (resuspended and dialyzed into TE) was applied to the column and eluted in a 20-column-volume sodium chloride gradient from 0 to 2 M. SIC eluted at around 0.3 M sodium chloride.

indirectly by the protection of other bacteria present, especially those producing β -lactamase. The interactions of SIC with SLPI and lysozyme are good targets for developing antimicrobial therapy directed against GAS, particularly on mucous surfaces. SIC is also the first inhibitor of the antibacterial action of SLPI to be described and may prove to be an important tool for investigating this activity in vivo. Investigations of the interactions of SIC with other mucosal antimicrobial peptides are in progress.

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