Some strains of *Streptococcus pyogenes* secrete a virulence factor called the streptococcal inhibitor of complement (SIC) function. SIC is a multifunctional protein that interacts with a number of host proteins and peptides, especially with those that are involved in host defense systems. In addition to inhibiting the complement-mediated lysis of cells, SIC inhibits lysozyme, secretory leukocyte protease inhibitor, and β-defensins. SIC also binds to proteins associated with the cytoskeleton and thereby may cause cytoskeletal derangement. The SIC molecule has three distinct structural domains constituting the N-proximal short repeat region (SRR), the central long repeat region (LRR), and the C-proximal proline-rich region (PRR). To map various functions to the structural domains, we have analyzed recombinant subclones expressing various parts of SIC and elastase-generated discrete fragments of SIC for binding to various ligands and for determining their biological properties. The results demonstrate the following. (a) SRR alone was sufficient to confer inhibition of complement function. (b) Anti-defensin and anti-lysozyme activities were mapped to the SRR plus LRR. (c) The LRR plus PRR harbored ezrin binding activity.

Some strains of *Streptococcus pyogenes* secrete a major protein, called “streptococcal inhibitor of complement” (SIC), which was originally identified as an inhibitor of the membrane attack complex (MAC) (1). Inhibition of MAC function is achieved by preventing the insertion of C567 into the cell membrane (2). The distribution of the gene for SIC is highly restricted among *S. pyogenes* serotypes, based on a classification of a major surface antigen called the M protein. Only M1 and M57 strains have been shown to contain the sic gene (1, 3), although in a recent population-based study Ma et al. (4) reported occurrences of the gene in several other M types.

SIC, although originally characterized as an inhibitor of complement function, has subsequently been shown to have other activities. It inhibits the antimicrobial activity of lysozyme, secretory leukocyte protease inhibitor (SLPI), α- and β-defensins, and LL-37, which are all components of the innate immune system (5–7). Additionally, SIC has been shown to bind ezrin and, thereby, may modulate functions of cytoskeleton proteins (8), as it has been shown to inhibit streptococcal attachment to host cells in vitro. SIC also binds to various blood glycoproteins such as clusterin (1). The presence of the sic gene in all *S. pyogenes* M1 isolates (generally considered as highly virulent), the high diversity of the sic gene (9), and the recovery of new SIC variants within an epidemic wave (10) suggest an important role for SIC in *S. pyogenes* virulence. SIC is highly immunogenic in humans, and the immune response occurs during natural infection (11, 12). It is thought that the high variability of SIC may be a response to the need for the molecule to mutate and escape immune pressure. Interestingly, these mutations occur throughout the length of the molecule; that is, they are not confined to any one domain, suggesting that mutations in the sic gene are not directed to overcome immune responses to any specific functional domain. The multitude of interactions between SIC and host proteins and the effects of SIC on a diverse range of host defense systems have hindered the definition of its principal role in virulence. One study in mice (13) reported that the knock-out of the sic gene from an M1 strain resulted in more rapid clearance of the pathogen after intranasal inoculation than with the wild type parent strain, which suggests that a SIC-positive strain is likely to be more pathogenic.

The sequence of SIC revealed three distinct regions (1, 3, 14) (Fig. 1) as follows: (a) an N-proximal, tryptophan-rich, short repeat region (SRR) called the DWS repeat region; (b) the central long repeat region (LRR); and (c) a C-proximal proline-rich region (PRR). The role of these regions in the diverse biochemical/biological functions outlined above is unknown, although some preliminary binding data using proteolytic fragments of the SIC protein show that only a fragment comprising most of the SRR plus part of the LRR binds to the β-defensins and SLPI (7, 15).

In this communication, we report on the construction of subclones that express these distinct regions of SIC and on the testing of their properties. Our results clearly define binding sites within these poly-functional protein. Implications of these properties in relation to pathogenesis are discussed.

**EXPERIMENTAL PROCEDURES**

*Bacterial Strains, DNA, Peptides, and Antibodies—* *S. pyogenes* 2031, an M1 serotype, was used to obtain clones of the sic gene and portions corresponding to one or more of structural domains of the protein. Streptococcus was grown in Todd Hewitt broth (Oxoid) supplemented with neopeptone. DNA manipulations and sequencing were as described previously (2, 6).

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FIG. 1. Diagrammatic representation of subclones and fragments used in this study. Panel A shows relative positions of primers used (numbered arrows) and the domains (SRR, LRR, and PRR). The primer sequences (based on sic gene sequence) (1) are as follows: primer 1, 5′-c-tactaggtacacacaa-3′; primer 2, 5′-actaggtag7tacaa-3′; primer 3, 5′-gtaggaaagtataaaacg-3′; primer 4, 5′-gggggtcatttcttctcatc-3′; primer 5, 5′-taactaggtacacacaa-3′; primer 6, 5′-cagtacaaatitatacag-3′. Panel B shows the full-length SIC protein (305 residues) (1) from which the fragments were derived. The first 32 residues of this sequence comprise the signal sequence. The fragments A, B, and C have 1–33, 34–126, and 127–273 residues, respectively, in the mature protein.

12344) was obtained from the National Collection of Type Cultures, Central Public Health Laboratory, London. This strain was grown in Todd Hewitt broth and 0.2% yeast extract (Oxoid Ltd., Basingstoke, Hants, UK) and maintained in the short term on selective Columbia agar plates with 5% horse blood (containing 5 μg/ml oxolinic acid and 10 μg/ml colistin sulfote; Sigma-Aldrich). The sequence of the sic gene in this strain is identical to that originally published from strain AP1 (GenBank™ accession number X92968) (1, 2).

A peptide corresponding to the last 35 amino acids of ezrin was synthesized (Mimotopes). This portion of the molecule has been shown to bind to SIC (8). Recombinant human β-defensin 3 (hBD-3) was obtained from PeproTech EC Ltd., London, UK.

Antibodies to SIC were raised in rabbits by immunizing with recombinant M1 SIC from strain 2031 (IMVS, Gilles Plains, Australia) or with M1 SIC (National Collection of Type Cultures number 8198) purified from bacterial culture supernatants as described (2). Anti-ezrin antibody was obtained from Santa Cruz Biotechnology and was raised against a recombinant protein corresponding to carboxy-terminal amino acid residues 511–586.

Cloning, Expression, and Purification of Recombinant SIC and SIC Subclones—The sic gene from 2031 was amplified by polymerase chain reaction using the primers shown in Fig. 1. The relative positions of these primers are also indicated. The polymerase chain reaction conditions, the cloning into the pBAD Thio vector, and the purification of the thioredoxin fusion proteins containing a C-terminal His tag were described previously (14). Control clones expressing only His-tagged thioredoxin were also obtained. DNA sequences for all subclones confirmed their identity. The majority of the fusion proteins are intact as seen on the Coomassie Blue-stained gel (Fig. 2). The anti-recombinant SIC antibody did not react with thioredoxin.

Purification of SIC and Proteolytic Subfragments—SIC was purified from 3-liter overnight culture supernatants of M1 group A streptococcus as described previously (5). Subfragments of SIC, obtained by digestion with elastase, were purified as described by Fernie-King et al. (15). These fragments were as follows: fragment A, amino acids 1–33 (part of the short repeat region); fragment B, amino acids 34–126 (the remainder of the short repeat region plus long repeats 1 and 2); and fragment C, amino acids 127–273 (long repeat 3 plus the proline-rich region). All residue numbers are from the start of the mature protein (Fig. 1).

Enzyme-linked Immunosorbent Assay (ELISA) for the Binding of SIC to the Complement Proteins C6 and C7—Assays for binding of SIC or its fragments to the complement proteins C6 and C7 were performed as described previously by indirect ELISA (16). Briefly, 96-well plates (Titertek) were coated with 100 μl of 20 μg/ml recombinant SIC proteins in phosphate-buffered saline (PBS) at 4°C overnight. After blocking with 5% skimmed milk in PBS and 0.05% Tween 20, purified C6 or C7 (1 μg/ml final; Sigma) was added and incubated for 1 h at 37°C in a final volume of 100 μl. The wells were then washed three times with PBS and 0.05% Tween 20. Binding was detected with primary goat anti-C6 or anti-C7 immunoglobulin (1:1000; ICN Biomedical) and hors eradish peroxidase-conjugated rabbit anti-goat secondary (1:1000; Sigma). The reaction was developed with o-phenylenediamine dihydrochloride (Sigma), and the absorbance read at 450 nm in a Bio-Rad benchmark microplate reader.

ELISA for the Binding of SIC to Ezrin—Assays for SIC-ezrin binding were performed by indirect ELISA as described for complement binding assays. In this case the microwell wells were coated with 5 μg of SIC or subclones per well and then reacted with 1 μg/well ezrin peptide. Binding was detected using polyclonal rabbit anti-ezrin Ig and subsequently with horseradish peroxidase-conjugated anti-rabbit Ig.

ELISA for the Binding of SIC to hBD-3—ELISA for the binding of SIC and its proteolytic or recombinant subfragments to triplicate wells coated with hBD-3 was performed essentially as described previously (15), except that all ligands were added at 5 μg/ml. Bound proteins were detected with a rabbit anti-SIC Ig fraction (2) followed by alkaline phosphatase-conjugated goat anti-rabbit Ig (Sigma-Aldrich) diluted 1:1200 in PBS and 0.05% Tween 20 with 0.1% gelatin. Control wells with either no ligand, no first antibody, or a second antibody only were set up together with matching sets of wells to which coating buffer only had been added as background controls. Incubations were for 1 h at 37°C, and all washes were in PBS and 0.05% Tween 20. The Sigma Fast pNPP substrate/buffer system was used as the final reagent (Sigma-Aldrich). Absorbance was read at 405 nm (reference wavelength 490 nm) in a Bio-Rad 3550 microplate reader.

Complement-mediated Hemolysis Assay—The detail of this assay was described previously (14). Sheep erythrocytes were activated with hemolysin (1:500 dilution; Virion) in gelatin veronal buffer plus MgCl2 and CaCl2 (GVB++) for 30 min at 37°C followed by 30 min at 4°C. Human serum, used in this study as the complement source, was titrated to usually 1:100–1:150 dilution in GVB++ to give ~30–50% lysis. For the SIC activity assay, the serum was pre-incubated with SIC or SIC fragments (4 μg), thioredoxin (control protein, 1.5 μg), or PBS for 30 min at 37°C. Osmolysis of erythrocytes with water was taken as 100% value. After removing the intact erythrocytes by centrifugation at 1700 × g, hemolysis was measured by reading the absorbance at 415 nm in a Bio-Rad benchmark microplate reader.

Complement “Reactive Lysis” Assays—Reactive lysis (17) is a technique for studying the terminal pathway of complement on its own. Lysis is initiated by C5b6, a complex generated in acute phase sera (which have an excess of C5 and C6 over C7), by activation of the alternative pathway with yeast cell walls. A egubulin fraction of such activated sera is used as a source of C5b6. When the C56 encounters C7
it forms the C567 complex, which briefly has the capacity to bind to lipid membranes. Lysis is then brought about by C8 and C9. In a reactive lysis assay, opposing wells are cut into agarose plates and filled with C56 euglobulin, which is functionally free of C7 (prepared from acute phase serum as described in Ref. 17) and normal human serum (as a source of C7, C8, and C9), and allowed to diffuse. Where the diffusing reagents meet, lines of lysis form. Agarose plates (1% agarose in complement fixation diluent) containing 0.75% washed guinea pig red cells (TCS Biosciences Ltd., Botolph Claydon, UK) were poured on 3 × 1-inch microscope slides (2.5 ml per plate). A pattern of 3-mm wells was cut, and 10 µl of the various reagents were added as indicated in Scheme 1. Up to five 10-µl aliquots of M1 SIC and proteolytic or recombinant subfragments of SIC (as potential inhibitors of lysis) were added to the wells and allowed to diffuse into the agarose (to achieve an equal amount of protein, ~2 nmol). 10 µl of normal human serum, C56 euglobulin, or PBS (as a negative control) were then added to the other wells. Plates were incubated overnight at room temperature in a moist box and then photographed.

**Titration of the Effect of SIC Subfragments on the Killing of M1 S. pyogenes by hBD-3**—Briefly, overnight cultures of M1 group A streptococcus were grown at 37 °C in Todd Hewitt broth with 0.2% yeast extract and used to inoculate fresh broth at 1:100. Bacteria were grown to mid log-phase, spun down, and washed twice in 10 ml Tris-HCl, pH 7.5, and 5 mM glucose (Tris-glucose) and resuspended to a concentration of 2 × 10^9/ml in Tris-glucose and 2 mg/ml bovine serum albumin. Doubling dilutions of SIC, recombinant SIC fragments, or recombinant thioredoxin control protein from 3.4 µM were combined with 3.4 µM hBD-3 (all in Tris-glucose) in a total volume of 20 µl (in duplicate) for 3 h at 37 °C. 20 µl of prepared bacteria were added and incubated for a further 1.75 h at 37 °C. Thus, final overall concentrations were SIC from 0.85 µM, 0.85 µM hBD-3, and 1 mg/ml bovine serum albumin, each with bacteria at 10^9/ml. The reaction mixtures were then diluted one-fifth with Tris-glucose and 100 µl spread on duplicate selective horse blood agar plates, and incubated overnight at 37 °C. Controls were bacteria in Tris-glucose, matching dilutions of SIC, SIC fragments, or control protein, or hBD-3 alone.

**The Effect SIC Subfragments on the Catalytic Activity of Lysozyme**—Sequential dilutions of three parts SIC or proteolytic subfragments A and C from 35 µM or subfragment B from 70 µM, plus one part dextran, were combined with 17.5 µM hen egg lysozyme in 20-µl volumes in 50 mM sodium phosphate buffer, pH 7, in duplicate and incubated overnight at 4 °C. Similar sequential dilutions of hen egg lysozyme alone from 35 µM were also made up and incubated as described above to provide a standard curve. 5 µl of each set of reaction mixtures together with a set of standard curve dilutions were added to 2-mm wells of duplicate agarose lysoplates containing Micrococcus lysodeikticus cell walls (Sigma-Aldrich) at 0.5 mg/ml. The plates were incubated at room temperature for ~4 h, and the diameters of the zones of bacterial cell wall clearance were measured using a magnifying graticule with divisions of 0.1 mm.

**RESULTS**

**Production of SIC Subclones**—Earlier studies have clearly defined three major structural domains within the SIC molecule, namely a short repeat region, a long repeat region, and a proline-rich region. As pointed out in the introduction, SIC is a multifunctional protein. To assign these functions to one or more of the structural domains we constructed various truncated recombinants containing the SRR (clone S1), the LRR (clone S2), the PRR (clone S3), the SRR plus LRR (clone S4), and the LRR plus PRR (clone S5). As evident in Fig. 2, top, the majority of the recombinant fusion proteins for the above clones and full-length mature protein were intact. Antibodies against full-length SIC, raised in rabbits, reacted specifically with all the subclones but not with the thioredoxin fusion partner (Fig. 2, bottom).

**Interference with the Complement-mediated Cell Lysis Is Largely Restricted to the N-terminal Third of the SIC Molecule**—Sensitized sheep erythrocytes were incubated with human sera as a complement source diluted sufficiently to result in ~50% lysis of erythrocytes. Pre-incubation of the sera with SIC or the SRR (clone S1) inhibited complement-mediated lysis of erythrocytes to a similar extent (45 and 57%, respectively; Fig. 3). The LRR alone did not show significant inhibition. However, whereas the PRR alone exhibited a low to moderate level of inhibition, LRR plus PRR did not inhibit complement function.

In reactive lysis assays, which more precisely reflect the established mode of action of SIC against complement (2), almost total inhibition of lysis was observed with the SRR
Dissection of Functional Domains of SIC

**TABLE I**

| SIC or fragment                  | Degree of inhibition |
|----------------------------------|----------------------|
| Recombinant SIC                  | ++ +                 |
| S1: SRR                          | ++ +                 |
| S2: LRR                          | 0                    |
| S3: PRR                          | (+)                  |
| S4: SRR + LRR                    | ++ +                 |
| S5: LRR + PRR                    | 0                    |
| Thioredoxin control              | (+)                  |
| Native M1 SIC                    | ++ +                 |
| Fragment A                       | 0                    |
| Fragment B                       | 0                    |
| Fragment C                       | 0                    |
| Fragments A+B                    | 0                    |
| Fragments B+C                    | 0                    |
| Fragments A+C                    | 0                    |
| Fragments A+B+C                  | 0                    |

* 0, (+), and ++ + represent no, low, and high activity, respectively.

**Fig. 4.** The SRR of SIC inhibits complement-reactive lysis. Plates shown from top to bottom had M1 SIC, clone S1 (SRR), and clone S2 (LRR), respectively. In all of the plates the top left and bottom right wells contain SIC or subfragment, the top right and bottom left wells contain PBS, the center row left and right wells contain normal human serum, and the center well contains C56 euglobulin. In the top two plates the truncation of the line of lysis can be seen, demonstrating the inhibition of reactive lysis, whereas in the bottom plate there is no inhibition.

In our previous studies we showed by ELISA that SIC binds to the complement proteins C6 and C7 and to C567 complexes (2, 14). In this study, we demonstrate that binding of these complement proteins to SIC is mediated by the SRR subclone S1 (Fig. 5, A and B). The subclone S2 containing the LRR did not bind at all. However, the PRR-containing subclones (S3 and S5) had low binding to C6 and C7. These results are consistent with our recent studies that show distantly related SIC (DRS) proteins, which have significant similarity with SIC only in the PRR, are also able to bind C6 and C7, albeit at a lower level (15). The results of the functional assays, together with the binding studies described above, clearly show that interference with complement function is largely restricted to the N-terminal third of the SIC molecule.

**Fig. 5.** Mapping of the C6 and C7 complement protein-binding site. In panels A and B microtiter wells were coated with SIC, truncated SIC polypeptides, or thioredoxin (Thio) as indicated. The proteins were subsequently incubated with C6(A) or C7(B), and binding was detected using C6- and C7-specific antibodies.

**The SRR Plus LRR Inhibits the Antimicrobial Activity of hBD-3 against M1 Group A Streptococci and Binds to hBD-3** as determined by ELISA—Harder et al. (18) showed that skin and tonsils are major tissues for production of hBD-3 and that oral epithelial cells and keratinocytes express this defensin. These tissues are primary sites of infection by *S. pyogenes*. Hence, studying the interaction between SIC and hBD-3 is relevant for streptococcal infection. Experiments were performed with the recombinant SIC fusion proteins to elucidate which region of SIC is responsible for inhibition of the antimicrobial activity of hBD-3. Equimolar and 1:2 molar ratios (clone/hBD-3) of clone S4 (the SRR plus the LRR) resulted in 100% inhibition of hBD-3, and ~80% inhibition was achieved at a 1:4 molar ratio. These values are consistent with the results of previous studies showing that the whole SIC molecule inhibited hBD-3 at a 1:2 molar ratio in Tris buffer (15). Somewhat surprisingly, equimolar concentrations of clone S3 (the C-terminal PRR) resulted in up to 50% inhibition. Clone S5 (LRR plus PRR) inhibited no better than the PRR alone. No inhibition of hBD-3 activity was exhibited by clone S1 (SRR), clone S2 (LRR), or the thioredoxin fusion partner alone (Fig. 6A). The ELISA also showed that binding to hBD-3 was confined to the whole molecule, clone S4 (SRR plus the LRR), and proteolytic fragment B, which is broadly similar to clone S4 but is truncated from both ends (Fig. 6B). The higher background observed with the recombinant proteins is probably due to residual Escherichia coli proteins present in the recombinant protein preparation.

**The SRR Plus LRR Inhibits the Enzymatic Activity of Hen Egg Lysozyme**—Experiments were performed with the proteolytic fragments of SIC, designated A, B and C, which are broadly similar to the recombinant fragments S1, S4, and S3, respectively. Fragment B, which comprises approximately two-thirds of the SRR plus the first two long repeats, inhibited ~50% of the enzymatic activity of hen egg lysozyme. This compares with the 80% inhibition that is achieved with the whole SIC molecule. However, it should be noted that a far higher concentration of fragment B is required for this amount of inhibition than that of the whole molecule. Fragment A (the first third of the SRR) and C (the third long repeat plus the PRR) showed no inhibitory activity whatsoever (Fig. 7).

**The SRR Plus LRR Inhibits the Enzymatic Activity of Hen Egg Lysozyme—**Experiments were performed with the proteolytic fragments of SIC, designated A, B, and C, which are broadly similar to the recombinant fragments S1, S4, and S3, respectively. Fragment B, which comprises approximately two-thirds of the SRR plus the first two long repeats, inhibited ~50% of the enzymatic activity of hen egg lysozyme. This compares with the 80% inhibition that is achieved with the whole SIC molecule. However, it should be noted that a far higher concentration of fragment B is required for this amount of inhibition than that of the whole molecule. Fragment A (the first third of the SRR) and C (the third long repeat plus the PRR) showed no inhibitory activity whatsoever (Fig. 7).
Final overall concentrations were 0.85 μM hBD-3 incubated for a further 1.75 h at 37 °C, and then diluted and plated. The killing of M1 group A streptococci by hBD-3 is inhibited by SIC. Earlier studies by Fernie-King et al. (2) showed that inhibition of the terminal complement complex by SIC is mediated by preventing the incorporation of the C567 complex into the cell membrane. In this study we showed, using two different assay systems, that the region of SIC responsible for the inhibition of complement-mediated cell lysis of erythrocytes resides within the N-terminal short repeat region of the protein. Because the proteolytic fragment A of SIC, comprising the first 32 amino acids, showed no inhibitory activity, it would appear that either the whole of the region is necessary for inhibition or that the binding point is in the vicinity of the elastase digestion site between amino acids 32 and 33 and has thus been destroyed. This same region is responsible for the binding of SIC to the terminal complement proteins by ELISA. Our recent studies (16) with DRS from an M12 strain show a reduced level of binding to C6 and no complement inhibitory activity. DRS purified from the culture supernatant of a different M12 strain containing a much longer variant of the protein was also unable to inhibit reactive lysis.2 This finding suggests that DRS may have a different type of interaction with complement proteins compared with that of SIC from type M1 strains. Furthermore, the addition of DRS to SIC did not interfere with complement-mediated cell lysis by the latter. Taken together, these results suggest that a specific interaction between the SRR domain and the terminal complement complex is required for the inhibition of complement-mediated lysis.

SIC has been shown to be an inhibitor of several bactericidal peptides, including hBD-3. The SRR plus LRR regions together form by far the most effective part of SIC in inhibiting hBD-3 activity against S. pyogenes and at the same molar ratios as that observed previously for the complete SIC molecule (15). The PRR on its own also showed some anti-defensin activity, suggesting that SIC may have distinct modes of anti-defensin activity.

Subclones containing the SRR and the LRR alone. However, whereas the PRR by itself showed a moderate level of binding to ezrin, the PRR together with the LRR (clone S5) showed as much binding as full-length SIC. These results suggest that multiple regions of SIC may have ezrin-binding domains and, overall, are consistent with the interpretation of Hoe et al. (8) based on competition assays using peptides spanning SIC. They found that two distinct non-overlapping peptides competed against ezrin binding and that these two peptides map within SRR and PRR.

**DISCUSSION**

Optimal SIC-ezrin binding requires multiple structural domains. Microtiter wells were coated with SIC, subclones, or thioredoxin control protein as indicated and then reacted with peptide corresponding to C-terminal region of ezrin. Binding was detected with anti-ezrin antibodies followed with the appropriate secondary antibody. The results were reproducible over multiple experiments.

**Fig. 6. Effect of SIC and its subclones on hBD-3.** Panel A shows that the killing of M1 group A streptococci by hBD-3 is inhibited by SIC and SRR plus LRR. Doubling dilutions of SIC or recombinant SIC fragments from 3.4 μM were combined with 3.4 μM hBD-3 and incubated for 3 h at 37 °C. Equal volumes of bacteria at 2 × 10^7/ml were added, incubated for a further 1.75 h at 37 °C, and then diluted and plated. Final overall concentrations were SIC from 0.85 μM plus 0.85 μM hBD-3 and bacteria at 10^7/ml. Results are expressed as the percentage of survival compared with bacteria incubated in the equivalent concentration of SIC fragment alone (no defensin controls). Panel B shows binding of SIC and subfragments to hBD-3. Coating protein was hBD-3, and the ligands were native (nSIC) or recombinant SIC (rSIC), proteolytic fragments (A, B, and C), or recombinant SIC fractions (S1–S5). Results are expressed as net OD^405 after the deduction of background values from matching uncoated wells ± S.E. Where no error bars are shown the S.E. is too small to be seen.

**Fig. 7. Inhibition of the catalytic activity of hen egg lysozyme (HEL) by SIC.** Dilutions (three in four) of SIC (circles), fragment A (triangles), and fragment C (inverted triangles) from 35 μM or fragment B (diamonds) from 70 μM were combined with 17.5 μM hen egg lysozyme and incubated overnight at 4 °C. Residual lysozyme activity was assayed by the lysoplate method. Data are from at least three experiments performed in duplicate and are expressed as the percentage of lysozyme activity remaining compared with controls of lysozyme alone, ± S.E. Where no error bars are shown, the S.E. is too small to be seen.

Binding to Ezrin Is Predominantly by the C-terminal Two-thirds of the SIC Molecule Containing the LRR and PRR—Binding of SIC and fragments thereof to ezrin, a cytoskeleton-associated protein, is shown in Fig. 8. The thioredoxin tag protein did not bind ezrin. A low level of binding was seen with subclones containing the SRR and the LRR alone. However, whereas the PRR by itself showed a moderate level of binding to ezrin, the PRR together with the LRR (clone S5) showed as much binding as full-length SIC. These results suggest that multiple regions of SIC may have ezrin-binding domains and, overall, are consistent with the interpretation of Hoe et al. (8) based on competition assays using peptides spanning SIC. They found that two distinct non-overlapping peptides competed against ezrin binding and that these two peptides map within SRR and PRR.

**Fig. 8. Optimal SIC-ezrin binding requires multiple structural domains.** Microtiter wells were coated with SIC, subclones, or thioredoxin control protein as indicated and then reacted with peptide corresponding to C-terminal region of ezrin. Binding was detected with anti-ezrin antibodies followed with the appropriate secondary antibody. The results were reproducible over multiple experiments.

![Image](http://www.jbc.org/)

**M. J. Binks, B. A. Fernie-King, D. J. Steilly, P. J. Lachmann, and K. S. Sriprakash, unpublished observations.**
activities. It should be borne in mind that these assays were performed in low ionic strength buffer, which facilitates ionic interactions, so it could be that although the specific interaction is between the SRR plus LRR region and hBD-3, there is a less specific ionic interaction between PRR and hBD-3. However, it cannot reflect simply the overall charge, as the relative charge of the SIC regions is SRR > LRR > PRR, and the SRR alone exhibited no inhibitory activity whatsoever.

Similar results have been obtained using the proteolytic fragments of SIC in that fragment B inhibited the bactericidal activity of hBD-3, albeit somewhat less efficiently than the recombinant SRR plus LRR. Total inhibition was obtained only with equimolar concentrations of fragment B and hBD-3. It seems, therefore, that the whole of the SRR plus LRR region is required for maximum inhibition. Overall, these results for anti-defensin activity are consistent with the ELISA binding studies for both proteolytic and recombinant fragments of SIC, which show that only the SRR plus LRR and fragment B bind to hBD-3 (this study and Ref. 15).

We have also shown that the same region of the molecule (fragment B) is responsible for inhibition of the enzymatic activity of lysozyme. The interaction between SIC and lysozyme has been shown to be ionic in nature (5), so it was somewhat surprising to find that inhibition was localized exclusively to fragment B (SRR plus LRR), indicating that there is an element of specificity to the interaction.

Finally, SIC is also shown to bind ezrin, a cytoskeleton-associated protein. This property is mainly attributed to the PRR, and the presence of the central LRR seems to have an additive effect. SIC-ezrin binding is thought to alter the ultrastructure of host cells, which, in turn, may result in changes in host-pathogen interaction.

As described in the introduction, the sic gene is extraordinarily variable throughout its entire length. Therefore, new variants of SIC could not have arisen simply to evade the neutralizing immune response to the SRR (which mediates complement inhibitory function) or the other two regions, which we have now shown to be responsible for inhibition of two classes of antimicrobial peptide and for binding to ezrin, respectively. The high mutation rate in sic may simply be a reflection of its chromosomal location at a hotspot, in this case the mga regulon in serotype M1 strains (1). This regulon harbors other highly mutable genes such as emm and fcrA in S. pyogenes (19).

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Attribution of the Various Inhibitory Actions of the Streptococcal Inhibitor of Complement (SIC) to Regions within the Molecule

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