Streptococcal IgA-binding proteins bind in the Cα2-Cα3 interdomain region and inhibit binding of IgA to human CD89*

Richard J. Pleass‡, Thomas Areschoug§, Gunnar Lindahl¶, and Jenny M. Woof‡¶

From the ‡Department of Molecular and Cellular Pathology, University of Dundee Medical School, Ninewells Hospital, Dundee, DD1 9SY, United Kingdom and the §Department of Laboratory Medicine, Lund University, Sölvegatan 23, S-223 62 Lund, Sweden

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¶ To whom correspondence should be addressed. E-mail: j.m.woof@dundee.ac.uk. or gunnar.lindahl@mmb.lu.se

xx These authors made equal contributions to this study

Running title: Bacterial IgA-Fc-binding proteins
Summary

Certain pathogenic bacteria express surface proteins that bind to the Fc-part of human IgA or IgG. These bacterial proteins are important as immunochemical tools and model systems but their biological function is still unclear. Here, we describe studies of three streptococcal proteins that bind IgA: the Sir22 and Arp4 proteins of *Streptococcus pyogenes* and the unrelated β protein of group B streptococcus. Analysis of IgA domain swap and point mutants indicated that two loops at the Cα2/Cα3 domain interface are critical for binding of the streptococcal proteins. This region is also used in binding the human IgA receptor CD89, an important mediator of IgA effector function. In agreement with this finding, the three IgA-binding proteins, and a 50 residue IgA-binding peptide derived from Sir22, blocked the ability of IgA to bind CD89. Further, the Arp4 protein inhibited the ability of IgA to trigger a neutrophil respiratory burst via CD89. Thus, we have identified residues on IgA-Fc that play a key role in binding of different streptococcal IgA-binding proteins, and we have identified a mechanism by which a bacterial IgA-binding protein may interfere with IgA effector function.
Introduction

Human IgA is abundant in the seromucous secretions which bathe mucosal surfaces, such as those lining the lungs, gut, and genitourinary tracts. These surfaces represent major potential sites of invasion and the immune protection offered by secretory IgA, as the predominant antibody at these sites, serves as a critical ‘first line of defence’ against many bacteria and viruses (1). Moreover, evidence is accumulating that IgA present in serum plays an important role in a ‘second line of defence’ against microorganisms that have penetrated the mucosal barrier (2, 3).

IgA performs the dual role of all antibodies, of both recognising foreign invaders and triggering their elimination. For IgA, this latter effector function involves the interaction of its Fc region with an Fcα receptor (FcαRI, CD89) expressed on neutrophils, eosinophils, or macrophages (4, 5). After binding to antigen, IgA can interact with CD89 and elicit an array of potent eradication mechanisms, including phagocytosis, superoxide generation, and release of enzymes and inflammatory mediators (5). The molecular basis of this important interaction between IgA and CD89 is now emerging, with the demonstration of the critical role played by two loops lying at the interface of the two domains of IgA-Fc (6, 7), and with the identification of the binding region in CD89 (8, 9).

Surface proteins that bind human IgA-Fc have also been identified in many strains of Streptococcus pyogenes (group A streptococcus; GAS) and group B streptococcus (GBS), two important human pathogens (10-12). Despite the importance of streptococci as pathogens, it is unclear what advantage the ability to bind IgA-Fc offers a bacterium during the establishment of an infection. However, even in the absence of information concerning their exact biological role, the IgA-binding proteins (IgA-BPs) are of considerable interest as immunochemical tools and model systems. A similar situation prevails for the well-known bacterial IgG-binding proteins (IgG-BPs), staphylococcal protein A and streptococcal protein G, which have been extensively characterised (13), but whose biological function is unknown.

In S. pyogenes, the two IgA-BPs that have been studied in most detail are the Arp4 and Sir22 proteins (14, 15), which are members of the heterogeneous M protein family (16, 17). These streptococcal proteins have 29-residue IgA-binding regions that are related, but
not identical, making comparisons of interest (18, 19). Importantly, these IgA-BPs bind human IgA of both subclasses and bind both serum IgA and secretory IgA (15, 20).

In GBS, binding of IgA is due to the β protein, which is unrelated to the IgA-BPs of S. pyogenes (21, 22) and has a 73-residue IgA-binding region that does not vary in sequence between strains (23). The β protein binds human serum IgA of both subclasses and has the remarkable property that it binds poorly to secretory IgA, the molecular form of IgA that predominates on mucous membranes (24).

Here, we report experiments aimed at analysing the interaction between IgA-Fc and streptococcal IgA-BPs. Experiments with domain swap antibodies and mutant IgAs indicate that binding of the different IgA-BPs, and a 50-residue synthetic IgA-binding peptide derived from the Sir22 protein (25), depend on almost identical sites in the Fc interdomain region of IgA, the binding region also used by CD89. In agreement with these results, we demonstrate that the streptococcal IgA-BPs inhibit interaction of IgA with CD89, a property that may allow IgA-BPs to inhibit IgA effector function.
Experimental Procedures

Bacterial strains

The *S. pyogenes* strain AL168 expresses the Sir22 protein (15). Strain AL168*mrp22 sir22* is a derivative of AL168 lacking expression of the two M proteins Mrp22 and Sir22 (26). The M-negative *S. pyogenes* strain JRS145 and its derivative JRS145/pJRS264, which expresses the Arp4 protein, have been described (27). The GBS strain A909, which expresses the β protein (11), was obtained from Dr J. Michel (Channing Laboratory, Boston, Mass.). A β-negative derivative of A909 was constructed by replacement of the β gene with a kanamycin resistance cassette². Streptococcal strains were grown in Todd-Hewitt broth (Oxoid, Basingstoke, Hampshire, U.K.) at 37°C, without shaking.

Purified bacterial proteins and peptides

The *S. pyogenes* proteins Arp4 and Sir22 were purified after expression in *E. coli* (15). Two deletion derivatives of Arp4 with non-overlapping deletions in the IgA-binding region, the Arp4Δ450 and Arp4Δ451 proteins, were constructed and purified as described (18, 28). The group B streptococcal β and Rib proteins were purified from streptococcal extracts (29). Protein G was purchased from Calbiochem-Novagen Sciences (Nottingham, U.K.). A synthetic 50-residue IgA-binding peptide, derived from the Sir22 protein, has been described (25). This peptide contains a C-terminal C residue, not present in Sir22, that promotes dimerization and thereby enhances IgA-binding (30). A 53-residue synthetic peptide (M5-N), derived from the N-terminal region of the non-IgA-binding streptococcal M5 protein, was purchased from the Department of Clinical Chemistry, Malmö General Hospital, Sweden. This peptide includes 50 residues derived from the M5 protein and also includes the sequence YYC added at the C-terminal end, allowing radiolabelling at the tyrosine residues and dimerisation via the cysteine residue.

Analysis of purified bacterial proteins by Western blot was performed as described (29), using rabbit anti-Sir22 serum and mouse anti-β serum for analysis of cross-reactivity. The blotting membranes were incubated with antiserum diluted 1000-fold, followed by
incubation with radiolabelled protein G (for rabbit IgG) or protein A (for mouse IgG) and autoradiography. Control membranes were incubated with preimmune serum.

**Human IgG/IgA domain swap and mutant IgA1 antibodies**

All the recombinant anti-NIP antibodies used were chimeric (heavy chains comprised human constant domains and murine variable domain, light chains (\(\lambda\)) were murine). They included previously described wildtype human IgA1 and IgG1 (7, 31), and the IgG1/IgA1 domain swap antibodies \(\alpha_1\alpha_2\gamma_3\) and \(\gamma_1\gamma_2\alpha_3\) (7). IgA1 mutants with single or double residue substitutions in the two interdomain loops (L257R, G259R, P440R, P440A, A442R, F443R and LA441-442MN) [numbering according to the commonly adopted scheme used for IgA1 Bur (32)] have also been described (7). Recombinant antibodies were purified from supernatants of CHO-K1 transfectants by affinity chromatography on NIP-Sepharose (7).

**Analysis of Ig binding by ELISA**

Nunc Maxisorp microtitre plates, coated overnight with 0.25 \(\mu\)g of appropriate streptococcal protein in fresh coating buffer (0.05M sodium carbonate buffer pH 9.6), were blocked with PBS containing 0.25% (w/v) gelatin and 0.1% (v/v) Tween-20. After three washes with water, 100 \(\mu\)l of appropriately diluted wildtype or mutant IgA1 or IgG1 in Glasgow minimum essential medium with 10% (v/v) FCS were added per well and incubated for 2 h at room temperature. Following washing, 100 \(\mu\)l rat monoclonal anti-mouse lambda (\(\lambda_1\) and \(\lambda_2\)) light chain-biotin conjugate (Pharmingen, San Diego, CA, USA) diluted 1:2000 in PBS with 0.1% Tween 20 (PBST) were added to each well and incubated 1 h at room temperature. This monoclonal was used, since the light chains of the Igs studied here were of mouse origin. After washing, wells were incubated for 1 h with 100 \(\mu\)l streptavidin-alkaline phosphatase (Dako) diluted 1:2000 in PBST. After further washing, reactions were developed by incubating with substrate (Sigma Fast p-Nitrophenyl phosphate tablets made up according to manufacturer’s instructions).

The binding of the detecting antibody in the absence of IgA or IgG was used as the value for non-specific binding and was subtracted from total binding to give specific binding.
values. Results from different experiments were normalised such that the fractional binding of wildtype IgA at 1 \times 10^{-6} \text{ M} = 1.0.

**Binding assays with whole bacteria**

Washed suspensions of bacteria (~5 \times 10^9/ml) were prepared as described (26), and identical samples (200 µl) were added to each of a series of tubes. Different amounts of a purified Ig (in a volume of 50 µl) were added, giving the final concentrations indicated. After incubation for 2 h, the bacteria were washed twice with PBSAT (PBS with 0.02% NaN_3 and 0.05% Tween-20) and presence of bound Ig was analyzed by the addition of \sim 15,000 \text{ cpm} of I^-labelled rat anti-mouse λ light chain (Pharmingen). After incubation for 1 h and two washes with PBSAT, the radioactivity associated with the pelleted bacteria was determined in a γ-counter. All incubations were performed at room temperature. Binding is expressed as a percentage of the radioactivity added to each tube. Binding to control bacteria (≤1%) has been subtracted. All determinations were made in duplicate.

Binding to Sir22 on the bacterial cell surface was analyzed with *S. pyogenes* strain AL168, using the non-IgA-binding mutant AL168*mrp22 sir22* as a negative control. Binding to Arp4 on the surface of *S. pyogenes* was performed with strain JRS145/pJRS264, with JRS145 as the negative control, and binding to β on the surface of GBS was analyzed with strain A909, using a β-negative mutant of this strain as the negative control.

**Inhibition of rosetting**

Human erythrocytes were derivatized with NIP and sensitized with wildtype IgA1 at 200 µg/ml as described (7). Neutrophils were isolated as described (7) and resuspended in PBS containing 0.1% (w/v) BSA (PBS/BSA). In the inhibition assay, which was essentially a modification of a previously described method (33), diluted coated erythrocytes and inhibitor protein (peptide, bacterial proteins, or their control peptide or proteins), both in PBS/BSA, were incubated at room temperature for 1 hour (except for β protein and protein G where overnight incubation was used) in wells of a V-bottomed microtiter plate. Neutrophils (~50,000) in PBS/BSA were added and mixed carefully, the plates incubated for 10 min,
centrifuged at 50 g for 5 min, and further incubated for 50 min. Following addition of acridine orange solution (6 µg/ml final concentration) to stain nucleated cells, the suspensions were examined by fluorescence microscopy, defining a rosette as a fluorescent neutrophil with three or more erythrocytes attached.

The results were normalised so that the mean rosetting level seen in the absence of inhibitor gave 0% normalised rosette inhibition. For each inhibitor, experiments were performed at least twice, each time using neutrophils from a different donor.

**Inhibition of neutrophil respiratory burst**

The inhibition assay was essentially a modification of a previously described chemiluminescence assay of respiratory bursts (7). Wells of a chemiluminescence microtiter plate (Dynex Technologies, Ashford, UK) were coated with NIP-BSA with subsequent incubation with wildtype IgA1 in PBS (100 µl/well at 50 µg/ml) for 1-2 h at room temperature. After washing, appropriately diluted inhibitor in Hanks’ buffered saline solution containing 20mM HEPES and 0.1% (w/v) globulin-free BSA (HBSS/BSA) was added. After incubation for 1 h at room temperature to allow pre-binding of the inhibitor to the IgA, neutrophils in HBSS/BSA containing 260 µg/ml luminol were added (giving a final suspension of 0.25 x 10^6/ml), the plate transferred to a Microlumat LB96P luminometer, and the chemiluminescence measured at regular intervals.

**Results**

**Immunochemical comparison of streptococcal IgA-binding proteins**

The ~40 kDa Arp4 and Sir22 proteins from *S. pyogenes* are both members of the M protein family and share structural similarities, including a high degree of residue identity in the 29-residue IgA-binding region (Figure 1). As expected, antibodies to Sir22 were found to cross-react with Arp4 (Figure 1). However, the ~125 kDa β protein of GBS lacks residue identity with Arp4 and Sir22 and did not react with anti-Sir22 antibody, nor did anti-β antibodies
recognise Arp4 or Sir22. These data confirm that the IgA-BPs of *S. pyogenes* and GBS are unrelated, underlining the interest in comparing their functional properties.

**Contribution of IgA-Fc domains to interaction with IgA-binding proteins**

To analyse the contribution of the Cα2 and Cα3 domains of IgA-Fc to the binding of streptococcal IgA-BPs, we used two domain swap antibodies in which homologous domains are exchanged between IgA1 and IgG1. These domain swaps are designated γ1γ2α3 (constant domain structure Cγ1, Cγ2, Cα3), and α1α2γ3 (constant domain structure Cα1, Cα2, Cγ3) (Table I). The ability of the constructs to bind the three streptococcal IgA-BPs, and the IgA-binding peptide derived from Sir22, was analysed by ELISA (Figure 2).

Because intact Sir22 binds both IgA and IgG (15), the IgA/IgG domain swap antibodies could not be used to provide information on the IgA binding requirements of Sir22. However, the Sir22-derived peptide, Arp4 and β protein did not bind wildtype IgG1 in the ELISA, so this approach was useful to illustrate the relative contributions of the two IgA-Fc domains to the interaction site. For all these three IgA-binding molecules, we observed that the γ1γ2α3 antibody bound with an apparent affinity generally comparable to wildtype IgA1 (Figure 2). In contrast, no binding was observed for the α1α2γ3 antibody. Several streptococcal proteins/peptides that do not bind IgA were used as controls, and all were unable to bind to either wildtype IgA1 or the swap antibodies. Together, these results suggest that the Cα3 domain makes a major contribution to the binding site for these IgA-binding molecules.

To analyse whether the findings using purified bacterial IgA-binding proteins give a true reflection of the reactivity of the proteins when expressed on the bacterial cell surface, we performed binding studies with whole bacteria. Due to the IgG-binding ability of the *S. pyogenes* strains expressing Sir22 and Arp4 (34), we were unable to analyse whole *S. pyogenes* bacteria for ability to bind the IgA/IgG domain swap antibodies, but could perform studies with whole GBS bacteria expressing the β protein (Figure 3). Neither IgG1 nor the domain swap α1α2γ3 bound to the β-expressing GBS, while both wildtype IgA1 and the γ1γ2α3 antibody bound to these bacteria. Indeed, the γ1γ2α3 construct bound even better
than IgA1 in this test. None of the constructs showed significant binding to an isogenic GBS mutant lacking expression of the β protein (data not shown). These results are consistent with a major role for the Cα3 domain of IgA in binding to the β protein.

Although the data described above indicate that the Cα3 domain is of major importance for the binding of streptococcal IgA-BPs to IgA, they do not rule out the possibility that the Cα2 domain also makes a contribution, since regions in the Cγ2 domain in the γ1γ2α3 antibody may be able to adequately substitute for the corresponding parts of the Cα2 domain in the binding process. Data reported below suggest that this is indeed the case.

Use of point mutations in the Cα2/Cα3 interdomain region of IgA-Fc for characterization of sites that bind streptococcal proteins

Since the binding regions for the IgG-binding bacterial proteins staphylococcal protein A and streptococcal protein G have been localised to the Fc interdomain region of IgG (35, 36), we analysed whether the interdomain region of IgA-Fc might be directly involved in interaction with the streptococcal IgA-binding proteins. Studies of this region in IgA were also of interest because recent work has implicated two loops at the Cα2/Cα3 interface in the binding of IgA to human CD89 (6, 7). We used a panel of IgA1 antibodies (7), each with a single or double amino acid substitution located in either of two predicted loops in the interdomain region, corresponding to Leu257-Gly259 in the Cα2 domain and Pro440-Phe443 in the Cα3 domain (Table I). These two predicted loops will be referred to as the LLG and PLAF loops. Molecular modelling (37) suggests that these two loops occupy positions in IgA analogous to interdomain loops in IgG that are essential for binding of staphylococcal protein A (35). The IgA proteins mutated in the two loops have Arg substitutions, since conversion to this bulky side chain in a critical residue was thought likely to be sufficient to ablate binding. However, several lines of evidence indicate that no gross conformational changes have been introduced into the IgA mutants (7).
**Effect of mutations in the predicted LLG loop of the Cα2 domain**

Two mutants with single point mutations in the LLG loop, the L257R and G259R mutants, were assessed by ELISA for binding to the four streptococcal IgA-binding molecules (Figure 4A). The G259R mutant had apparent affinities similar to those of wildtype IgA1. In contrast, the L257R mutant displayed slightly decreased binding to the Sir22-derived peptide, and more markedly decreased binding to intact Sir22 and to β protein, but was not affected in its ability to bind the Arp4 protein. Together, these data suggest that residues in the Cα2 region may play a role in the binding of streptococcal IgA-BPs and that the Arp4 and Sir22 proteins, which are closely related, may not have completely identical IgA-binding properties, when tested in purified form.

When the two Cα2 mutants were analysed for binding to IgA-BPs expressed on the bacterial cell surface, a similar pattern emerged (Figure 4B). Thus, G259R bound to bacteria expressing Sir22, Arp4, or β with apparent affinities similar to those of wildtype IgA1. In contrast, L257R displayed strongly decreased ability to bind each strain. The binding observed in this analysis with whole bacteria was due to the IgA-BPs, since bacterial mutants lacking expression of the different IgA-BPs were completely unable to bind any of the IgA proteins (data not shown). Together, these results suggest that the Gly259 residue does not play a role in interaction with any of the streptococcal IgA-BPs when they are expressed on the bacterial cell surface, but that residue Leu257 may be involved. It is noteworthy that the L257R mutant showed strongly reduced binding to Arp4 expressed on bacteria, but not to purified Arp4 (Figure 4A), stressing the importance of comparing binding tests performed with purified proteins and tests performed with whole bacteria. However, it should be noted that L257R is the only protein for which we have noted a clear difference between results obtained with purified proteins and with whole bacteria.

**Effect of mutations in the predicted PLAF loop of the Cα3 domain**

The IgA antibodies with mutations in the Cα3 interface-proximal PLAF loop all had single amino acid substitutions with the exception of LA441-442MN (Table I). The effects of the substitutions on binding to purified proteins were similar for the three molecules originating
from *S. pyogenes*, *i.e.* Sir22, the Sir22-derived peptide, and Arp4 (Figure 5A). The A442R mutant bound all three molecules with affinity generally similar to wildtype IgA1, while the LA441-442MN mutant displayed binding consistent with a decrease in affinity of around 10-fold for Sir22 and the peptide, and around 2-5-fold for Arp4. Mutant P440R showed only weak binding to the three IgA-binding molecules originating from *S. pyogenes*, with apparent reductions in affinity of greater than 100-fold, while the P440A and F443R mutants showed essentially no binding. Analysis of binding of these mutants to Sir22 or Arp4 expressed on the surface of *S. pyogenes* produced a similar picture (Figure 5B). Thus, the binding of A442R to whole *S. pyogenes* bacteria was similar to that observed with wildtype IgA1. In contrast, P440R and F443R displayed markedly reduced binding, whilst no binding was observed for P440A, *i.e.* the effect on binding was even more dramatic for P440A than for P440R. Together, these results suggest that the PLAF loop in Cα3, and residues Pro440 and Phe443 in particular, are critical for binding of Sir22, its peptide derivative, and Arp4. As described above, some contribution to binding is also apparently made by the close-lying Leu257 residue in the LLG loop of Cα2.

Mutations in the PLAF loop of Cα3 also had dramatic effects on binding to β protein. The results were reminiscent of those obtained with the *S. pyogenes* proteins/peptide, but with important distinctions. As observed for the *S. pyogenes* proteins, mutant A442R had an apparent affinity similar to that of wildtype IgA1, and LA441-442MN showed decreased binding consistent with a drop in affinity of around 10-fold (Figure 5A). However, unlike the *S. pyogenes* proteins, mutant P440R was almost completely negative, while mutant F443R retained some binding, with an apparent reduction in affinity of around 10-fold. Thus, both of the P440R and P440A mutations appeared to virtually ablate binding to β protein, suggesting that the Pro440 residue plays a highly critical role in binding of β to IgA. Binding tests with β expressed on the bacterial cell surface confirmed the ELISA data (Figure 5B).
**Streptococcal IgA-BPs inhibit binding of IgA to human CD89**

The data reported above revealed that the PLAF and LLG loops at the IgA-Fc interdomain region, which are critical for CD89 binding (6, 7) also appear to be important for binding to the bacterial IgA-BPs. Therefore we investigated the capacity of the bacterial proteins to inhibit the ability of IgA to bind to and activate CD89.

Using a rosetting assay, we found that Sir22 and the Sir22-derived peptide inhibited binding of IgA1 to human CD89, the former producing half maximal inhibition at concentrations of $\sim 1 \times 10^{-8}$ M and the latter at $\sim 1 \times 10^{-7}$ M (Figure 6A). This effect appears to be specific since a control peptide (M5-N) did not display inhibitory ability at concentrations up to $5 \times 10^{-6}$ M. Similarly we found that Arp4 was capable of inhibiting the IgA1-CD89 interaction, with half maximal inhibition at $\sim 1.6 \times 10^{-8}$ M (Figure 6B). This inhibition required the presence of an intact IgA-binding region in Arp4, since two non-IgA-binding Arp4-derivatives with short deletions in the IgA-binding region, Arp4Δ450 and Arp4Δ451, did not cause any inhibition. The β protein also appeared able to inhibit IgA binding to CD89, but much greater concentrations were required with half maximal inhibition only being reached at $\sim 2 \times 10^{-6}$ M (Figure 6C). However, the protein G control did not influence rosette formation at equivalent concentrations. Together, these data indicate that all four of the IgA-binding bacterial molecules studied here are able to block the binding of IgA-Fc to CD89.

As an additional, physiologically relevant test for function, we assessed the ability of the bacterial IgA-BPs to inhibit the IgA-mediated triggering of a respiratory burst in neutrophils, characterised by NADPH oxidase activation and degranulation which can be assessed in a chemiluminescence assay. The oxidative burst is a biological response of major importance in host defence, and an ability to inhibit it would clearly be advantageous to a pathogen. Although we were unable to perform such assays with Sir22, the Sir-22 derived peptide, or β protein due to unexpected generalised effects on neutrophil bursts, we did observe that Arp4 was capable of inhibiting the IgA-triggered oxidative burst at concentrations greater than $5 \times 10^{-8}$ M (Figure 7). This inhibition appears to be due to the ability of Arp4 to bind IgA since the two non-IgA-binding Arp4 deletion mutants Arp4Δ450...
and Arp4Δ451 caused little or no inhibition. Moreover, Arp4 did not inhibit a PMA-stimulated respiratory burst (data not shown). Together these data indicate that Arp4 can inhibit a respiratory burst triggered by the binding of IgA-Fc to CD89.
Discussion

Surface proteins that bind to the Fc-part of human IgA or IgG are expressed by many pathogenic bacteria, in particular by Gram-positive pathogens (38). While IgA-binding proteins have been less extensively studied than those binding IgG, more is known about their biological properties. For example, the IgA-binding Sir22 and Arp4 proteins of GAS are known to be important virulence factors (26) and the IgA-binding β protein of GBS has been shown to be a target for protective antibodies (39). However, the exact role of IgA-BPs in streptococcal pathogenesis remains unknown. This situation, and the potential usefulness of IgA-BPs as immunochemical tools and model systems, prompted us to characterize the binding site in IgA-Fc for different IgA-BPs.

To analyse regions in IgA-Fc critical for interaction with streptococcal IgA-BPs, we employed domain swaps and point mutants. As discussed before (7), these mutant proteins are unlikely to have undergone any gross structural aberrations, allowing conclusions to be drawn on the relative contributions of different domains and mutated residues to the binding of streptococcal proteins. Our results indicate that the Cα3 domain of IgA-Fc makes a major contribution to binding for all the streptococcal proteins, with the Cα2 domain possibly playing a less important role. This result is in agreement with a previous study, which implicated the Cα3 domain in the binding of the Arp4 protein (40). The present study indicates that the PLAF loop (residues 440-443), predicted to lie on the surface of the Cα3 domain, is of particular importance for the binding of the bacterial IgA-BPs, but the LLG loop in Cα2 also appears to contribute. The data on proteins/peptide from S. pyogenes suggest that they bind to identical or very similar sites in IgA-Fc, and binding of the β protein from GBS appears to depend on essentially the same residues. However, Phe 443 is less critical for interaction with β than with the IgA-binding molecules from S. pyogenes. Thus, the IgA-BPs of S. pyogenes bind to a site that appears to be very similar to the site used by the unrelated β protein of GBS, but the sites are probably not identical. This conclusion is in good agreement with previously reported inhibition experiments, which indicated that the Arp4 and β proteins bind to the same region in IgA (24).
It may be argued that mutations in the PLAF loop of Cα3 and the LLG loop of Cα2 produced their effects either by perturbing direct binding interactions, or by triggered alterations in the conformation of close-lying residues which provide the binding contacts. The finding that mutation of Gly259 in the LLG loop and of Ala 442 in the PLAF loop did not reduce binding, whilst that of adjacent residues produced marked effects, may indicate that the latter possibility is less likely. In either case, these interdomain loops may be considered as important markers of the binding sites for the streptococcal IgA-BPs.

The PLAF loop in Cα3 and the LLG loop in Cα2 are predicted to lie close in three-dimensional space, as highlighted on a molecular model of IgA based on solution structural studies (37) (Figure 8). These loops are proposed to play a key role for binding of IgA-Fc to CD89 (6, 7) and to streptococcal proteins (this study), but there are clear differences between the sites interacting with the human and bacterial proteins. In particular, domain swap experiments indicated that binding of CD89 to IgA-Fc requires both of the Cα2 and Cα3 domains, while the Cα2 domain can be replaced with the Cγ2 domain without affecting binding of the streptococcal proteins. Further, mutation in the PLAF loop of Ala442 to Arg was associated with loss of detectable binding to CD89 (7), whilst it had no impact on binding to the bacterial proteins. Together, these data indicate that CD89 and the bacterial IgA-BPs have overlapping, but not identical, binding sites in IgA-Fc.

The proposed overlapping nature of the binding sites in IgA-Fc for CD89 and bacterial IgA-BPs is strongly supported by the observed ability of the IgA-BPs to inhibit the binding of IgA to CD89, as measured by rosetting, and by the ability of the Arp4 protein to inhibit an IgA-triggered respiratory burst in CD89-expressing neutrophils. It could be argued that the observed blockade is rather a gross effect since intact IgA-BPs might be expected to mask an appreciable area of the IgA-Fc surface due to their molecular size. However, we found that the much smaller 50-residue IgA-binding peptide, representing an isolated IgA-binding domain (25), was still capable of specifically blocking interaction with CD89. This peptide would be anticipated to adopt a three-dimensional structure of relatively small size, and so its ability to inhibit CD89 binding is most likely explained by close proximity of their respective binding sites on IgA-Fc.
The ability of streptococcal IgA-BPs to inhibit binding of IgA to CD89 suggests that such disruption may also occur during a bacterial infection. We can therefore propose a mechanism by which possession of an IgA-BP may confer on a bacterium the ability to evade clearance mediated by specific IgA antibodies. According to this mechanism, specific binding of an IgA molecule to a bacterial surface antigen is followed by binding of the Fc-part of the IgA molecule to a bacterial IgA-BP also present on the bacterial surface, thereby allowing the bacterium to evade the elimination processes that would normally be triggered via binding of IgA-Fc to CD89 (3). Such bridging of a bound Ig molecule has also been proposed to explain the mechanism of action of the IgG-Fc receptor of herpes simplex virus type 1 (HSV-1), but it is not known if this viral Fcγ receptor blocks binding of IgG to human Fcγ receptors or if it exerts its function by some other mechanism (41, 42).

The finding that unrelated IgA-BPs, expressed by *S. pyogenes* or GBS, bind to similar sites in IgA-Fc is reminiscent of the situation described for the two unrelated bacterial IgG-binding molecules, staphylococcal protein A and streptococcal protein G, both of which bind to the Fc domain interface in IgG (13, 35, 36, 43). Taken together, these data imply that convergent evolution has favoured the appearance of bacterial proteins that bind to the CH2/CH3 interdomain region in IgA or IgG. Interestingly, accessibility and sequence comparison analyses, and a recent study exploiting random peptides, indicate that the interdomain region of IgG-Fc has intrinsic properties that favour binding to other proteins (44, 45). This conclusion is supported by evidence that the Fcγ receptor of HSV-1 binds at the Cγ2/Cγ3 domain interface (46, 47) and by localisation of the binding site for CD89 to the interdomain region of IgA-Fc (6, 7). These data raise the question whether one biological function of protein A, protein G, and the Fcγ receptor of HSV-1 might be to inhibit IgG effector function in a manner analogous to that proposed here for bacterial IgA-BPs, *i.e.* by interfering with the binding of IgG to Fcγ receptors on phagocytic cells. However, the majority of available evidence appears to argue against such a mechanism since protein A has been shown not to inhibit the binding of IgG to human FcγRI and FcγRII (48) and all FcγR receptors bind at the N-terminal end of the Fc, well away from the Fc interdomain region (49-
54). Further work will be required to clarify the various mechanisms which may afford evolutionary advantage to microbes that possess proteins that bind IgA or IgG.

In summary, we have demonstrated that unrelated bacterial IgA-BPs bind in the interdomain region of IgA-Fc, at sites overlapping with that used by the human IgA-receptor CD89. These findings have allowed us to propose a possible mechanism by which bacterial IgA-BPs may interfere with IgA effector function, thereby contributing to bacterial virulence. This study also highlights the potential of the bacterial IgA-BPs, and the IgA-binding peptide in particular, as tools for studies of the structure and function of IgA.
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References


Footnotes

1 The abbreviations used are: FcαR, Fcα receptor; GAS, group A streptococcus; GBS, group B streptococcus; IgA-BP, IgA-binding protein; IgG-BP, IgG-binding protein; NIP, 3-hydroxy-4-nitro-5-iodophenylacetate; CHO, Chinese hamster ovary; PBST, phosphate-buffered saline containing 0.1% Tween 20; PBSAT, phosphate-buffered saline containing 0.02% NaN₃ and 0.05% Tween-20; BSA, bovine serum albumin; HBSS, Hanks’ buffered saline solution; ELISA, Enzyme-linked immunosorbent assay; PMA, phorbol 12-myristate 13-acetate.

2 T. Areschoug, in preparation

3 The atomic coordinates for the molecular model of human IgA1 are available in the Research Collaboratory for Structural Bioinformatics Protein Databank under PDB accession number 1iga.
Legends to figures

FIG. 1. **Comparison of streptococcal IgA-binding proteins.** (A) Western blot analysis of purified preparations of the Sir22 and Arp4 proteins of *S. pyogenes*, and the β protein of group B streptococcus. Blotting membranes were incubated with anti-Sir22 serum or anti-β serum, as indicated, and bound antibodies were detected with radiolabelled protein A or protein G, as described in Materials and methods. No bands were seen in control blots incubated with preimmune serum. An equivalent Coomassie-stained SDS gel is shown on the left. (B) Alignment of the IgA-binding regions of Sir22 and Arp4.

FIG. 2. **Binding of wildtype IgA1, wildtype IgG1 and domain swaps to streptococcal IgA-binding molecules.** The antibodies were diluted as indicated and analysed by ELISA for ability to bind to the Sir22-derived IgA-binding peptide and to the Arp4 and β proteins, immobilized in microtiter wells. Results were normalised by expressing ELISA absorbance as a fraction of mean absorbance seen with wildtype IgA1 at 1 x 10^{-6} M. The controls for the IgA-binding peptide was the M5-N peptide, the control for Arp4 was the Arp4Δ451 mutant, and the control for β was the Rib protein from GBS. All of these control molecules lack ability to bind IgA. The control binding shown is in each case the mean of the fractional binding seen for the panel of antibodies, all at 1 x 10^{-6} M. This value varied very little from antibody to antibody (standard deviations of ± 0.02 for M5-N, ± 0.06 for Arp4Δ451, and ± 0.19 for Rib). The experiment was performed twice with very similar results.

FIG. 3. **Use of Ig domain swap mutants to analyse binding of IgA to β protein expressed on the surface of GBS.** The analysis was performed with whole A909 bacteria and purified Ig proteins, as described in Materials and methods. Each value represents the average of duplicate determinations. This experiment was performed twice, with very similar results.
FIG. 4. **Binding of IgA1s with point mutations in the predicted LLG loop in Cα2.** (A) Binding to the streptococcal Sir22, Arp4 and β proteins, and the Sir22-derived IgA-binding peptide. The results were normalised by expressing ELISA absorbance as a fraction of mean absorbance seen with wildtype IgA1 at 1 x 10^{-6} M. The following non-IgA-binding streptococcal peptide/proteins served as negative controls: M5-N peptide for the IgA-binding peptide, Arp4Δ451 for Arp4, and Rib for β protein. The control binding shown is in each case the mean of the fractional binding seen for the panel of antibodies, all at 1 x 10^{-6} M. This value varied very little from antibody to antibody (standard deviations of ± 0.01 for M5-N, ± 0.04 for Arp4Δ451, and ± 0.08 for Rib). The experiment was performed twice with very similar results. (B) Binding to whole streptococci expressing one of the IgA-binding proteins Sir22, Arp4 or β protein. The protein expressed and the bacterial species are indicated above each panel. Each panel shows results obtained with the IgA1 wild type protein (IgA1) and different mutant proteins, as indicated. Each value represents the average of duplicate determinations and each experiment was performed at least twice, with very similar results.

FIG. 5. **Binding of IgA1s with point mutations in the predicted PLAF loop in Cα3.** (A) Binding to the streptococcal Sir22, Arp4 and β proteins, and the Sir22-derived IgA-binding peptide. The results were normalised by expressing ELISA absorbance as a fraction of mean absorbance seen with wildtype IgA1 at 1 x 10^{-6} M. Controls were as for Figure 4. The control binding shown is in each case the mean of the fractional binding seen for the panel of antibodies, all at 1 x 10^{-6} M. This value varied very little from antibody to antibody (standard deviations of ± 0.007 for M5-N, ± 0.02 for Arp4Δ451, and ± 0.08 for Rib). The experiment was performed twice with very similar results. (B) Binding to whole streptococci expressing one of the IgA-binding proteins Sir22, Arp4 or β protein. The protein expressed and the bacterial species are indicated above each panel. Each value represents the average of duplicate determinations and each experiment was performed at least twice, with very similar results.
FIG. 6. **Inhibition of binding of IgA1 to CD89 on neutrophils, assessed by rosette formation.** (A) Inhibition test with Sir22 and the Sir22-derived IgA-binding peptide, and with the non-IgA-binding M5-N peptide. (B) Inhibition test with Arp4, and with the non-IgA-binding deletion mutants Arp4Δ450 and Arp4Δ451. (C) Inhibition test with β protein, and with the non-IgA-binding protein G. The results were normalised and expressed such that rosette formation in the absence of IgA1-coating was used to provide the value for 0% rosettes (equivalent to 100% inhibition), while that in the absence of inhibitor was used as the value for 100% rosettes (= 0% inhibition).

FIG. 7. **Analysis of inhibition of an IgA1-mediated neutrophil respiratory burst by Arp4, and its non-IgA-binding deletion mutants Arp4Δ450 and Arp4Δ451.** Chemiluminescence (CL, arbitrary units) was induced by IgA1 alone (■) or in the presence of bacterial inhibitor, as indicated. The negative control (△) shows chemiluminescence observed in the absence of IgA1 and inhibitor. Inhibitor at ● 5 x 10^{-10} M, ▲ 5 x 10^{-9} M, □ 5 x 10^{-8} M, ○ 5 x 10^{-7} M. The results of representative experiments are shown. The experiments were performed at least twice with very similar results.

FIG. 8. **Molecular model of human IgA1 Fc, highlighting residues at the Cα2/Cα3 interface critical for binding to streptococcal IgA-binding proteins.** This model of IgA1 Fc is adapted from ref. 37^3^. The two heavy chain backbones are shown as blue ribbons, with the LLG loop (Leu257 - Gly259) of the Cα2 domain and the PLAF loop (Pro440 - Phe 443) of the Cα3 domain represented in yellow. Residues Leu257, Pro440 and Phe443, which are predicted to play particularly important roles for binding of the streptococcal proteins, are highlighted in green. The C-terminal tailpieces are omitted.
Table I
Mutant antibodies used

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<thead>
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<th>Antibody</th>
<th>Structural notes</th>
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<tr>
<td><strong>Domain swap antibodies</strong></td>
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<tr>
<td>α1α2γ3</td>
<td>CH1, hinge and CH2 of human IgA1 and CH3 of human IgG1</td>
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<td>(IgA derived sequence ends at residue Ser341)</td>
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<tr>
<td>γ1γ2α3</td>
<td>CH1, hinge and CH2 of human IgG1 and CH3 of human IgA1</td>
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<td><strong>Cα2 domain LLG loop mutants</strong></td>
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<tr>
<td>L257R</td>
<td>Leu257 of human IgA1 replaced by Arg</td>
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<td>G259R</td>
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<td><strong>Cα3 domain PLAF loop mutants</strong></td>
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<tr>
<td>LA441-442MN</td>
<td>Leu441 and Ala442 replaced by Met and Asn respectively</td>
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Figure 1

A  

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<tr>
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<td>Arp4</td>
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<td>Anti-β</td>
</tr>
<tr>
<td>β</td>
<td><img src="image.png" alt="Image" /></td>
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</tbody>
</table>

B

Sir22  45 EPRYKALRGENQDLREKERKYQDKIKKLE73

Arp4  45 DPQYRALGENQDLRKREGQYQDKIEELE73
Figure 2

Sir22-derived peptide

Fractional Binding (normalised)

- IgA1
- IgG1
- γ1γ2α3
- α1α2γ3
- control

Fractional Binding (normalised)

Arp4

Fractional Binding (normalised)

β protein

log[antibody]
Figure 3

![Graph showing binding percentage vs. log concentration of antibody. The graph compares IgA1, IgG1, γ1γ2α3, and α1α2γ3.](image-url)
Figure 5

A

Fractional Binding (normalised)

Sir22

Sir22-derived peptide

Fractional Binding (normalised)

Arp4

β protein

log[antibody]

log[antibody]

B

Sir22 (S. pyogenes)

Arp4 (S. pyogenes)

β protein (Group B streptococcus)

Binding (%)

log[antibody] (M)

log[antibody] (M)

log[antibody] (M)
Streptococcal IgA-binding proteins bind in the Cα2-Cα3 interdomain region and inhibit binding of IgA to human CD89
Richard J. Pleass, Thomas Areschoug, Gunnar Lindahl and Jenny M. Woof

J. Biol. Chem. published online November 28, 2000

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