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Cell Host & Microbe
Short Article

Nonimmunodominant Regions Are Effective as Building Blocks in a Streptococcal Fusion Protein Vaccine

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DOI 10.1016/j.chom.2007.10.003

SUMMARY

Identification of antigens that elicit protective immunity is essential for effective vaccine development. We investigated the related surface proteins of group B *Streptococcus*, Rib and α, as potential vaccine candidates. Paradoxically, nonimmunodominant regions proved to be of particular interest as vaccine components. Mouse antibodies elicited by Rib and α were directed almost exclusively against the C-terminal repeats and not against the N-terminal regions. However, a fusion protein derived from the nonimmunodominant N-terminal regions of Rib and α was much more immunogenic than one derived from the repeats and was immunogenic even without adjuvant. Moreover, antibodies to the N-terminal fusion protein protected against infection and inhibited bacterial invasion of epithelial cells. Similarly, the N-terminal region of *Streptococcus pyogenes* M22 protein, which is targeted by opsonic antibodies, is nonimmunodominant. These data indicate that nonimmunodominant regions of bacterial antigens could be valuable for vaccine development.

INTRODUCTION

When a microbial surface protein elicits an antibody response, some epitopes are usually immunodominant, while other ones are nonimmunodominant, i.e., elicit poor or no immune responses. Little is known about the molecular basis for immunodominance, but it is often assumed that immunodominant epitopes are particularly attractive as vaccine targets (Holtappels, 2005). However, for a pathogen it should be an advantage if a protein region, which potentially is an important target for protective immunity, is nonimmunodominant (Nara and Garrity, 1998). In this case, the nonimmunodominant region may be of particular interest as a vaccine target, if conditions can be found that allow a host immune response to that region. We analyzed this hypothesis for two surface proteins of group B *Streptococcus* (GBS; *Streptococcus agalactiae*) and present evidence that a fusion protein, derived from subregions that are poorly immunogenic within the intact proteins, is highly immunogenic and elicits good protective immunity.

GBS is the major cause of invasive bacterial infections, including meningitis, in the neonatal period (Edwards and Baker, 2001). Recent vaccine studies have focused on surface proteins (Larsson et al., 1996; Brodeur et al., 2000; Lindahl et al., 2005; Maione et al., 2005), and it has been suggested that a combination of four different proteins, including three pilus components and the previously described Sip (Brodeur et al., 2000), may be used as a universal vaccine (Maione et al., 2005). However, the potential of this vaccine remains unclear, because subsequent studies (Buccato et al., 2006) showed that the major pilus component was not immunogenic without CFA, an adjuvant that cannot be used in humans.

Most clinically important strains of GBS express either Rib or α, two related surface proteins with repetitive sequence that elicit antibodies protecting against strains expressing the corresponding protein (Michel et al., 1992; Wästfelt et al., 1996; Lindahl et al., 2005). Importantly, Rib is expressed by most serotype III strains, which cause almost all cases of meningitis, and by all strains of a hypervirulent type III clone (Stålhammar-Carlemalm et al., 1993; Edwards and Baker, 2001; Brimil et al., 2006; Brochet et al., 2006). Because Rib and α elicit protective immunity when administered with alum, an adjuvant accepted for human use (Larsson et al., 1999), these proteins are of interest for vaccine development.

With the long-term goal to develop a GBS vaccine based on a single protein, our work was focused on analyzing whether a fusion protein derived from Rib and α would elicit protective immunity. The large size of Rib and α, and the genetic instability of the repeat regions (Michel et al., 1992; Wästfelt et al., 1996), implied that a fusion protein should be derived from subregions, but it was not obvious which regions to chose, because studies of α have indicated that the antibody response to this protein is complex (Gravekamp et al., 1996, 1997; Kling et al., 1997).

Using a mouse model, we analyzed antibodies elicited by intact Rib and α and present evidence that these

antibodies, which are protective, are directed almost exclusively against the repeats. Thus, the repeat regions of Rib and α are immunodominant and contain protective epitopes, suggesting that a fusion protein vaccine should be derived from the repeats. However, the possibility that the immunodominance of the repeats reflects an immune evasion mechanism prompted us to also study the N-terminal regions. We show that a fusion protein derived from the nonimmunodominant N-terminal regions of the two proteins elicited good protective immunity and had properties much superior to a fusion protein of similar size derived from the repeats. Analysis of the immune response to another bacterial surface protein, the well-known M protein of *Streptococcus pyogenes* (group A streptococcus) supported the results obtained with GBS proteins. These data focus interest on nonimmunodominant protein regions for vaccine development.

RESULTS AND DISCUSSION

The Repeat Regions of Rib and α Are Immunodominant

The tandem repeats of Rib and α are identical within each protein, but the sequences of the repeats differ between Rib and α (Michel et al., 1992; Wästfelt et al., 1996) (Figure 1A). For both Rib and α, the number of repeats varies between bacterial isolates, but except for this variation the available evidence indicates that Rib proteins expressed by different GBS isolates have identical sequences, and the same is true for α (Kong et al., 2002; Lindahl et al., 2005). In spite of the considerable aa residue identity, the two proteins show little or no antigenic crossreactivity (Larsson et al., 1996). The studies described here employed the intact proteins and a series of recombinant proteins (Figures 1B and 1C).

An inhibition test was used to analyze whether mouse anti-Rib antibodies, elicited with alum as adjuvant, were directed against the N-terminal region and/or the repeat region (Figure 1D). Of these antibodies, >90% were IgG (data not shown). Binding to Rib was completely inhibited by Rib, as expected, and almost complete inhibition was also observed with Rib2R, while RibN had a very small effect. Thus, almost all antibodies to intact Rib were directed against the repeats, i.e., this region was immunodominant. The inhibition by Rib2R was specific, because Rib2R did not inhibit binding of antibodies against an unrelated GBS protein (data not shown). In the α system, a dot blot analysis showed that anti-α reacted with intact α but not with αN (Figure 1E, left), indicating that the repeats are immunodominant also in this protein, as previously suggested (Gravekamp et al., 1997). The lack of reactivity of αN was not an inherent property of that construct, because anti-αN reacted with both α and αN (Figure 1E, right).

The reason for the immunodominance of the repeats in Rib and α is not known. Multivalent interactions between the repeats and Ig receptors on B cells may contribute, but Rib and α are probably not T cell-independent antigens, because they elicit good IgG responses in rabbits

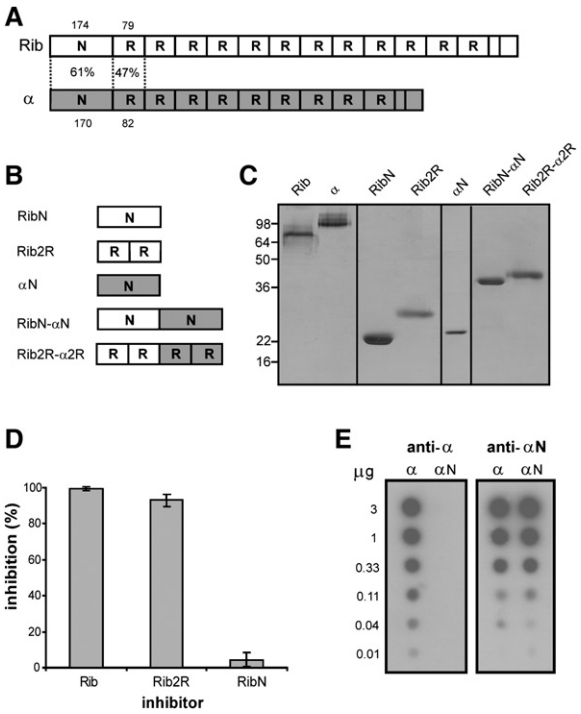

Figure 1. GBS Proteins Used and Identification of Immunodominant Regions

(A) Each of the Rib and α proteins includes a unique N-terminal region (N) and a long repeat (R) region. The proteins expressed by strains BM110 and A909 have 12 and 9 repeats, respectively, as indicated. The number of aa residues in different regions and residue identity (in percent) are indicated. The wall anchoring regions are located at the C-terminal ends.

(B) Recombinant proteins derived from Rib and α.

(C) Analysis of purified proteins by SDS-PAGE. The figure is combined from two gels. Numbers to the left indicate molecular mass in kD. Because Rib and α migrate aberrantly in gels (Wästfelt et al., 1996), the apparent sizes of the proteins do not correspond to those deduced from aa sequences.

(D) Inhibition test with mouse anti-Rib antibodies. The antibodies, elicited with alum as adjuvant, were used to detect pure Rib immobilized in microtiter wells, and binding was inhibited by addition of the pure protein indicated (2 μg). SDs are indicated. Very similar results were obtained with two mouse sera raised with alum and one rabbit antiserum raised with CFA.

(E) Dot blot analysis. The mouse antisera indicated (prepared with alum) were used to detect pure α and αN immobilized on membranes. The amounts of α and αN applied are indicated (in μg) to the left. Bound mouse antibodies were detected by incubation with rabbit anti-mouse Ig, followed by radiolabeled protein G and autoradiography.

and mice (Stålhammar-Carlemalm et al., 1993; Larsson et al., 1999). Importantly, the poor immune response to the N-terminal regions was not due to masking, because these regions are available to antibodies (see below). Possibly, the repeats directly interfere with the immune response to the N-terminal region.

Fusion Proteins Derived from Rib and α Elicit Protective Antibodies

Because antibodies to Rib and α are directed almost exclusively against the repeats and are protective, it would

appear that a fusion protein vaccine should be derived from the repeats. However, the available data did not exclude the possibility that the isolated N-terminal regions might be more protective than the repeats and would be suitable for the construction of a fusion protein. To analyze this hypothesis, we used the Rib system to directly compare the protective ability of antibodies directed against the N-terminal region or the repeats. The analysis employed rabbit antibodies elicited by RibN or Rib2R and a mouse model of passive vaccination. Both antisera reacted with Rib-expressing bacteria, but not with a Rib-negative mutant, i.e., they recognized epitopes exposed on the native form of Rib (Figure S1A in the Supplemental Data available with this article online). Because anti-RibN had an ~7-fold higher titer than anti-Rib2R, it was diluted accordingly, to allow direct comparisons in the vaccination model. Each antiserum protected against lethal infection (Figure S1B), showing that protective epitopes are present in the N-terminal region and in the repeat region of Rib, as previously reported for the α protein (Kling et al., 1997).

Our data suggested that a fusion protein vaccine might be derived either from the N-terminal regions or from the repeats of Rib and α, making it of interest to compare the two types of fusion protein. However, it was not clear that it was necessary to derive a fusion protein from the N-terminal regions, because these regions exhibit 61% residue identity (Figure 1A), suggesting that they might crossreact. Crossreactivity could have gone unnoticed in previous studies, which employed antibodies against the intact proteins, i.e., antibodies directed mainly against the repeats. This hypothesis was analyzed with anti-RibN and anti-αN (Figure 2A). Each of these antisera reacted with the corresponding intact protein but not with the heterologous protein, indicating that the N-terminal regions lack crossreactivity. The fusion protein RibN-αN was therefore constructed and compared with a fusion protein of similar size derived from the repeats, Rib2R-α2R.

In the rabbit, RibN-αN elicited better antibody responses than Rib2R-α2R, as judged by reactivity with Rib- or α-expressing bacteria (Figure 2B). For comparisons in the mouse model of passive protection, anti-(RibN-αN) was therefore diluted to the same titer as anti-(Rib2R-α2R). Each antiserum protected against a Rib-expressing strain of capsular type III and an α-expressing strain of type Ia (Figure 2C). Thus, each of the two fusion proteins elicited protective antibodies directed against Rib and α.

Antibodies to RibN-αN Protect against GBS of Multiple Serotypes

Further work was focused on RibN-αN, for several reasons. First, the analysis of rabbit antisera suggested that this fusion protein was most immunogenic. Second, the N-terminal regions of Rib and α are probably located farthest from the bacterial surface, suggesting that these regions may be of particular importance for interactions with the host. Third, studies of the RibN-αN protein were of general interest for analysis of the hypothesis that nonimmunodominant protein regions may be of particular importance as targets for protective antibodies.

The passive vaccination model was used to analyze whether protection provided by anti-(RibN-αN) is independent of capsular serotype. Good protection was observed in experiments with a Rib-expressing type II strain and an α-expressing type Ib strain (Figure S2A). Thus, anti-(RibN-αN) protected against Rib- and α-expressing strains of the four classical GBS serotypes, Ia, Ib, II, and III. This protection was not unspecific, because anti-(RibN-αN) did not protect against a Rib-negative mutant (Figure S2B).

Antibodies to RibN-αN also recognized strains expressing two proteins related to Rib and α, the R28 and ε proteins, which are expressed by many strains of serotypes V and Ia, respectively (Lindahl et al., 2005; Brimil et al., 2006). However, anti-(RibN-αN) did not protect against these strains (data not shown), suggesting that protection against strains expressing R28 or ε may require construction of a fusion protein including the N-terminal regions of these proteins.

Active Immunization

In active immunizations with pure RibN-αN, this protein was equally immunogenic for mice when administered with CFA, alum, or PBS (Figure 2D). In contrast, the Rib2R-α2R protein elicited antibodies in only one of four CFA mice and no antibodies in mice that received antigen with alum or PBS (data not shown). Thus, Rib2R-α2R was poorly immunogenic for mice, although intact Rib and α elicited good immune responses to the repeats.

Importantly, active immunization with RibN-αN and alum protected mice against Rib- and α-expressing strains (Figure 2E). Thus, RibN-αN elicited protective immunity with an adjuvant accepted for human use. These data corroborate the conclusion that RibN-αN is of interest as a vaccine component. The antibodies elicited by RibN-αN were almost exclusively of the IgG class (data not shown), implying that a fetus may be protected by maternal anti-(RibN-αN) antibodies. This conclusion is supported by the finding that IgG antibodies to intact Rib and α are transferred over the human placenta (Larsson et al., 2006).

Antibodies to RibN-αN Prevent Invasion of Epithelial Cells

Studies in a primate model have indicated that GBS invades epithelial cells during an infection (Rubens et al., 1991). Because α promotes invasion of GBS in vitro (Bolduc et al., 2002), we compared the role of Rib and α in invasion, using GBS mutants (Figure 3A). Invasion of human ME180 cells was reduced 20-fold for the Rib mutant and 4-fold for the α mutant, as compared to the parental strains. Thus, Rib and α share the ability to promote invasion. This potentially important function was efficiently blocked by anti-(RibN-αN) (Figure 3B). The reduction in invasion was not due to antibody-mediated bacterial clumping, which did not occur under the conditions used (data not shown). These results suggest that the presence of antibodies to RibN-αN may prevent

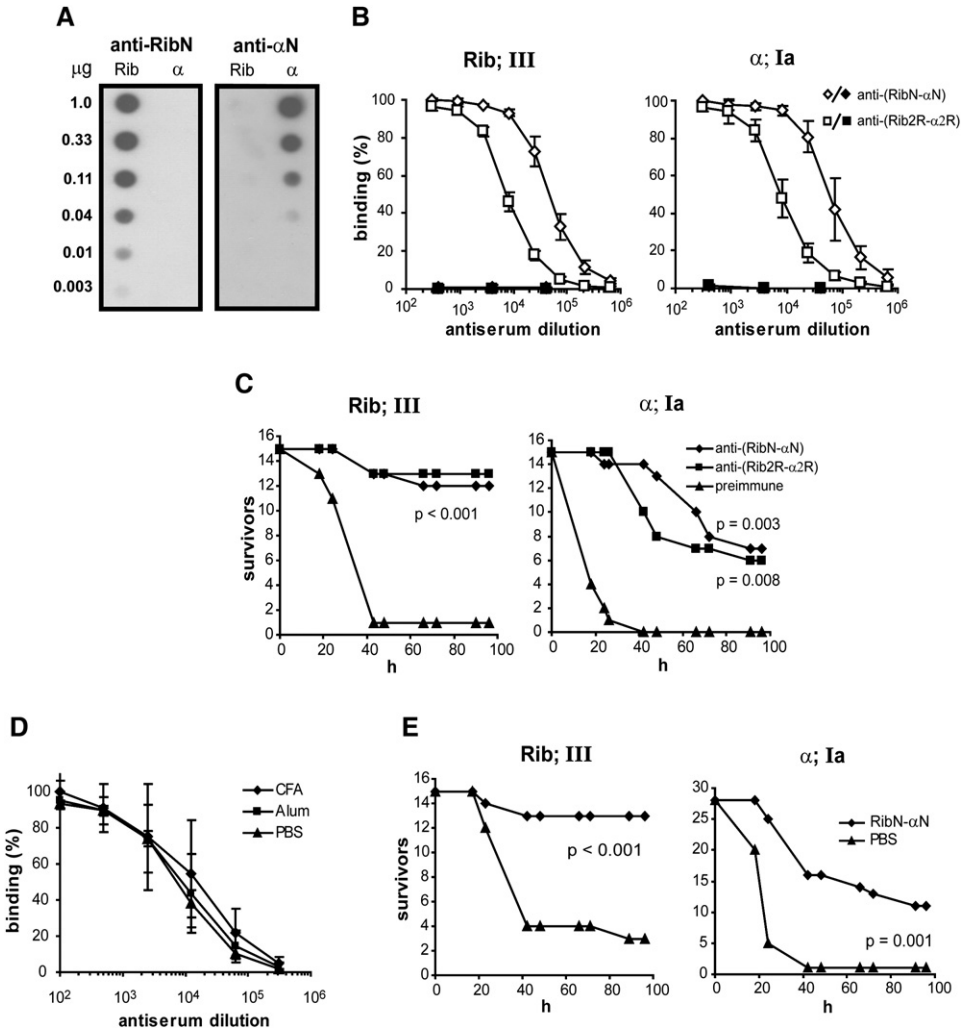

Figure 2. Fusion Proteins Derived from Rib and α Elicit Protective Antibodies

(A) Dot blot analysis of crossreactivity between the N-terminal regions of Rib and α. Mouse anti-RibN and anti-αN were used to detect immobilized Rib and α, as indicated.

(B) Characterization of rabbit antibodies against RibN-αN and Rib2R-α2R. Each antiserum reacted with whole bacteria of the Rib-expressing type III strain BM110 (left, open symbols) but not with a Rib-negative mutant (left, closed symbols). Similarly, each antiserum reacted with bacteria of the α-expressing type Ia strain A909 (right, open symbols) but not with an α-negative mutant (right, closed symbols). SDs are indicated. Similar data were obtained with two rabbit sera of each type.

(C) Passive vaccination of mice with antibodies to the fusion proteins RibN-αN and Rib2R-α2R, followed by challenge with the Rib-expressing type III strain BM110 or the α-expressing type Ia strain A909, as indicated. Control mice received preimmune serum. Survival after challenge was recorded during a 96 hr period. Because the titer of anti-(RibN-αN) was higher than that of anti-(Rib2R-α2R) (B), it was diluted 6-fold, giving the two sera the same titer. The p values refer to comparisons with the preimmune control at 96 hr.

(D) Immunogenicity of the RibN-αN protein when administered with or without adjuvant. Groups of four mice were immunized with RibN-αN mixed with CFA, alum, or PBS, boosted after 4 weeks, and bled 2 weeks later. The mouse sera were analyzed for reactivity with the pure antigen immobilized in microtiter wells. Bound mouse antibodies were detected by incubation with rabbit anti-mouse Ig, followed by radiolabeled protein G. SDs are indicated.

(E) Active vaccination with RibN-αN. Mice (number indicated on the y axis) were immunized with pure RibN-αN mixed with alum, boosted after 4 weeks, and challenged 2 weeks later with the Rib-expressing type III strain BM110 (left) or the α-expressing type Ia strain A909 (right). Control mice received PBS and alum. The data for the α strain are pooled from two experiments. The p values refer to comparisons at 96 hr.

Rib- and α-expressing GBS from invading human cells in vivo. Of note, it was not of relevance to study the effect of the antibodies on epithelial cell adhesion, which is not affected by Rib or α (Bolduc et al., 2002; Lindahl et al., 2005).

The N-Terminal Region of Streptococcal M22 Protein Is Nonimmunodominant

The results obtained with Rib and α suggested that regions, which are important targets for protective antibodies, might be nonimmunodominant also in other

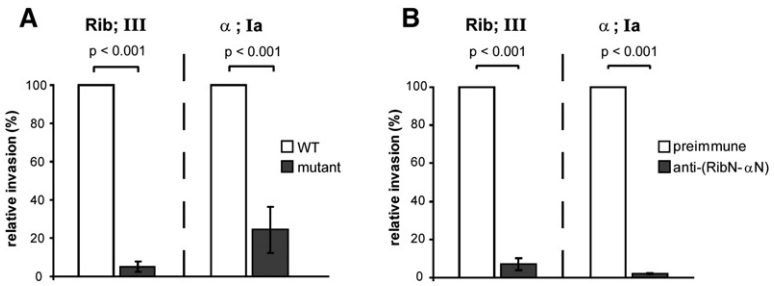

Figure 3. Antibodies to RibN-αN Prevent Invasion of Human Epithelial Cells

(A) Role of Rib and α in epithelial cell invasion. A Rib-negative mutant of strain BM110 (left) and an α-negative mutant of strain A909 (right) were compared with the corresponding wild-type (WT) bacteria for ability to invade cells of the human cervical cell line ME180.

(B) Inhibition of epithelial cell invasion by anti-(RibN-αN). Bacteria of strain BM110 (left) or A909 (right) were preincubated with rabbit anti-(RibN-αN) or with preimmune serum before use in the invasion assay.

All data in (A) and (B) are based on three different experiments. SDs and p values are indicated.

microbial surface proteins. To analyze this hypothesis, we studied *Streptococcus pyogenes* M protein, a major virulence factor with a variable N-terminal region targeted by opsonizing antibodies and a conserved C repeat region (Kehoe, 1994). Our analysis was focused on the extensively studied M22 protein.

Antibodies elicited by intact pure M22 were analyzed for reactivity with different parts of the protein. For this purpose, we used three long peptides derived from M22: the M22-N, Sap22, and C22 peptides (Figure 4A). Previous work had shown that antibodies to M22-N and Sap22 promoted opsonization, while antibodies to C22 did not (Carlsson et al., 2003). Interestingly, antibodies to intact M22 reacted well with C22 but showed little or no reactivity with M22-N or Sap22 (Figure 4B, left). This lack of reactivity with M22-N and Sap22 was not caused by a technical problem, because anti-(M22-N) reacted with M22-N, as expected (Figure 4B, right) and anti-Sap22 reacted with Sap22 (data not shown). Importantly, the M22-N and Sap22 peptides retain the structure of the corresponding domains in M22, as shown by their ability to bind ligands (Johnsson et al., 1999; Morfeldt et al., 2001), so absence of antibodies to these peptides reflects absence of antibodies to the N-terminal part of M22. These data indicate that the N-terminal part of M22, which is targeted by opsonizing antibodies, is nonimmunodominant. This conclusion is supported by a study of the M6 protein, which has a centrally located B repeat region that apparently is immunodominant (Fischetti and Windels, 1988). Thus, the data obtained with M22 corroborate those obtained with the GBS proteins Rib and α, and they focus interest on nonimmunodominant regions as targets for protective antibodies.

Of note, the immunodominance of the C repeat region in M22 cannot be explained by a molar excess of repeats, because the size of the repeat region is similar to that of the nonimmunodominant N-terminal part of M22. Similarly, the data on Rib and α in Figure 1 indicate that the immunodominance of the repeats in these proteins cannot simply be explained by molar excess. An interesting alternative hypothesis predicts that both M protein and Rib/α exploit a specific mechanism, by which the repeat regions of these proteins actively interfere with the formation of antibodies to the N-terminal regions.

Concluding Remarks

The work reported here shows that the N-terminal regions of the GBS proteins Rib and α are nonimmunodominant when present within the intact proteins but elicit good

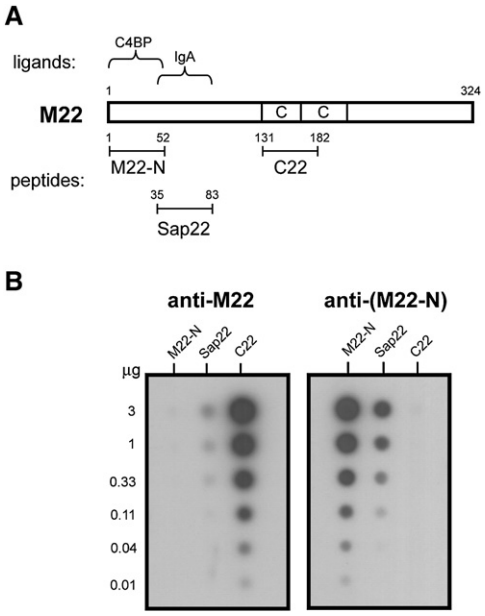

Figure 4. The N-Terminal Part of the *S. pyogenes* M22 Protein Is Nonimmunodominant

(A) Schematic representation of the M22 protein (Carlsson et al., 2003). The N-terminal hypervariable region of M22 binds the human complement inhibitor C4BP, while an adjacent semivariable region binds human IgA. The C-terminal part of M22 includes the conserved C repeat region. Three long peptides (M22-N, Sap22, and C22) were derived from these regions, as indicated. The two peptides M22-N and Sap22 specifically bind C4BP and IgA, respectively.

(B) Dot blot analysis. The rabbit antisera indicated were used to detect M22-derived peptides immobilized on membranes. The amounts of peptide applied are indicated to the left. Rabbit anti-M22 reacted almost exclusively with the C22 peptide (left blot). This lack of reactivity was not due to a technical problem, because antiserum raised to M22-N reacted with M22-N and also showed some reactivity with the overlapping Sap22 peptide, as expected (right blot). Bound antibodies were detected by incubation with radiolabeled protein G, followed by autoradiography. Very similar results were obtained with two rabbit antisera of each type.

protective immune responses when used to construct a fusion protein. Remarkably, this fusion protein was much more immunogenic than one of similar size derived from the C-terminal repeat regions, which are immunodominant within the intact proteins. The paradoxical conclusion from these studies is that nonimmunodominant protein regions may be of particular interest as vaccine components.

The function of the N-terminal region in Rib and α during infections in humans is not known, but it seems possible that this region protrudes beyond the bacterial capsule at some stages of an infection. This location may allow the N-terminal region to play a key role in pathogenesis, but could also make it particularly important as the target for human protective antibodies. A mechanism may therefore have evolved that allows the repeat regions of Rib and α to interfere with the immune response to the adjacent N-terminal regions. Characterization of this mechanism may provide information that is of general interest with regard to microbial immune evasion mechanisms, vaccine development, and immunosuppression.

With regard to GBS vaccines, our data indicate that the RibN-αN fusion protein is immunogenic even without adjuvant, making it a promising component of a vaccine for human use. A vaccine including this fusion protein may elicit protective immunity against many clinically important GBS strains, including almost all strains causing meningitis. The RibN-αN protein may also be suitable as carrier in a polysaccharide conjugate, allowing the development of a single vaccine that protects against different pathogens (Reddin et al., 2001; Lindahl et al., 2005).

The conclusion that nonimmunodominant regions may be of particular interest as targets for antibodies is supported by our analysis of the *S. pyogenes* M22 protein. Immunization with that M protein did not elicit antibodies to the N-terminal part, which is targeted by opsonizing antibodies, but elicited antibodies to the conserved C repeat region, a region found in all M proteins. This finding indicates that *S. pyogenes* evades antibodies to the N-terminal region of M protein not only through the well-known sequence variability, but also through nonimmunodominance. This nonimmunodominance of the N-terminal region might explain early observations that opsonizing anti-M antibodies appear late during an infection (Denny et al., 1957; Fischetti and Windels, 1988).

Vaccine development has recently been revolutionized by the advent of reverse vaccinology, in which many surface proteins, identified from genome sequences, are evaluated as potential vaccine components (Rappuoli, 2000). This approach has allowed the identification of a number of promising vaccine candidates (Serruto and Rappuoli, 2006), but it is noteworthy that a microbial surface protein may not be identified by reverse vaccinology, if protective epitopes are nonimmunodominant, when present within the intact protein. As a complement to reverse vaccinology, it may therefore become important to analyze major surface proteins for the presence of subregions that elicit protective immunity.

EXPERIMENTAL PROCEDURES

Bacterial Strains

GBS strains used were A909 (α; type Ia), SB35*sed*1 (α; type Ib), 1954/92 (Rib; type II), and BM110 (Rib; type III) (Stålhammar-Carlemalm et al., 1993; Larsson et al., 1996). Strain BM110 is a member of the hypervirulent ST-17 clone (Brochet et al., 2006). All GBS strains were grown in Todd-Hewitt broth at 37°C without shaking.

Fusion Proteins and Other Derivatives of Rib and α

Genes encoding fusion proteins and protein fragments were constructed by standard procedures, using primers listed in Table S1. Fragments of the *rib* gene in strain BM110 (encoding Rib) and the *bca* gene in strain A909 (encoding α) were cloned into pGEX-6P-2 (Amersham) and used for preparation of GST-fusions. After removal of the GST moiety, the purified derivatives had the N-terminal sequence GPLGS. RibN and Rib2R correspond to aa residues 1–174 and 175–332, respectively, of Rib, while αN corresponds to residues 1–170 of α (numbering of Wästfelt et al., 1996). RibN-αN contains aa 1–174 of Rib and aa 1–170 of α, while Rib2R-α2R contains aa 175–332 of Rib and aa 171–334 of α. Due to the procedures used, each fusion protein included the sequence EF between the two regions.

Other Proteins, Peptides, Antisera

Rib and α were purified from GBS strains BM110 and A909, respectively (Stålhammar-Carlemalm et al., 1993; Larsson et al., 1996). The *S. pyogenes* M22 (Sir22) protein was purified after expression in *E. coli*, as described (Stenberg et al., 1994). The synthetic ~50 residue peptides M22-N, Sap22, and C22, all derived from M22, have been described (Johnsson et al., 1999; Morfeldt et al., 2001; Carlsson et al., 2003). These peptides were dimerized via a C-terminal Cys residue, not present in M22, to promote ligand binding and formation of a coiled-coil structure (Morfeldt et al., 2001). Rabbit antisera to proteins derived from Rib and α were raised by subcutaneous (s.c.) immunization with ~35 μg protein in CFA, followed by two boosters with ~18 μg protein in IFA. Rabbit antiserum to M22 was raised by immunization s.c. with pure protein (100 μg) mixed with alum; three injections were given at intervals of 3–4 weeks, and the rabbits were bled 2 weeks later. Rabbit antiserum to the M22-N peptide has been described (Carlsson et al., 2003). Mice were immunized s.c. with 25 μg protein with or without adjuvant, as indicated, boosted after 4 weeks with 12 μg protein, and bled 2 weeks later. For the CFA mice, the booster was administered with IFA.

Antibody-Binding Assays with Immobilized Proteins or Whole Bacteria

Antibody-binding and inhibition tests were performed essentially as described (Stålhammar-Carlemalm et al., 1993; Wästfelt et al., 1996). Bound rabbit antibodies were detected with radiolabeled protein G, and bound mouse antibodies were detected by incubation with rabbit anti-mouse Ig followed by radiolabeled protein G. Binding was calculated in % of protein G bound at the lowest antiserum dilution. Under these conditions, ~70% of the added protein G was bound; this is the maximal binding that can be achieved, because some radiolabeled protein G molecules have lost ability to bind IgG. The sensitivity of inhibition tests (Figure 1D) was optimized by using a coating solution at 0.05 μg/ml and mouse serum diluted 1000-fold. All tests were performed at least three times, and SDs are indicated. Dot blot analysis with proteins immobilized on membranes was performed essentially as described (Stålhammar-Carlemalm et al., 1993). When a membrane was incubated with a mouse antiserum, bound antibodies were detected by incubation with rabbit anti-mouse Ig, followed by radiolabeled protein G and autoradiography. After incubation with a rabbit antiserum, bound antibodies were directly detected by incubation with protein G.

Mouse Vaccinations

Passive vaccinations were performed as described (Stålhammar-Carlemalm et al., 1993), using C3H/HeN mice, rabbit antiserum, and an $\sim LD_{90}$ dose of log-phase bacteria (10^5–10^6 CFU, depending on the strain used). Survival was recorded during a 96 hr period. For active vaccinations, mice were immunized s.c. with 10 μg protein, mixed with alum. A 5 μg booster was given after 4 weeks, with alum. Control mice received PBS and alum. Two weeks after the booster, the mice were challenged with an $\sim LD_{90}$ dose of bacteria, and survival was recorded. All animal experiments were approved by the Lund/Malmö review board on animal studies and conformed to the relevant regulatory standards.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, one supplemental table, and two supplemental figures and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/2/6/427/DC1/.

ACKNOWLEDGMENTS

This work was supported by the Swedish Research Council; the U.K. Meningitis Research Foundation; the Swedish Government Support for Clinical Research (ALF); the Royal Physiographic Society in Lund; and the Trusts of Cornell, Crafoord, Golje, Kock, Lundström, McDonald, and Österlund. We thank Fredrik Nilsson for help with statistical analysis.

Received: May 16, 2007
Revised: August 27, 2007
Accepted: October 12, 2007
Published: December 12, 2007

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