Protein Rib: A Novel Group B Streptococcal Cell Surface Protein that Confers Protective Immunity and Is Expressed by Most Strains Causing Invasive Infections

By Margaretha Stålhammar-Carlemalm, Lars Stenberg, and Gunnar Lindahl

From the Department of Medical Microbiology, University of Lund, S-22362 Lund, Sweden

Summary

The group B *Streptococcus*, an important cause of invasive infections in the neonate, is classified into four major serotypes (Ia, Ib, II, and III) based on the structure of the polysaccharide capsule. Since the capsule is a known virulence factor, it has been extensively studied, in particular in type III strains, which cause the majority of invasive infections. Two cell surface proteins, α and β, have also been studied in detail since they confer protective immunity, but these proteins are usually not expressed by type III strains. We describe here a cell surface protein, designated protein Rib (resistance to proteases, immunity, group B), that confers protective immunity and is expressed by most strains of type III. Protein Rib was first identified as a distinct 95-kD protein in extracts of a type III strain, and was purified to homogeneity from that strain. Rabbit antiserum to protein Rib was used to demonstrate that it is expressed on the cell surface of 31 out of 33 type III strains, but only on 1 out of 25 strains representing the other three serotypes. Mouse protection tests showed that antiserum to protein Rib protects against lethal infection with three different strains expressing this antigen, including a strain representing a recently identified high virulence type III clone. Protein Rib is immunologically unrelated to the α and β proteins, but shares several features with the α protein. Most importantly, the NH_2-terminal amino acid sequences of the Rib and α proteins are identical at 6 out of 12 positions. In addition, both protein Rib and the α protein are relatively resistant to trypsin (and Rib is also resistant to pepsin) and both proteins vary greatly in size between different clinical isolates. Finally, both protein Rib and the α protein exhibit a regular ladderlike pattern in immunoblotting experiments, which may reflect a repetitive structure. Taken together, these data suggest that the Rib and α proteins are members of a family of proteins with related structure and function. Since protein Rib confers protective immunity, it may be valuable for the development of a protein vaccine against the group B *Streptococcus*, an encapsulated bacterium.

During the last three decades, the group B *Streptococcus* has emerged as a major cause of neonatal disease in the Western world. In the United States alone, there are about 10,000 cases per year of septicemia, meningitis, and neonatal pneumonia caused by this bacterium (1, 2). These invasive infections have an overall mortality of about 20%, and many of the infants that survive meningitis have permanent neurological sequelae (1, 2). In view of these findings, a large effort has been made to analyze the pathogenetic mechanisms in group B streptococcal infections, to identify infants at risk, and to find methods of prevention and treatment (1–4).

Group B streptococcal strains are divided into four major serotypes (Ia, Ib, II, and III) based on the structure of the polysaccharide capsule (5, 6). Serotypes I, II, and III occur in roughly equal proportions among strains in the normal flora, but type III accounts for about two thirds of all isolates from invasive infections (1). Since the capsule is a known virulence factor (1, 5–7), it has been studied in considerable detail, in particular in type III strains (1, 7–9). Efforts are under way to develop a vaccine, in which the type III capsule would be an essential component (3, 8).

In addition to the polysaccharide capsule, some group B streptococcal strains also express a cell surface antigen of protein nature, the c antigen (10–12). This antigen is composed of at least two polypeptides with unrelated sequences, the α and β proteins (11–16), which can be expressed independently of each other (13, 17, 18) and elicit protective immunity (10, 19, 20). The α protein has recently been shown

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/06/1593/11 $2.00
Volume 177 June 1993 1593–1603

to have a highly repetitive structure, which may be of importance for its biological function (16). The β antigen, which has also been referred to as protein Bac (14), binds to the Fc part of IgA (21, 22), a property that may allow it to interact with the immune system of the infected host. Although the α and β proteins confer protective immunity, the available evidence indicates that they are of limited importance for group B streptococcal disease, since they are usually not expressed by strains of type III, which cause most cases of invasive infections (1). However, it has been suggested that a combination of the α and β proteins with the type III polysaccharide may be used as a vaccine against the group B *Streptococcus* (11).

In this report we describe a group B streptococcal cell surface protein, designated protein Rib (resistance to proteases, immunity, group B), that elicits protective immunity and is expressed by most strains of type III. Protein Rib is immunologically unrelated to the α and β proteins, but it shares several properties with the α protein.

Materials and Methods

Bacterial Strains and Media. Four group B streptococcal strains representing the four main capsular serotypes were used as reference strains: A909, type Ia (10); SB35, type Ib (22); B1284, type II (gift from Dr. C. Schalén, University of Lund); and BS30, type III, described here. The BS30 strain was isolated at Lund University Hospital from a boy with neonatal infection. A collection of 58 group B streptococcal strains, all isolated from cases of invasive infections, were used to study the expression of cell surface proteins (see Table 1). 43 of these 58 strains were isolated from samples of blood or cerebrospinal fluid sent to the Clinical Microbiology Laboratory of Lund University Hospital during a 5-yr period. Of these 43 strains, 29 were of serotype III. Since this series of 43 strains included only two type II strains, another 11 strains of this serotype, obtained from Dr. C. Schalén, were later added to the collection. In addition, four type III strains of known electrophoretic type (23), obtained from Dr. S. Mattingly (University of Texas, San Antonio, TX), were included among the strains studied. Among these four type III strains, two (BM110 and BM122) are members of the high-virulence clone in phylogenetic division I, and two (BM177 and BM181) are members of division II (23). Typing of group B streptococcal strains was performed in the Clinical Microbiology Laboratory of Lund University Hospital, using the coagglutination technique (24). For several of the strains, the typing was kindly confirmed by Dr. N. E. Jensen (State Veterinary Laboratory, Copenhagen, Denmark), using the double diffusion technique with HCl-extracted antigens (25). The SB35*sed1* strain is a mouse-virulent mutant of SB35 showing reduced autoaggregation, possibly due to increased capsule production (Lindahl, G., unpublished results). Three group A streptococcal strains were used: D58X, an M3 strain expressing R protein (26), received from Dr. E. Falsen (Culture Collection of the University of Gothenburg, Sweden); AL168, an M22 strain (27); and AL368, an M28 strain expressing R antigen (27; typing kindly performed by Dr. G. Colman, Central Public Health Laboratory, London, UK). All bacterial strains were grown in Todd-Hewitt broth (Oxoid, Basingstoke, Hampshire, UK) at 37°C, without shaking.

Purification of Protein Rib. The bacteria in a 10-liter overnight culture of strain BS30 were spun down, washed twice with 50 mM Tris, pH 7.3, and resuspended to 20% (vol/vol) in the same buffer. Mutanolysin (Sigma Chemical Co., St. Louis, MO), dissolved to 5,000 U/ml in 10 mM potassium phosphate, pH 6.2, was then added to the bacterial suspension (125 ml) to give a final concentration of 350 U/ml. The digestion was allowed to proceed for 17 h at 37°C with gentle shaking, and protease inhibitors were then added to the following final concentrations: benzamidine chloride, 5 mM; iodoacetic acid, 5 mM; and PMSF, 2 mM. The suspension was centrifuged and the supernatant was dialyzed against 10 mM Tris, pH 8.0. This dialyzed preparation was immediately subjected to two consecutive steps of ion exchange chromatography, a procedure that allowed the best recovery of pure protein Rib, as shown by preliminary experiments. The presence of protein Rib was analyzed by SDS-PAGE and visual inspection of the gels for presence of the 95-kD band (see Results). In the first chromatography step, the dialyzed preparation (110 ml) was mixed with the same volume of 0.4 M NaCl in 10 mM Tris, pH 8.0, and 30 ml of DEAE Bio-Gel A (Bio-Rad Laboratories, Richmond, CA), equilibrated with 10 mM Tris, pH 8.0. This mixture was stirred gently at 4°C for 1 h, and unabsorbed material (containing protein Rib) was recovered by filtration through a glass filter. For the second chromatography step (see Fig. 2 *A*), the filtrate containing protein Rib was diluted 20-fold with distilled water, to reduce the ionic strength, and mixed with 30 ml of DEAE Bio-Gel A, equilibrated as described above. After gentle stirring at 4°C for 16 h, the gel was recovered by filtration and washed with 10 mM Tris, pH 8.0. Absorbed proteins (including protein Rib) were eluted with an 800-ml linear salt gradient (0–0.2 M NaCl in 10 mM Tris, pH 8.0), followed by 1 M NaCl (60 ml). Fractions (10 ml) were collected and those containing protein Rib were pooled, concentrated, and subjected to gel filtration in a column of Sepharose CL6B (4.2 × 90 cm) in PBSA (0.12 M NaCl, 0.03 M phosphate, 0.02% NaN_3, pH 7.2) (see Fig. 2 *B*). Fractions (10 ml) containing protein Rib were pooled and frozen. The yield of protein Rib was about 6 mg from 25 g of bacteria. To ensure the purity of the protein Rib preparations used for immunochemical analysis, the protein used for such work was further purified by SDS-PAGE, followed by electroelution of the 95-kD band. However, SDS-PAGE analysis did not demonstrate any difference in purity between this electroeluted material and that recovered from the gel filtration step.

Purification of the α Protein. The α protein was purified from strain SB35, a type Ib strain expressing both the α and β proteins. The procedure used was similar to that used for purification of protein Rib from strain BS30. Fractions were analyzed for the presence of α protein by dot-blot analysis, using rabbit anti-α serum (kindly provided by Dr. L. Bevanger, University of Trondheim, Trondheim, Norway) and protein G (Calbiochem-Novabiochem, San Diego, CA) radiolabeled with ^{125}I. In the ion exchange and gel filtration steps, the behavior of the α protein was similar to that of protein Rib (cf Fig. 2) The alpha protein recovered from the gel filtration step was present in a sharp peak. Analysis of this material with different antisera indicated that it contained trace amounts of contaminating β protein, which was removed by passage of the preparation through a small column of IgA–Sepharose. The purified α protein had a molecular weight of about 110,000, according to SDS-PAGE analysis (cf Fig. 6). The yield of α protein was 12 mg from 39 g of bacteria. The α protein used for immunochemical work was further purified by electroelution from SDS-PAGE gels, as described above for protein Rib.

Purification of the β Protein (Protein Bac). The IgA-binding β protein (21), which has also been referred to as protein Bac (14), was previously purified by affinity chromatography on IgA–Sepharose (22), but the high cost of this method led to the development of another purification method. The starting material was obtained

by incubating washed SB35 bacteria in 50 mM glycine-NaOH buffer, pH 11.0 (final pH in suspension, 9.7), as described (14, 22). The major protein species in such an extract is the β protein (22). The extract (222 ml) was dialyzed against 10 mM Tris, pH 8.0, immediately diluted 20-fold with distilled water, and mixed with 40 ml of DEAE Bio-Gel A (equilibrated with 10 mM Tris, pH 8.0). After gentle stirring at 4°C for 2 h, the gel was transferred to a column and eluted with an 800-ml linear salt gradient (0–0.2 M NaCl in 10 mM Tris, pH 8.0). A dot-blot procedure was used to test fractions (10 ml) for presence of β protein, using radiolabeled IgA or anti-β serum and radiolabeled protein G for the analysis. The β protein was eluted in the first part of the gradient. Appropriate fractions were pooled, concentrated, and subjected to gel filtration on a column (4.2 × 100 cm) of AcA34 (Pharmacia-LKB, Uppsala, Sweden) in PBSA. The β protein was eluted in a well-defined peak. Appropriate fractions were pooled, concentrated, and frozen. The yield was 9 mg of pure protein from 23 g of bacteria. The major protein species in such a preparation had a molecular weight of about 130,000, according to SDS-PAGE, but small amounts of degradation products of lower molecular weight were also seen when the protein was subjected to Western blot analysis (cf Fig. 6).

Analysis of Streptococcal Strains for Cell Surface Expression of the α, β, and Rib Proteins. The bacteria in a 10-ml overnight culture were washed twice with PBSAT (PBSA supplemented with 0.05% Tween 20) and a 1% suspension in PBSAT was prepared. A sample (180 μl) of this bacterial suspension was mixed with 20 μl of rabbit antiserum that had been diluted in PBSAT, and the mixture was incubated at 23°C for 1 h. 2 ml of PBSAT was then added, the bacteria were spun down, washed once with 2 ml of PBSAT, and resuspended in 200 μl of PBSAT. For detection of bound IgG, 25 μl of radiolabeled protein G (about 10^4 cpm in PBSAT) was then added, and incubation was continued at 23°C for 1 h. After addition of 2 ml of PBSAT, the bacteria were spun down and the pellet was then washed by addition of 2 ml of PBSAT. After a final centrifugation, the supernatant was discharged and the radioactivity in the pellet was determined. Selected strains were tested at different antiserum dilutions as indicated (see Fig. 3). When many strains were tested for expression of the α, β, and Rib proteins (see Table 1 and Fig. 4), a single final antiserum dilution of 1:1,000 was used. This dilution was chosen on the basis of the curves shown in Fig. 3. Controls with preimmune rabbit antiserum were always included and were completely negative in all cases.

Mouse Protection Tests. C3H/HeN mice, bred in our department, were used at an age of 10–20 wk. The mice were injected intraperitoneally with 0.1 ml of rabbit serum (diluted with PBS to a total volume of 0.5 ml), and infected 4 h later by i.p. injection of 0.5 ml of log-phase bacteria diluted in Todd-Hewitt broth. The number of bacteria used, which was estimated to be the 90% lethal dose (LD_{90}), was 2×10^6 CFU for strains BM110, BE210, and SB35*sed1*, and 2×10^7 CFU for BS30 and L25. Dead animals were counted daily for 4 d. Control animals usually died within 24 h.

Antisera. All antisera were produced in rabbits, which were immunized subcutaneously on the back. For preparation of antiserum to protein Rib, slices corresponding to several 95-kD bands in SDS-PAGE gels were cut out, divided into small pieces, and mixed with CFA. For the initial immunization, six slices (about 60 μg of protein) in 1 ml of PBS were mixed with 1 ml of adjuvant. Three bands (30 μg of protein) were used for booster injections. The first booster was given after 4 wk and three additional boosters were given at 2-wk intervals. The rabbit was then bled three times at 3-wk intervals. The serum obtained from these three bleedings was pooled and used for the experiments reported here.

Two different antisera to the α protein were used. The first of these anti-α sera, used for purification of the α protein expressed by strain SB35, was the kind gift of Dr. L. Bevanger. The α protein purified from strain SB35 was then used for preparation of a second anti-α serum, used for all experiments reported in this paper. The procedure used was similar to that used for preparation of antiserum to protein Rib, i.e., slices from SDS-PAGE gels were used as antigen, but only two booster injections were given. 2 wk after the second booster injection, the rabbit was exsanguinated and the anti-α serum obtained from this bleeding was used for all experiments reported here. Antiserum to the β protein was prepared from protein purified by affinity chromatography on IgA–Sepharose, as described (22).

Electrophoresis, Western Blotting, and Dot-Blot Analysis. SDS-PAGE was performed with standard techniques, using a total polyacrylamide concentration of 10% and a crosslinking of 3.3%. Samples were boiled for 3 min in a solution containing 2% SDS and 5% 2-ME before electrophoresis. The separated proteins were stained with Coomassie brilliant blue R-250 or transferred by electroblotting to a membrane of methanol-activated polyvinylidene difluoride (Immobilon-P; Millipore Corp., Molsheim, France), using a Semi-Dry Electroblotter (Ancos, Vig, Denmark). The Immobilon membranes were blocked as described (27) and then incubated with the indicated type of rabbit antiserum (diluted 1000-fold), followed by radiolabeled protein G and autoradiography, as described (28). For dot-blot analysis, samples were applied with a Bio-Dot apparatus (Bio-Rad Laboratories) and the Immobilon membranes were analyzed with rabbit antiserum and radiolabeled protein G, as described above.

Other Methods. Small-scale mutanolysin extracts of streptococcal strains were prepared as described for the large-scale extracts used for protein purification, but cultures of only 50 ml were used to prepare 20% bacterial suspensions, of which 1-ml samples were digested with the enzyme.

For analysis of protease sensitivity (see Fig. 8), 200-μl samples of purified α, β, or Rib protein (0.5 mg/ml) were incubated for 1 h at 37°C with trypsin, pepsin, or proteinase K (0.2 mg/ml). Trypsin digestion was performed in 0.25 M sodium phosphate, pH 7.5, pepsin digestion in 0.25 M sodium acetate, pH 4.0, and proteinase K digestion in 0.25 M Tris, pH 7.4. The samples were neutralized before analysis by SDS-PAGE.

Proteins were radiolabeled with carrier-free ^{125}I (Amersham International, Amersham, Bucks, UK), using the chloramine T method (29). Total protein concentrations were determined with a protein assay reagent (MicroBCA; Pierce, Rockford, IL). Electroelution of protein from SDS-PAGE gels was performed with an Electro-Eluter (model 422; Bio-Rad Laboratories).

Automated amino acid sequence analysis of protein bands transferred to Immobilon was performed directly on the membranes, using a gas–liquid solid-phase sequenator (model 470A; Applied Biosystems, Inc., Foster City, CA). The membranes were lightly stained with Coomassie brilliant blue to localize the protein bands, which were then cut out for sequencing. The SwissProt Data Bank was used for analysis of protein sequences.

Results

Identification and Purification of Protein Rib. While studying group B streptococcal cell surface proteins, we prepared mutanolysin extracts of several strains of different serotypes. These extracts were analyzed by SDS-PAGE and by immunoblotting, using antisera to the α and β proteins. Results

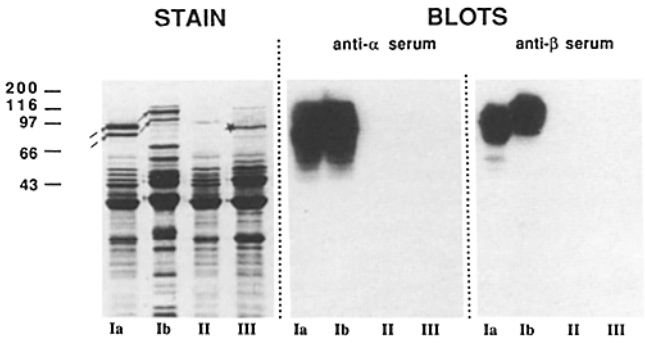

Figure 1. Western blot analysis of mutanolysin extracts prepared from group B streptococcal strains representing the four main serotypes. The positions of three proteins relevant to this report are indicated on the stained gel: the α protein (*bottom arrow*) and the β protein (*top arrow*) expressed by the strains of types Ia and Ib, and the 95-kD protein (*star*) expressed by the strain of type III. The relative positions of the α and β proteins had been determined in preliminary experiments. The autoradiograms of the blots were deliberately overexposed, to demonstrate that the antisera to the α and β proteins completely lack reactivity with extracts of the type II and type III strains used here. The four strains studied here were A909 (type *Ia*), SB35 (type *Ib*), B1284 (type *II*), and BS30 (type *III*). (*Left*) Molecular mass markers are in kilodaltons.

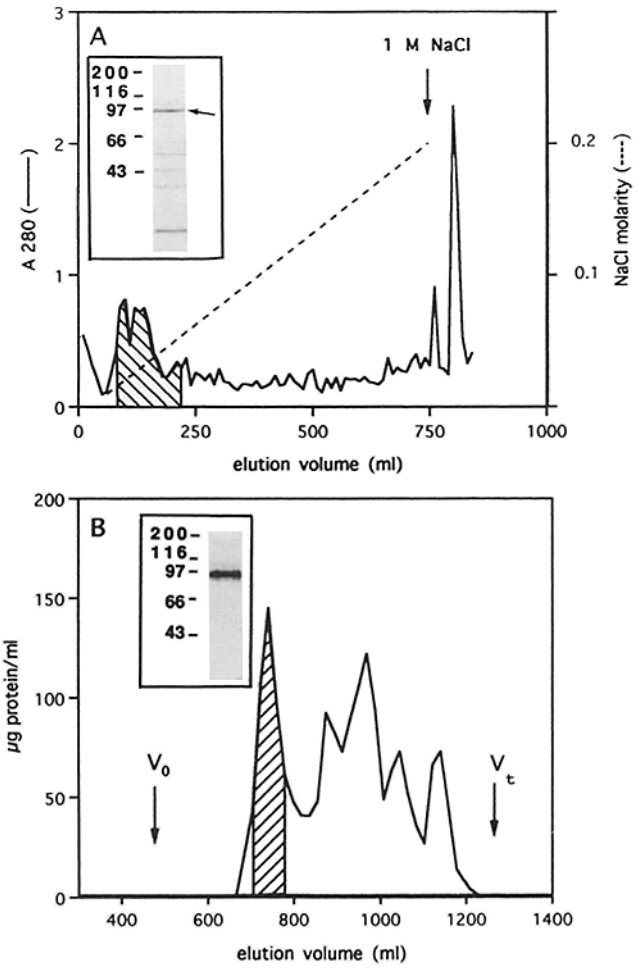

Figure 2. Purification of protein Rib from the type III strain BS30. (*A*) A mutanolysin extract, partially purified through a previous step of DEAE ion exchange chromatography (see Materials and Methods) was subjected to ion exchange chromatography on a 30-ml column of DEAE Bio-Gel A, which was eluted with a linear gradient (800 ml) of NaCl in 10 mM Tris, pH 8.0, followed by 1 M NaCl (60 ml). (*Shaded area*) Fractions containing protein Rib. (*Inset*) Pool of the protein Rib–containing fractions analyzed by SDS-PAGE. (*Left*) Molecular mass markers, are in kilodaltons. (*Arrow*) Position of protein Rib (95 kD). (*B*) The pool of protein Rib–containing fractions from the ion exchange chromatography were subjected to gel filtration on a column (4.2 × 90 cm) of Sepharose CL6B. (*Shaded area*) Fractions containing protein Rib. (*Inset*) Pool of these fractions analyzed by SDS-PAGE. (V_o) Void volume; (V_t) total volume.

obtained with four strains representing the four major serotypes are shown in Fig. 1. The α and β proteins, which are expressed by both the type Ia and Ib strains gave rise to distinct bands in the high molecular weight region of the stained gel and vary in size between the two strains, in agreement with previous results (21, 22, 30, 31, and our unpublished data). Antisera to these two proteins did not detect any proteins in the extracts of the type II and III strains, as shown in the blot (which was deliberately overexposed to demonstrate this lack of reactivity). A major protein species in the high molecular weight region was present also in the extract prepared from the type III strain, although this strain does not express the α protein or the β protein. Such a distinct protein species of high molecular weight was also observed in extracts of other type III strains, and the protein appeared to vary in size between different strains. These similarities to the α and β proteins made it of interest to study the high molecular weight proteins of type III strains in more detail. Strain BS30 was chosen for this work, because it was known to be mouse virulent. The 95-kD protein expressed by this strain (Fig. 1) was purified from mutanolysin extracts, using two consecutive steps of ion exchange chromatography, followed by gel filtration (Fig. 2). Fractions were analyzed by SDS-PAGE for presence of the 95-kD protein. When appropriate fractions from the gel filtration were pooled and analyzed, only two protein species were detected: a major 95- and a minor 90-kD protein (see *inset*, Fig. 2 *B*). The 90-kD protein most likely represents a degradation product of the 95-kD protein, since these two proteins have the same NH_2-terminal sequence (see below). The purified protein will be referred to as protein Rib (resistance to proteases, immunity, group B; see below). Antiserum to the 95-kD form of protein Rib was prepared by immunizing rabbits with slices cut out from SDS-PAGE gels.

Protein Rib Is Expressed on the Cell Surface of Most Group B Streptococcal Strains of Type III. To analyze whether protein Rib is a cell surface protein, strains representing the four major serotypes were tested for ability to bind anti-Rib serum (Fig. 3). The five strains studied included the four strains described above and an additional type III strain, BM110, which is a member of the high-virulence type III clone described by Musser et al. (23). For comparison, these five strains were also tested for expression of the α and β proteins, using antisera to highly purified preparations of these proteins.

The anti-α serum reacted strongly with the Ia and Ib strains, as expected, and it also reacted weakly with the two strains

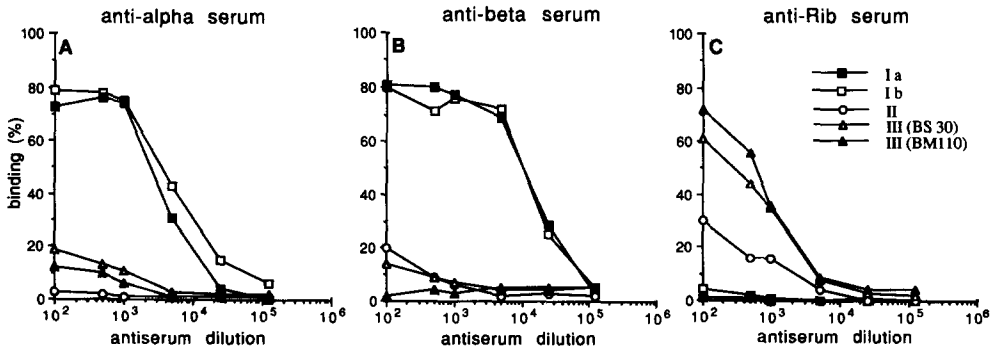

Figure 3. Analysis of group B streptococcal strains of the four major serotypes for cell surface expression of the α, β, and Rib proteins. Five strains were tested: A909 (type Ia); SB35 (type Ib); B1284 (type II); BS30 (type III), and BM110 (type III). (*C*) Symbols used for these five strains. Bacterial suspensions were incubated with different dilutions of rabbit antiserum to the α, β, or Rib protein, as indicated. The numbers on the x-axis refer to final antiserum dilution in the bacterial mixture. Bound antibodies were detected by incubation with radiolabeled protein G, and the numbers on the y-axis refer to the percentage of protein G bound of that added. Controls with preimmune rabbit serum were included in all experiments and were completely negative in all cases.

of type III (Fig. 3 *A*). However, mutanolysin extracts of the type III strains did not contain any detectable α protein, when analyzed in a Western blot. It therefore seems likely that this weak reactivity of anti-α serum with whole bacteria of type III represents a crossreactivity with some other cell wall component. These data show that reactivity with anti-α serum can be used to unequivocally analyze whether a strain expresses the α antigen on the cell surface. Similar data were obtained with anti-β serum (Fig. 3 *B*).

The antiserum to protein Rib reacted with the two type III strains, but not with the strains of types Ia and Ib (Fig. 3 *C*). An intermediate level of binding was observed for the type II strain. When mutanolysin extracts of the five strains were analyzed in a Western blot experiment, using anti-Rib serum for the analysis, the extracts of the type III strains reacted strongly, giving major blotting bands at 95 kD, but the extracts of the three other strains completely lacked reactivity (data not shown). This result indicates that the intermediate reactivity of anti-Rib serum with the type II strain was due to a crossreactivity, which disappeared under the conditions of the Western blot. We conclude that protein Rib is expressed on the cell surface of the two type III strains, but not on the other three strains.

A comparison of *A–C*, Fig. 3, indicates that the titer of the anti-Rib serum is lower than the titer of the other two antisera. Several factors could contribute to such a difference, e.g., the concentration of specific antibodies in the sera, the affinity of these antibodies, the amount of antigen per bacterial cell, etc. To study whether the concentration of antibodies in the sera contributed to the difference, we measured the level of such antibodies, using a solid-phase RIA with the purified antigens immobilized in microtiter wells. The data indicated that the concentration of specific antibodies was about threefold lower in the anti-Rib serum than in the other two antisera (data not shown). This result can at least partially explain the difference in titer between the three sera in the binding assay (Fig. 3).

Since the data in Fig. 3 indicated that protein Rib is found on the surface of two different type III strains, it was of interest to study a larger number of group B streptococcal strains for expression of this protein. A total of 58 strains of known serotype, all of which had been isolated from invasive infections, were therefore tested for ability to bind antibodies to protein Rib (Table 1). Each strain was also tested for binding of antibodies to the α and β proteins. To simplify the study of many strains, each antiserum was tested at a single 1,000-fold dilution, chosen on the basis of the data shown in Fig. 3. The results obtained with antiserum to protein Rib are presented in Fig. 4, which shows that the strains can be divided into two populations with 0–15% and 21–60% binding, respectively. Western blot analysis of several bacterial extracts confirmed that these two populations correspond to strains that do or do not express protein Rib, except that a single strain showing 21% binding did not express the protein (data not shown). As shown in Table 1, protein Rib was found

Table 1. *Cell Surface Expression of the α, β, and Rib Proteins by 58 Group B Streptococcal Strains Isolated from Patients with Invasive Infections*

Protein expressed	Capsular type			
	Ia (n = 9)	Ib (n = 3)	II (n = 13)	III (n = 33)
α	6	0	4	0
β	1	0	0	0
α and β	1	3	5	0
Rib	0	0	1	31
None	1	0	3	2

The cell surface expression of the α, β, and Rib proteins was analyzed with specific antisera, and bound antibodies were detected with radiolabeled protein G, as shown in Figs. 3 and 4. The 58 strains studied here were all isolated from cases of invasive infections, but do not represent a random collection of such strains, since most of the type II strains were later added to the collection originally studied, which included only two type II strains (see Materials and Methods).

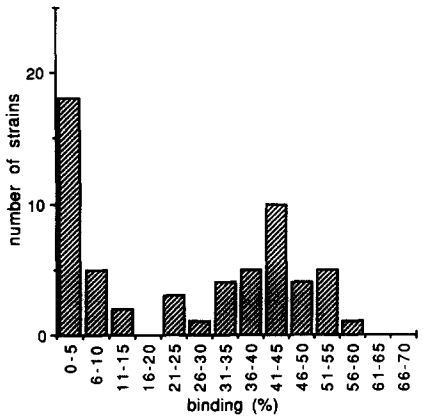

Figure 4. Binding of anti-Rib serum to 58 group B streptococcal strains isolated from patients with invasive infections. Each strain was tested for ability to bind anti-Rib antibodies at a single antiserum dilution (1:1,000). Bound antibodies were detected with radiolabeled protein G. Controls with preimmune serum were completely negative in all cases.

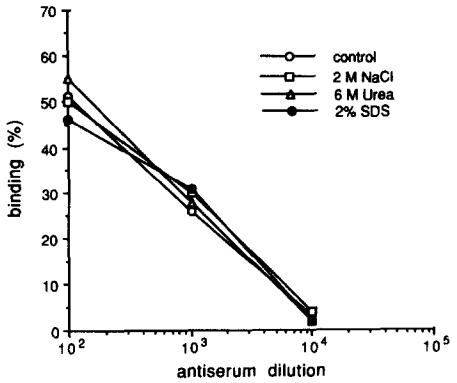

Figure 5. Binding of anti-Rib antibodies to the type III strain BS30 treated with different agents before the binding assay. The procedure used was the same as that used for the experiments shown in Fig. 3, except that the BS30 bacteria was incubated in one of the solutions indicated before the binding assay. After incubation for 20 min at room temperature, the bacteria were washed three times with PBSAT and analyzed for ability to bind anti-Rib serum.

on the cell surface of 31 out of 33 type III strains and on 1 out of 13 type II strains, but not on any of the 12 strains of types Ia and Ib. The tests with antisera to the α and β proteins also gave unequivocal results, and are summarized in Table 1.

It seemed possible that strains that do not express protein Rib on the cell surface excrete the protein into the medium. Culture supernatants of the 58 strains listed in Table 1 were therefore analyzed in a dot-blot experiment, using anti-Rib serum for the analysis. Protein Rib was not detected in the supernatants of any of the 26 strains that do not express the protein on the cell surface, but was found in the supernatants of 26 of the 32 strains expressing the protein on the cell surface (data not shown). Analysis of concentrated culture supernatants from two different type III strains showed that protein Rib was one of the major proteins in these supernatants, but >80% of the protein was estimated to be cell bound (data not shown). The presence of protein Rib in culture supernatants was not unexpected, since it is known that the α and β proteins are often found in supernatants of strains expressing these proteins (11, 22, 32). In agreement with these reports, we found that most of the strains expressing the α and β proteins (Table 1) released these proteins into the culture medium (17/19 and 8/10, respectively).

The data reported above indicate that protein Rib is a cell surface antigen, like the α and β proteins. However, the possibility remained that Rib is secreted into the medium and unspecifically bound to the bacterial cell surface, giving a positive result in the binding assay. To test this hypothesis, we treated strain BS30 with different chemicals before the binding assay. The result (Fig. 5) shows that not even 6 M urea had any effect on the ability of the cells to bind antibodies to protein Rib. Additional evidence that protein Rib is a cell surface molecule was obtained in an experiment in which strain BS30 was treated with proteinase K before mutanolysin extraction. Such protease treatment completely removed protein Rib from the extract, as shown by Western blot analysis (data not shown). Taken together, all of these data strongly suggest that protein Rib is a cell surface protein.

We have previously shown that the β protein is released in almost pure form from bacteria incubated at high pH (22). When strains expressing protein Rib or the α protein were tested in the same way, such release was not observed (data not shown).

Protein Rib Confers Protective Immunity. A mouse protection model was used to study whether rabbit antibodies to protein Rib can protect against lethal infection with the group B *Streptococcus* (Table 2). Control animals received antiserum to the α protein or preimmune serum, as indicated. The antiserum to the α protein had been prepared by the same procedure as that used for protein Rib, i.e., by immunizing rabbits with gel slices containing highly purified protein.

The data in Table 2 demonstrate that antiserum to protein Rib protects against lethal infection with BS30, the type III strain from which the protein had been purified. This protection is not unspecific, as shown by the experiments with control sera. The anti-Rib serum also protected against lethal infection with another type III strain, BM110, a member of the high-virulence clone of group B streptococcal strains (23). In contrast, the anti-Rib serum did not protect against infection with L25, a type III strain that does not express protein Rib. The protective effect of anti-Rib serum was not limited to type III strains, as shown by the experiments with a type II strain expressing protein Rib. Taken together, the data in Tables 1 and 2 strongly suggest that protein Rib confers protective immunity to almost all type III strains and to some type II strains, i.e., to most group B streptococcal strains causing invasive infections.

As expected, anti-Rib serum did not protect against infection with a type Ib strain, but protection was obtained with anti-α serum (Table 2). Since the SB35 strain used for this experiment expresses both the α and β proteins, this result indicates that anti-α serum can protect against lethal infection even if the infecting strain also expresses the β protein.

Table 2. *Rabbit Antiserum to Protein Rib Protects Mice against Lethal Infection with Group B Streptococcal Strains Expressing this Protein*

Strain	Capsular type	Relevant cell surface protein	Mice surviving after pretreatment with‡ Anti-Rib serum	Anti-α serum	Normal serum
BS30	III	Rib*	29/32§	1/15	4/20
BM110	III	Rib	15/24§	0/15	0/15
L25	III	–	0/15	2/14	ND
BE210	II	Rib	10/15‖	0/14	ND
SB35*sed 1*	Ib	α	1/15	10/15¶	ND

C3H/HeN mice were injected intraperitoneally with 0.1 ml of rabbit antiserum (diluted to 0.5 ml with PBS) and challenged 4 h later with an LD_{90} dose of log-phase bacteria, diluted into 0.5 ml of Todd-Hewitt broth. The survival data were analyzed by the χ^2 test.

* Expression of protein Rib or the α protein, the two antigens relevant to these experiments.

‡ No. of mice surviving for 4 d/total no. of infected mice.

§ $P < 0.001$ when compared with the controls receiving anti-α or normal serum.

‖ $P < 0.001$ when compared with the controls receiving anti-α serum.

¶ $P < 0.01$ when compared to the controls receiving anti-Rib serum.

Immunochemical Comparison of Protein Rib with the α and β Proteins. Since protein Rib confers protective immunity, like the α and β proteins, it was of interest to compare the properties of these three proteins. A Western blot experiment was first performed, using antisera to the purified proteins for the analysis (Fig. 6). Although the staining gel showed that the three proteins were highly purified, with one major species in each preparation, the immunoblots revealed additional bands in each of the three proteins. For the α protein, the blot showed a ladderlike pattern with regularly spaced intervals, as previously described (33–35). This pattern probably reflects the repetitive structure of the protein, but the mechanism by which the pattern arises is not known (16). A ladder pattern was not seen in the blot of the β protein, but there were a few bands corresponding to proteins of lower molecular weight, as noted previously (14). Interestingly, the blotting analysis of protein Rib revealed a ladderlike pattern, very similar to that seen for the α protein. Protein Rib extracted from the bacterial cell wall and that present in culture supernatants gave rise to similar ladder patterns (data not shown). The distance between the individual steps in the ladder, which was similar for protein Rib and the α protein, corresponds to a molecular weight difference of about 9,000. In spite of this similarity, there was no serological cross-reaction between the α and Rib proteins in the Western blot. Since this analysis was made under denaturing conditions, a dot-blot test was also performed, in which the native proteins were tested for crossreactivity. This experiment confirmed that the α, β, and Rib proteins are serologically unrelated (Fig. 7).

Protein Rib Is Protease Resistant. The α and β proteins were originally distinguished as two separate components in the c antigen, because of a difference in protease sensitivity (11, 12). The α protein is resistant to trypsin but sensitive to pepsin, whereas the β protein is sensitive to both of these proteases. An experiment with the purified α and β proteins confirmed this difference and also demonstrated that protein Rib is resistant to pepsin and relatively resistant to trypsin (Fig. 8). There was a shift in the protein Rib pattern after treatment with trypsin, indicating a sensitive site at one end of the molecule, but a comparison with the β antigen indicates that Rib can be classified as relatively trypsin resistant. As expected, all three proteins were sensitive to degradation by proteinase K (data not shown). The protease resistance of protein Rib was not due to the presence of an inhibitor, since β protein was

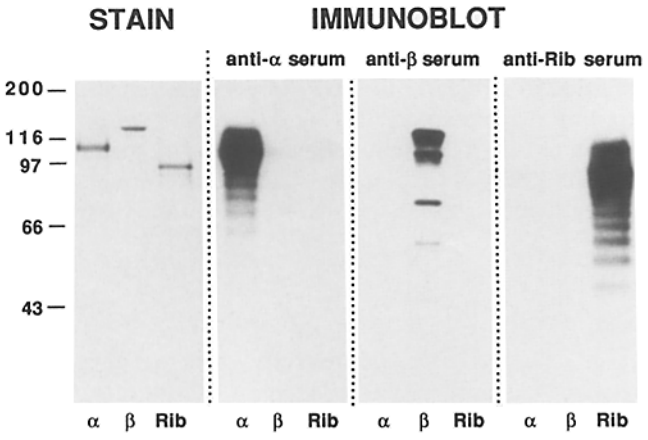

Figure 6. Western blot analysis of purified α, β, and Rib proteins with rabbit antisera raised against the purified proteins. The protein preparations used for this analysis had been electroeluted from SDS-PAGE gels (see Materials and Methods). Antisera were used at a 1:1,000 dilution, and bound antibodies were detected with radiolabeled protein G. The autoradiograms were deliberately overexposed, to visualize the ladder pattern in the immunoblots of the α and Rib proteins. (*Left*) Molecular mass markers are in kilodaltons.

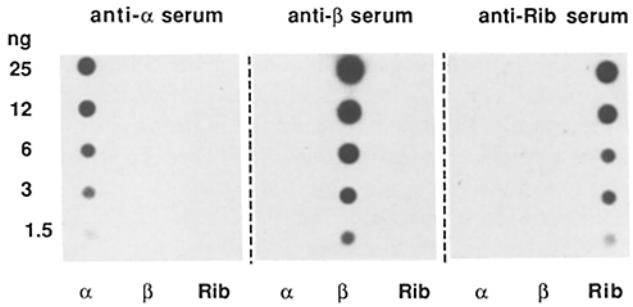

Figure 7. Dot-blot analysis of purified α, β, and Rib proteins with rabbit antisera raised against the purified proteins. Bound antibodies were detected with radiolabeled protein G. The protein preparations used for this analysis were the native proteins recovered after the gel filtration step in the purification procedures. Antisera were used at a 1:1,000 dilution.

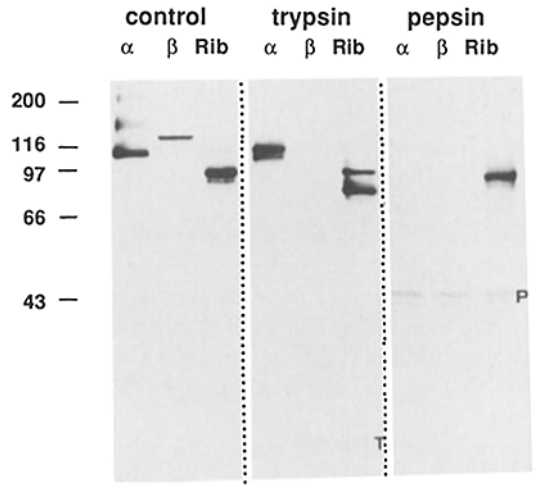

Figure 8. SDS-PAGE analysis of the purified α, β, and Rib proteins treated with trypsin or pepsin. The trypsin treatment was performed at pH 7.5, the pepsin treatment at pH 4.0. The samples were neutralized before the SDS-PAGE analysis. Controls were treated in the same way as the samples containing trypsin or pepsin, but no enzyme was added. Such treatment did not cause degradation of the proteins. The protein preparations used for this analysis were the native proteins recovered after the gel filtration step in the purification procedures. (*P*) Pepsin; (*T*) trypsin. (*Left*) Molecular mass markers are in kilodaltons.

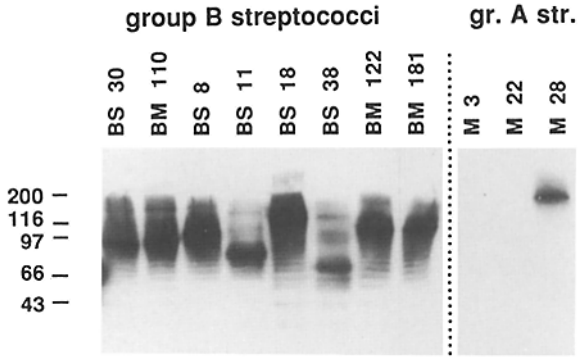

Figure 9. Western blot analysis with anti-Rib serum of extracts prepared from different streptococcal strains. The stained gels (not shown) showed that all extracts contained similar amounts of protein. Bound antibodies were detected with radiolabeled protein G. (Lanes *1–8*) Mutanolysin extracts of eight different group B streptococcal strains of type III. (Lanes *9–11*) Mutanolysin extracts of three group A streptococcal strains of different M types, of which the M3 and M28 strains express different R antigens (see Materials and Methods). The autoradiograms were deliberately overexposed, to visualize the ladder pattern in the blots of the group B streptococcal extracts. (*Left*) Molecular mass markers are in kilodaltons.

completely degraded by both trypsin and pepsin even in the presence of protein Rib (data not shown).

Protein Rib Varies in Size between Different Group B Streptococcal Strains. Both the α and β proteins vary in size between different bacterial strains (Fig. 1). The α protein has been reported to occur in forms ranging in size from 63 to 200 kD (30, 35), and two sequenced variants of the β protein vary in size by about 3 kD (14, 15, 31). Protein Rib also varies in size between different strains, as shown by an immunoblotting experiment with extracts made from eight different type III strains (Fig. 9, lanes *1–8*). The size of the major protein Rib species varied between 65 and 125 kD in these strains. A ladder pattern similar to that seen with the purified protein was observed for protein Rib expressed by each of these different strains.

To study whether the size of protein Rib varies at high frequency within an individual strain, five different colonies of strain BM110 were picked and reisolated 10-fold. Culture extracts were then prepared and analyzed by immunoblotting. In all cases, the size of the major protein Rib species was identical in the reisolated strain and in the original strain. Similar results were obtained with four other type III strains (data not shown).

NH_2-terminal Sequences of Protein Rib and the α Protein. The NH_2-terminal sequences of protein Rib and the α protein are shown in Fig. 10. The 95- and 90-kD polypeptides in purified protein Rib (Fig. 2 *B*) were found to have the same NH_2-terminal sequence, suggesting that the smaller molecule is a degradation product of the larger one. A data search showed that the NH_2-terminal sequence of protein Rib is unique.

The NH_2-terminal sequence of the α protein (Fig. 10) corresponds to residues 57–68 in the published amino acid sequence, which was deduced from the nucleotide sequence of the gene in strain A909 (16). This result indicates that the NH_2-terminal sequence of the α protein is identical in different strains, and it also suggests that the α protein has an unusually long signal peptide, 56 residues.

A comparison of the NH_2-terminal sequences of the Rib and α proteins revealed considerable sequence homology, with identical amino acid residues at 6 out of 12 positions (Fig. 10). In contrast, a comparison of the NH_2-terminal sequence of protein Rib with that of the β protein (14, 15) did not reveal any homology.

Relationship of Protein Rib to Streptococcal R Antigens. The R antigens are a heterogeneous group of cell surface antigens, unrelated to virulence, that are expressed by certain strains of group A streptococci (26, 36, 37). Antiserum to the R antigen of type M28 strains also detects an antigen in some strains of group B streptococci. This group B streptococcal antigen, which is also designated R, is trypsin resistant, like

```
Rib:     Ala-Glu-Val-Ile-Ser-Gly-Asp-Ala-Val-Thr-Leu-Asn-
              |   |   |   |   |   |   |   |   |   |
Alpha:       Ser-Thr-Ile-Pro-Gly-Ser-Ala- X -Thr-Leu-Asn-Thr-
```

Figure 10. NH_2-terminal sequences of protein Rib and the α protein. For the α protein, residue no. 8 could not be identified and is denoted by *X*. The NH_2-terminal sequence of the α protein shown here was determined on material purified from strain SB35 and corresponds to residues 57–68 in the published amino acid sequence (16), which was deduced from the nucleotide sequence of the gene in strain A909. The latter sequence includes the signal peptide and has an Ala residue at the position marked by *X* here. (|) Residue identities. The one-residue gap at the beginning of the α sequence was introduced to maximize homology.

protein Rib (36). However, the group B streptococcal R antigen is not identical to protein Rib, since the R antigen is sensitive to pepsin and does not confer immunity to type III strains, unlike protein Rib (36, 38). On the other hand, we have noted that antiserum to protein Rib detects a high molecular weight protein in extracts of M28 strains, but not in extracts of an M3 strain expressing another R antigen (Fig. 9). The blotting signal obtained with the M28 extract was weaker than those obtained with extracts prepared from group B streptococci and the blot did not show a ladder pattern even after prolonged exposure of the film. The antigen of the M28 strain had a molecular weight of about 180,000, larger than protein Rib expressed by any of the group B strains tested, and it was of similar size in two different M28 strains tested. These results indicate that protein Rib crossreacts with a large antigen expressed by M28 strains, possibly the R antigen of those strains. Further analysis of this crossreactivity has not yet been possible, since the R antigens have not been purified and monospecific antisera to these antigens are not available. However, an antiserum to whole M28 bacteria reacted with protein Rib, as expected (data not shown).

Discussion

Previous work has shown that two different cell surface proteins, the α and β proteins, confer protective immunity to group B streptococcal infection (10, 11, 19, 20), but the importance of these proteins in serious infections has remained unclear, since they are only rarely expressed by strains of serotype III (10, 17, 18). In contrast, the novel cell surface protein described here, protein Rib, is expressed by almost all type III strains, including two strains that are known to be members of a high-virulence clone (23). This finding allows us to conclude that most invasive strains of the group B *Streptococcus* express two different antigens that confer protective immunity: the type-specific polysaccharide capsule (1) and a cell surface protein. The relative importance of these two antigens is not known, but it is noteworthy that the presence of antibodies to only one of the two antigens is sufficient to protect against lethal infection (10), which suggests that both the polysaccharide and the protein antigen may be required for virulence.

Protein Rib is a cell surface protein of high molecular weight, like the α and β proteins, but it is immunologically unrelated to these two proteins. However, protein Rib and the α protein share several other properties. First, both the α and Rib proteins are relatively resistant to trypsin digestion. For the α protein, this resistance is not due to a lack of potentially trypsin-sensitive sites, but must be due to some other property of the molecule (16). Protein Rib is actually even more protease resistant than the α protein, since it is also resistant to pepsin. This protease resistance of the Rib and α proteins may contribute to the ability of the group B *Streptococcus* to avoid the unspecific defense mechanisms of the host. A second similarity between the α and Rib proteins is the great variation in size between proteins expressed by different bacterial isolates (30, 35). This size variation can most simply be explained by a variation in the number of repeating units (16), as described for the group A streptococcal M6 protein (39). Finally, both the α and Rib proteins exhibit a ladderlike pattern in immunoblotting experiments. For the α protein, which is a molecule with a highly repetitive structure (16), the distance between the steps in this ladder corresponds to the deduced molecular weight of the repeat region, but the mechanism by which the ladder pattern is generated is not understood (16). The similarity between the immunoblots of the α and Rib proteins suggests that both of these proteins may have a highly repetitive structure.

The Rib and α proteins exhibit considerable sequence homology in the NH_2-terminal region, which indicates that they have evolved from a common ancestor. This sequence similarity and the other similarities between the two molecules suggest that the Rib and α proteins are members of a family of proteins with related structure and properties, although they are immunologically unrelated. An analogous situation has recently been described in the group A streptococcal M protein family, which can be divided into two major classes of immunologically unrelated molecules (40). In this connection it should also be noted that protein Rib crossreacts with a protein expressed by M28 strains of the group A *Streptococcus*, possibly the R antigen of those strains (Fig. 9). This crossreacting protein might also be a member of the hypothetical family that includes the Rib and α proteins. Work is in progress to make a more detailed comparison of the structure and properties of these different proteins.

About 20% of all women are vaginal carriers of the group B *Streptococcus*, and vertical transmission from the maternal genital tract is probably the most common source of infection in neonatal disease caused by this bacterium (1). However, only 1–2% of the infants that are colonized by the group B *Streptococcus* at birth are afflicted by serious infection (1, 2). Other factors than exposure to the bacterium during birth must therefore contribute to the development of neonatal disease. An understanding of these factors will be essential to finding ways of prevention of group B streptococcal disease. It has been reported that mothers of infected infants have significantly lower levels of antibodies to the type III capsule, which implies that these antibodies are important for protection against neonatal disease (41), but this issue is controversial (2, 3). It has also been suggested that maternal antibodies to bacterial cell surface proteins may be important for protective immunity (42, 43). The properties of protein Rib indeed suggest that the level of antibodies to this protein may affect the susceptibility to group B streptococcal disease in the neonate. Studies are in progress to test this hypothesis.

In summary, we have found a novel cell surface protein that is expressed by almost all group B streptococcal strains of serotype III and that confers protective immunity. The available evidence indicates that protein Rib is structurally related to the α antigen, and a more detailed comparison of these two cell surface proteins is therefore of interest for the analysis of pathogenetic mechanisms in group B streptococcal infections. Characterization of the Rib and α proteins may also yield information that is of interest for studies of cell

surface proteins expressed by other encapsulated bacteria, e.g., the *Pneumococcus* (44). With regard to prevention of group B streptococcal disease, it seems possible that a combination of the Rib and α proteins could be used for the development of a vaccine, since the large majority of virulent strains express one of these two proteins. One advantage with a vaccine based on proteins, rather than on polysaccharides, might be absence of immunological crossreactions with human glycoproteins (45).

We are indebted to Dr. J. L. Michel for information about the α protein before publication. The amino acid sequence analysis was performed by Ms. Ingrid Dahlqvist.

Financial support was received from the Swedish Medical Research Council (project 9490), the Medical Faculty of the University of Lund, the Royal Physiographic Society in Lund, the Swedish Society for Medical Research, the Foundations of Crafoord, Kock and Österlund, and from King Gustaf V's 80-year Foundation.

Address correspondence to Dr. Gunnar Lindahl, Department of Medical Microbiology, University of Lund, Solvegatan 23, S-22362 Lund, Sweden.

Received for publication 29 December 1992 and in revised form 22 February 1993.

References

1. Baker, C.J., and M.S. Edwards. 1990. Group B streptococcal infections. *In* Infectious Diseases of the Fetus and Newborn Infant. J.S. Remington and J.O. Klein, editors. W.B. Saunders Company, Philadelphia. 742–811.
2. Hill, H.R. 1990. Group B streptococcal infections. *In* Sexually Transmitted Diseases. 2nd ed. K.K. Holmes, P.-A. Mårdh, P.F. Sparling, P.J. Wiesner, W. Cates, S.M. Lemon, and W.E. Stamm, editors. McGraw-Hill Inc., New York. 851–861.
3. Baker, C.J. 1990. Immunization to prevent group B streptococcal disease: victories and vexations. *J. Infect. Dis.* 161:917.
4. Walsh, J.A., and S. Hutchins. 1989. Group B streptococcal disease: its importance in the developing world and prospect for prevention with vaccines. *Pediatr. Infect. Dis. J.* 8:271.
5. Lancefield, R.C. 1934. A serological differentiation of specific types of bovine hemolytic streptococci (group B). *J. Exp. Med.* 59:441.
6. Lancefield, R.C. 1938. Two serological types of group B hemolytic streptococci with related, but not identical, type-specific substances. *J. Exp. Med.* 67:25.
7. Rubens, C.E., M.R. Wessels, L.M. Heggen, and D.L. Kasper. 1987. Transposon mutagenesis of type III group B *Streptococcus*: correlation of capsule expression with virulence. *Proc. Natl. Acad. Sci. USA.* 84:7208.
8. Wessels, M.R., L.C. Paoletti, D.L. Kasper, J.L. DiFabio, F. Michon, K. Holme, and H.J. Jennings. 1990. Immunogenicity in animals of a polysaccharide-protein conjugate vaccine against type III group B *Streptococcus*. *J. Clin. Invest.* 86:1428.
9. Marques, M.B., D.L. Kasper, M.K. Pangburn, and M.R. Wessels. 1992. Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B streptococci. *Infect. Immun.* 60:3986.
10. Lancefield, R.C., M. McCarty, and W.N. Everly. 1975. Multiple mouse-protective antibodies directed against group B streptococci. Special reference to antibodies effective against protein antigens. *J. Exp. Med.* 142:165.
11. Michel, J.L., L.C. Madoff, D.E. Kling, D.L. Kasper, and F.M. Ausubel. 1991. C proteins of group B streptococci. *In* Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci. G.M. Dunny, P.P. Cleary, and L.L. McKay, editors. American Society for Microbiology, Washington, DC. 214–218.
12. Wilkinson, H.W., and R.G. Eagon. 1971. Type-specific antigens of group B type Ic streptococci. *Infect. Immun.* 4:596.
13. Bevanger, L., and J.A. Maeland. 1979. Complete and incomplete Ibc protein fraction in group B streptococci. *Acta Pathol. Microbiol. Scand. Sect. B.* 87:51.
14. Hedén, L.-O., E. Frithz, and G. Lindahl. 1991. Molecular characterization of an IgA receptor from group B streptococci: sequence of the gene, identification of a proline-rich region with unique structure and isolation of N-terminal fragments with IgA-binding capacity. *Eur. J. Immunol.* 21:1481.
15. Jerlström, P.G., G.S. Chhatwal, and K.N. Timmis. 1991. The IgA-binding beta antigen of the c protein complex of group B streptococci: sequence determination of its gene and detection of two binding regions. *Mol. Microbiol.* 5:843.
16. Michel, J.L., L.C. Madoff, K. Olson, D.E. Kling, D.L. Kasper, and F.M. Ausubel. 1992. Large, identical, tandem repeating units in the C protein alpha antigen gene, *bca*, of group B streptococci. *Proc. Natl. Acad. Sci. USA.* 89:10060.
17. Bevanger, L. 1983 Ibc proteins as serotype markers of group B streptococci. *Acta Pathol. Microbiol. Immunol. Scand. Sect. B.* 91:231.
18. Johnson, D.R., and P. Ferrieri. 1984. Group B streptococcal Ibc protein antigen: distribution of two determinants in wild-type strains of common serotypes. *J. Clin. Microbiol.* 19:506.
19. Bevanger, L., and A.I. Naess. 1985. Mouse-protective antibodies against the Ibc proteins of group B streptococci. *Acta. Pathol. Microbiol. Scand. Sect. B.* 93:121.
20. Michel, J.L., L.C. Madoff, D.E. Kling, D.L. Kasper, and F.M. Ausubel. 1991. Cloned alpha and beta c-protein antigens of group B streptococci elicit protective immunity. *Infect. Immun.* 59:2023.

21. Russell-Jones, G.J., E.C. Gotschlich, and M.S. Blake. 1984. A surface receptor specific for human IgA on group B streptococci possessing the Ibc protein antigen. *J. Exp. Med.* 160:1467.
22. Lindahl, G., B. Åkerström, J.-P. Vaerman, and L. Stenberg. 1990. Characterization of an IgA receptor from group B streptococci: specificity for serum IgA. *Eur. J. Immunol.* 20:2241.
23. Musser, J.M., S.J. Mattingly, R. Quentin, A. Goudeau, and R.K. Selander. 1989. Identification of a high-virulence clone of type III *Streptococcus agalactiae* (group B *Streptococcus*) causing invasive neonatal disease. *Proc. Natl. Acad. Sci. USA.* 86:4731.
24. Christensen, P., G. Kahlmeter, S. Jonsson, and G. Kronvall. 1973. New method for the serological grouping of streptococci with specific antibodies adsorbed to protein A-containing staphylococci. *Infect. Immun.* 7:881.
25. Jensen, N.E. 1979. Production and evaluation of antisera for serological type determination of group-B streptococci by double diffusion in agarose gel. *Acta. Pathol. Microbiol. Scand. Sect. B.* 87:77.
26. Lancefield, R.C. 1958. Occurrence of R antigen specific for group A type 3 streptococci. *J. Exp. Med.* 108:329.
27. Stenberg, L., P. O'Toole, and G. Lindahl. 1992. Many group A streptococcal strains express two different immunoglobulin-binding proteins, encoded by closely linked genes: characterization of the proteins expressed by four strains of different M-type. *Mol. Microbiol.* 6:1185.
28. Åkerström, B., T. Brodin, K. Reis, and L. Björck. 1985. Protein G: a powerful tool for binding and detection of monoclonal and polyclonal antibodies. *J. Immunol.* 135:2589.
29. Greenwood, F.C., W.M. Hunter, and J.S. Glover. 1963. The preparation of ^{131}I-labelled human growth hormone of high specific activity. *Biochem. J.* 89:114.
30. Madoff, L.C., S. Hori, J.L. Michel, C.J. Baker, and D.L. Kasper. 1991. Phenotypic diversity in the alpha C protein of group B streptococci. *Infect. Immun.* 59:2638.
31. Lindahl, G., L.-O. Hedén, and L. Stenberg. 1992. Streptococcal IgA-receptors. *In* Molecular Recognition in Host–Parasite Interactions. T.K. Korhonen, T. Hovi, and P.H. Mäkelä, editors. Plenum Publishing Corp., New York. 77–83.
32. Ferrieri, P., D.R. Johnson, and A.E. Flores. 1985. Ibc protein antigens in culture supernatants of group B streptococci: comparison to HCl-extracted antigens. *In* Recent Advances in Streptococci and Streptococcal Diseases. Proceedings of the IXth Lancefield International Symposium on Streptococci and Streptococcal Diseases. Y. Kimura, S. Kotami, and Y. Shiokawa, editors. Reedbooks Ltd., Berkshire, England. 204–206.
33. Russell-Jones, G.J., and E.C. Gotschlich. 1984. Identification of protein antigens of group B streptococci, with special reference to the Ibc antigens. *J. Exp. Med.* 160:1476.
34. Madoff, L.C., J.L. Michel, and D.L. Kasper. 1991. A monoclonal antibody identifies a protective c-protein alpha-antigen epitope in group B streptococci. *Infect. Immun.* 59:204.
35. Bevanger, L., O.-J. Iversen, and A.I. Naess. 1992. Characterization of the alpha antigen of the c proteins of group B streptococci (GBS) using a murine monoclonal antibody. *Acta Pathol. Microbiol. Immunol. Scand.* 100:57.
36. Lancefield, R.C., and G.E. Perlmann. 1952. Preparation and properties of a protein (R antigen) occurring in streptococci of group A, type 28 and in certain streptococci of other serological groups. *J. Exp. Med.* 96:83.
37. Wiley, G.G., and P.N. Bruno. 1970. Cross-reactions among group A streptococci. III. The M and R antigens of type 43 and serologically related streptococci. *J. Immunol.* 105:1124.
38. Lindén, V. 1983. Mouse-protective effect of rabbit anti-R-protein antibodies against group B streptococci type II carrying R-protein. Lack of effect on type III carrying R-protein. *Acta Pathol. Microbiol. Scand. Sect. B.* 91:145.
39. Fischetti, V.A. 1989. Streptococcal M protein: molecular design and biological behavior. *Clin. Microbiol. Rev.* 2:285.
40. O'Toole, P., L. Stenberg, M. Rissler, and G. Lindahl. 1992. Two major classes in the M protein family in group A streptococci. *Proc. Natl. Acad. Sci. USA.* 89:8661.
41. Baker, C.J., and D.L. Kasper. 1976. Correlation of maternal antibody deficiency with susceptibility to neonatal group B streptococcal infection. *N. Engl. J. Med.* 294:753.
42. Christensen, K.K., P. Christensen, G. Duc, W.H. Hitzig, V. Lindén, B. Müller, and R.A. Seger. 1984. Human IgG antibodies to carbohydrate and protein antigens in mouse protection tests with group B streptococci. *Pediatr. Res.* 18:478.
43. Kim, K.S., C.A. Wass, J.K. Hong, N.F. Conception, and B.F. Anthony. 1988. Demonstration of opsonic and protective activity of human cord sera against type III group B Streptococcus that are independent of type-specific antibody. *Pediatr. Res.* 24:628.
44. Yother, J., L.S. McDaniel, M.J. Crain, D.F. Talkington, and D.E. Briles. 1991. Pneumococcal surface protein A: structural analysis and biological significance. *In* Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci. G.M. Dunny, P.P. Cleary, and L.L. McKay, editors. American Society for Microbiology, Washington, DC. 88–91.
45. Pritchard, D.G., B.M. Gray, and M.L. Egan. 1992. Murine monoclonal antibodies to type Ib polysaccharide of group B streptococci bind to human milk oligosaccharides. *Infect. Immun.* 60:1598.