The alpha C protein mediates internalization of group B Streptococcus within human cervical epithelial cells

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Summary
Group B Streptococcus (GBS) is the leading cause of bacterial chorioamnionitis and neonatal pneumonia, sepsis, and meningitis. Deletion of the alpha C protein gene (bca) attenuates the virulence of GBS in an animal model; significant survival differences in the first 24 h of infection suggest a pathogenic role for the alpha C protein early in the infection process. We examined the role of alpha C protein in the association between GBS and mucosal surfaces using a human cervical epithelial cell line, ME180. Fluorescent and confocal microscopy and flow cytometry demonstrated that 9-repeat alpha C protein binds to the surface of ME180 cells. Isolated N-terminal region of this protein also binds to these cells and competitively inhibits binding of the full protein. Wild-type GBS strain A909 and the bca-null isogenic mutant JL2053 bound similarly to the surface of ME180 cells. However, A909 entered these cells threefold more. Internalization of A909 was inhibited with 2- and 9-repeat alpha C and with N-terminal region alone but not by repeat region-specific peptide. Translocation across polarized ME180 membranes was fivefold greater for A909 than for JL2053. These findings suggest a role for the alpha C protein in interaction with epithelial surfaces and initiation of infection.

Introduction
Group B streptococci (Streptococcus agalactiae, GBS) are a leading cause of invasive bacterial diseases in neonates, pregnant women, and non-pregnant adults with underlying medical conditions (Baker and Edwards, 1995; Bliss et al., 2002). Group B streptococci colonize the human gastrointestinal and genitourinary tracts and may cause urinary tract infection, chorioamnionitis, endometritis and sepsis during the peripartum period. Group B streptococci may be transmitted to neonates during labour and delivery (Schuchat, 1998); one or two infants per 1000 live births develop invasive GBS disease, with 6% of neonatal infections ending in death (Zangwill et al., 1992; Baker and Edwards, 1995; Schrag et al., 2000). In vitro, GBS have been shown to adhere to (Zawaneh et al., 1979; Tamura et al., 1994) and invade (Rubens et al., 1992; Hulse et al., 1993; Valentini-Weigand et al., 1997) epithelial cells and it has been postulated that adherence to and invasion of epithelial cells are the first steps in invasive infection.

Little is known about the bacterial factors and structures required for binding and entry of GBS into eukaryotic cells. The capsular polysaccharides, which play an important role in GBS virulence by preventing complement activation, do not influence adherence of GBS to epithelial cells but do reduce internalization (Tamura et al., 1994). A role for bacterial surface proteins in these processes is suggested by the findings that adherence of GBS to epithelial cells is reduced by as much as 75% after the bacteria are treated with proteases, including trypsin, pronase, pepsin and proteinase K (Bulgakova et al., 1986; Tamura et al., 1994).

A protein that may mediate interactions with epithelial cells is the GBS alpha C protein, which is commonly expressed on the surface of GBS of serotypes Ia, Ib and II (Michel et al., 1991). The alpha C protein is the prototype for a family of long tandem repeat containing surface proteins that includes the Rib, Alp2 and Alp3 proteins in other GBS serotypes, R28 in group A streptococci (GAS) (whose sequence is identical to Alp3 in GBS) and Esp in Enterococcus faecalis. These proteins share considerable sequence homology, as well as common structural elements, including an N-terminal region, a series of tandem repeats of approximately 80 amino acids each and a C-terminal region containing a cell-wall anchor LPXTG motif common to several Gram-positive species. Deletion of the gene (bca) encoding the alpha C protein attenuates the virulence of GBS sevenfold in the neonatal mouse model, with significant survival differences noted during the first
24 h of infection (Li et al., 1997). In GAS, deletion of the gene encoding R28 resulted in a marked decrease in binding to ME180 cells (Stalhammar-Carlemalm et al., 1999). An E. faecalis strain deleted for expression of Esp had diminished virulence in a urinary tract infection model, suggesting decreased adherence to urinary epithelium (Shankar et al., 2001). Together, these data provide strong evidence for a role of this protein family in adherence to and internalization by epithelial cells.

In this study, we investigated the role of the alpha C protein in the interaction of GBS with cervical epithelial cells in polarized and non-polarized configurations and demonstrated adherence, internalization and transcytosis of intact GBS expressing alpha C protein. Further, we aimed to identify the regions of the alpha C protein involved in these activities by adding purified protein products, corresponding to regions of the alpha C protein, to these assays and assessing for inhibition of activity.

**Results**

**Alpha C protein binds to cervical epithelial cells**

We investigated a role for the alpha C protein in mediating bacterial–epithelial cell interaction using ME180 cells, a cervical carcinoma cell line that has been previously shown to bind to the related GAS R28 protein (Stalhammar-Carlemalm et al., 1999). This cell line is relevant to GBS disease because these organisms are known to colonize the human genital tract.

**Microscopy.** Purified 9-repeat alpha C protein or N-terminal region was fluorescently labelled and incubated with ME180 cells for 2 h and then observed by micros-
Alpha C protein mediates internalization of GBS

Presence of the alpha C protein does not affect binding of GBS to human cervical epithelial cells but does increase internalization

Adhesion and internalization of wild-type GBS strain A909, which expresses 9-repeat alpha C protein, or the isogenic alpha C protein-deficient mutant, JL2053, were determined to be linear with ME180 cells at MOI's (multiplicity of infection) ranging from 0.5 to 10. All subsequent assays were performed using an MOI = 5 (10^6 cfu). The numbers of cell-associated (surface-bound and internalized) and internalized-only GBS were calculated after a 2 h incubation with ME180 monolayers (Table 1). The number of cell-associated GBS was similar for the two strains of GBS (P = 0.5166), but internalization of strain A909 was approximately threefold greater than that of JL2053 (P = 0.0048), demonstrating that alpha C protein contributes to GBS internalization of cervical epithelial cells.

A potential role of the alpha C protein in the survival of GBS inside epithelial cells was also investigated. Following a 2 h incubation with A909 or JL2053, the ME180 cells were incubated in the presence of antibiotics for 1, 2, 4, 18 and 24 h. Viable internalized GBS were detected for up to 24 h (Fig. 2). The amount of live internalized GBS decreased at similar rates regardless of expression of alpha C protein. Thus, although the expression of alpha C protein increases internalization of GBS within ME180 cells, it does not affect the rate of survival of internalized GBS.

Alpha C protein expression is associated with increased transcytosis of GBS across polarized human cervical epithelial cells

Group B streptococci were previously shown to transcytose intact, polar brain microvascular endothelial cells grown on transwell membranes (Nizet et al., 1997). We examined strains A909 and JL2053 for their ability to transcytose intact ME180 cells under similar conditions. Both A909 and JL2053 migrated from the apical side (top chamber) of the ME180 cells to the basal side (lower chamber). However, by three hours strain A909 exhibited fivefold greater transcytosis of the epithelial cell layers than did alpha-negative strain JL2053 (Fig. 3).

Maximal inhibition of binding of the full 9-repeat alpha C protein to ME180 cells is achieved with the N-terminal region. To investigate which region of the alpha C protein is involved in epithelial cell interaction, we added unlabelled N-terminus, 9-repeat region, or BSA at varying concentrations to ME180 cells in the presence of AlexaFluor 488-labelled full 9-repeat alpha C protein. Inhibition of binding of the labelled alpha C protein to the

### Table 1. Number of viable GBS associated with human epithelial cells.

|                  | A909 (bca⁺) | JL2053 (bca⁻) |
|------------------|-------------|---------------|
| Cell associated  | 1.00 × 10⁷ ± 7.28 × 10⁶ | 7.80 × 10⁶ ± 3.96 × 10⁵ |
| Internalized     | 3.52 × 10³ ± 6.28 × 10² | 1.24 × 10³ ± 6.01 × 10² |

Each value represents the mean and standard deviation calculated from three experiments. The number of cell-associated A909 and JL2053 are similar (P = 0.5166), whereas the observed difference in internalization between the two strains is statistically significant (P = 0.0048).

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Fig. 2. Survival of internalized GBS strains A909 and JL2053 and E. coli DH5α in ME180 cells over time. The cfu values were averaged from three separate experiments. The error bars represent the standard deviation. E. coli was included as a non-internalized control strain.
ME180 cells was analysed by flow cytometry. There was dose-dependent inhibition of binding in the presence of unlabelled N-terminus protein to a maximum of 75% inhibition (Fig. 4). Addition of 3 µM of unlabelled 9-repeat region resulted in 50.6% inhibition, which did not increase with higher concentration (20 µM). Addition of 30 µM of BSA did not significantly inhibit binding.

Maximal inhibition of internalization of GBS by ME180 cells is achieved with the N-terminal region

To investigate which region of the alpha C protein is involved in GBS internalization, ME180 monolayers were preincubated for 1 h with purified complete 2- or 9-repeat alpha C protein or with purified N-terminal or 1-repeat regions before the addition of A909 or JL2053 (Fig. 5). Internalization of uninhibited JL2053 was 78% less than that of uninhibited strain A909. Fifty per cent of internalization of GBS strain A909 was inhibited with 0.1, 1.6 and 3.3 µM of 9-repeat alpha C protein, N-terminus, and 2-repeat alpha C proteins respectively. Higher concentrations of 9-repeat alpha C protein and N-terminal region reduced the levels of internalized A909 to that of strain JL2053. Internalization of A909 was not inhibited by the highest concentration of 1-repeat region tested (13 µM) or by BSA. Internalization of the mutant strain JL2053 was not inhibited by addition of either of the alpha C proteins or any of the individual regions. Together, these results strongly suggest that the N-terminal region of the alpha C protein is involved in internalization of GBS within ME180 cells.

Discussion

Group B streptococci colonize mucosal surfaces and cause infection after breaching these surfaces and entering normally sterile sites such as the bloodstream, the central nervous system and the fetal membranes. However, little is known about the nature of GBS structures involved in adhering to and penetrating epithelial cell barriers. Here we present data indicating that purified alpha C protein, the prototype of the GBS alpha-like protein family, binds to cervical epithelial cells in culture. The alpha C protein was not necessary for adherence of GBS to ME180 cells, as there was no difference in binding between wild-type strain A909 and the JL2053 mutant, which lacks the alpha C protein, but the wild-type strain entered more efficiently into ME180 cells than the mutant strain. Moreover, the wild-type strain showed greater transcytosis across a layer of ME180 cells than did the mutant strain. Thus, the importance of alpha C protein in GBS infection may lie in facilitation of processes subsequent to adhesion, including invasion.

Inhibition studies suggest that the N-terminal region of the alpha C protein, in particular, is involved in adherence and entry into the cervical epithelial cells. The exact role of the repeat region of the alpha C protein in the events associated with colonization, adherence and invasion is less clear. The isolated repeat domain alone was not sufficient to affect internalization at the concentrations used. However in a separate assay, an isolated repeat construct was able to partially inhibit binding of the
labelled alpha C protein to ME180 cells. Previous work from this laboratory showed that variations in the number of repeats within the alpha C protein alters the antigenic structure of alpha C protein, such that 2-repeat alpha C protein lacks conformational epitopes present on 9-repeat alpha C protein (Gravekamp et al., 1996). In the presence of specific antibody, the loss of repeats confers a selective advantage and allows evasion of host immunity. There is also evidence that the repeat region can serve as a scaffold that alters the availability of the protein for binding on the bacterial cell surface. Because the alpha C protein displayed on most wild-type clinical isolates contain 9 or 10 repeats, there must be some conditions selecting for a higher number of repeats. Based on our current observations, it is possible that higher numbers of repeats are required for optimal interaction with eukaryotic cells.

Once inside human epithelial cells alpha C protein does not appear to play a role in GBS survival. The number of internalized A909 within ME180 cells decreased over a 24 h time period, with a more dramatic change measured between 4 and 18 h. Similar survival rates were reported with strain A909 using human chorion cells (Winram et al., 1998). The reduction of internalized isogenic alpha-C protein-negative strain JL2053 occurred at the same rate. A possible explanation for this decrease is that GBS exit ME180 cells during the antibiotic protection assay and are killed by the antibiotics in the medium.

Nearly all GBS express an alpha-like protein. Because the alpha C protein and other members of the Alp family are so similar in primary structure, it is probable that the other Alp proteins also play a role in promoting bacterial internalization by eukaryotic cells (Lachenauer et al., 2000). Sequence alignments of the N-terminal regions of the alpha C protein and other alpha-like proteins show amino acid homology of 60% or greater at the amino acid level (Lachenauer et al., 2000). There are both conserved and variable domains within the Alp N-termini, and it is likely that functional activity lies within the conserved domains. The GAS protein R28, which is nearly identical to the GBS protein Alp3, promotes binding to ME180 cells (Stalhammar-Carlemalm et al., 1999), but its role in internalization has not been reported.

Similar primary structural organization was described in a family of surface-anchored proteins known as MSCRAMM (microorganism surface component recognizing adhesive matrix molecules) expressed by Gram-positive organisms. The MSCRAMMs are classified by their ability to adhere to extracellular matrix components. Identified binding regions within this group of proteins vary in structure. Fibronectin-binding MSCRAMMs consist of binding regions that undergo ligand-induced conformational changes, whereas collagen-binding MSCRAMMs consist of transversing β-sheets forming a trench where collagen binds (House-Pompeo et al., 1996; Foster and Hook, 1998; Rich et al., 1999). It is not known if alpha C protein is a member of this family, as its receptor is not known. Group B streptococci are known to bind to cytokeratin 8 and fibronectin (Tamura and Rubens, 1995; Tamura and Nittayajarn, 2000). It is possible the alpha C protein may interact with epithelial cells via a component of serum or the extracellular matrix. Further work towards identification of the receptor involved in interaction with cellular microorganisms.
members of the Alp family may provide a useful target for intervention in the prevention of invasive bacterial infections. Specifically, small molecule analogues or antibodies directed at such a molecule might be effective in inhibiting the interactions leading to bacterial invasion of human host tissues.

Experimental procedures

Bacterial strains and epithelial cell line

GBS type Ia/C (at+ l+) strain A909 and JL2053, an alpha deletion mutant derived in the A909 background, have been described (Li et al., 1997). Escherichia coli strains BLR (Novagen), BL21(DE3) (Novagen), and DH5α (GibcoBRL) were purchased. ME180 cells (HTB33) (Sykes et al., 1970), a human cervical epithelial carcinoma cell line, were purchased from the American Type Culture Collection.

Plasmids

Plasmids pT7LM2, pT7LM16 and pT7LM39 have been described (Gravekamp et al., 1996) and consist of the 9-, 2- and 1-repeat alpha C protein genes, respectively, cloned into the pT7 expression vector under the control of the T7 promoter. Protein purification was facilitated by recloning the 9- and 2-repeat alpha C protein genes into the pTrcHisA expression vector (Invitrogen) as follows. Polymerase chain reaction (PCR) primers 5′-CGGGATCCCTCTACCAATCCAGGAGGAT-3′ and 5′-GGAAT TCTTAATCTCTTTTTCATT-3′ were used to amplify the 2-repeat alpha C protein gene, minus its signal sequence, from pT7LM16. The PCR product was cloned into pCR2.1 (Invitrogen) and the insert was excised with BamHI and EcoRI and cloned into pTrcHisA. The resulting construct was named pLM3. Plasmid pT7LM2 was fully digested with BpiI followed by partial digestion with AflII. The 2870 bp fragment encoding for all 9-repeats flanked by partial N-terminal and C-terminal sequences was gel purified. Plasmid pLM3 was digested with BpiI and AflII to remove the nucleotides encoding for the 2-repeats and ligated with the 9-repeat containing sequence obtained above. The new construct, pCL1, consists of the complete 9-repeat alpha C protein gene, minus the signal sequence, cloned into the pTrcHisA expression vector.

The 9-repeat region (excluding the N- and C-terminal encoding regions) of bca (Michel et al., 1992) was amplified from GBS strain A909 chromosomal DNA by PCR using the primers 5′-GTAAATGTTGAGGTTAATTGTT-3′ and 5′-GGATATTATTTCC TTACCAG-3′. The PCR product was cloned into pCR2.1, excised with EcoRI and ligated into CIP-treated pTrcHisA, which had also been digested with EcoRI. The resulting construct was named pMJB1.

DNA encoding for the 1-repeat region of alpha C protein PCR amplified from pT7LM39 using the primers 5′-TTTCTGCGATCCGTACCGAGAATAA-3′ and 5′-TTCTGAAGCTTACCTTG TTG-3′. The DNA product was digested with BamHI and HindIII and ligated into pET24a previously digested with the same enzymes. This construct was named pET24RR.

Plasmid pDEK14 has been described (Kling et al., 1997) and consists of the alpha C protein N-terminus DNA cloned into pET24a (Novagen).

Protein expression

The 2- and 9-repeat alpha C proteins were expressed in E. coli strain BLR transformed with pLM3 and pCL1 respectively. The N-terminus and 1-repeat regions were expressed in E. coli strain BL21(DE3) transformed with pDEK14 and pET24RR respectively. The 9-repeat construct (without the N- and C-termini) was expressed in DH5α transformed with pMJB1. For each protein, a 2 litre culture of each transformant was rotated (200 r.p.m.) at 37°C to an OD600 equal to 0.6. Protein expression was induced with 1 mM IPTG for 16 h at 37°C rotating. The cells were harvested by centrifugation at 4000 g for 10 min at 4°C, resuspended in 200 ml of Bind buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) containing 1% protease inhibitor cocktail set III (Invitrogen) and lysed in a French pressure chamber. The lysate was centrifuged at 20 000 g for 20 min at 4°C and the supernatant was filtered through a 0.45 µm sterile membrane. A 15 ml His-Blend resin (Novagen) column charged with 50 mM NiSO4 was equilibrated in Bind buffer and the filtrate was loaded onto the column and allowed to pass through the resin by gravity flow. The column was washed with 90 ml of Wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and eluted with 90 ml of Elute buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9); 2 ml fractions were collected and analysed by SDS-PAGE. Fractions containing recombinant protein were pooled and dialysed at 4°C in 20 mM Tris, pH 8.0. The 2-repeat alpha C protein, N-terminus and 1-repeat region preparations were further purified on a 6 ml Resource Q anion exchange column equilibrated in 20 mM Tris, pH 8.0 and eluted with a 0–0.5 M NaCl continuous gradient. Fractions containing recombinant protein immunoreactive with alpha C protein specific sera eluted from the column at approximately 0.10–0.14 M NaCl. They were pooled and dialysed in 20 mM Tris, pH 7.2.

Adhesion and internalization assays

The method of Stahlhammar-Carlemalm et al. (1999) was adapted as follows. ME180 cells were grown to confluence in 24-well culture plates (Costar 3524) containing 1 ml of RPMI medium (RPMI 1640 with L-glutamine, 10% FCS, 25 mM Heps). GBS strains A909 and JL2053 and E. coli strain DH5α were grown to mid-log phase (OD600 = 0.3) in Todd–Hewitt broth (THB), pelleted, washed and resuspended in phosphate-buffered saline (PBS). The strains were diluted 1 : 100 in RPMI medium for a final concentration of approximately 2 x 10⁹ cfu ml⁻¹. The exact concentration was determined by plating aliquots of the bacteria onto Todd–Hewitt agar (THA) plates. Bacterial suspension (0.5 ml) was added to wells containing 0.5 ml of RPMI medium (MOI = 5 bacteria cell⁻¹). The plates were incubated at 37°C with 5% CO₂ for 2 h. The monolayers were washed three times per well with 1 ml of PBS to remove external non-adherent bacteria. The monolayers were incubated with 1 ml of RPMI medium containing 100 µg ml⁻¹ gentamicin and 5 µg ml⁻¹ penicillin G per well for 2 h at 37°C with 5% CO₂ and subsequently washed three times with 1 ml of PBS. The ME180 cells were detached from the wells by adding 0.1 ml of 0.05% trypsin, 0.035 mM EDTA (GibcoBRL) per well for 10 min at 37°C. They were then lysed by adding 0.4 ml of 0.025% Triton X-100 to the wells at room temperature with repeated pipetting. The number of viable internalized bacteria per well was determined by plating serial dilutions of the epithelial lysates on THA plates and incubating at 37°C for 16 h.

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The percentage of internalized bacteria was calculated as \([\text{cfu/well after antibiotic treatment}}/\text{cfu originally added to the well}] \times 100\). The number of cell-associated GBS (surface-adherent plus internalized GBS) was determined in separate wells not treated with gentamicin and penicillin. Statistical analysis was performed using unpaired \(t\)-test (www.graphpad.com/calculators/ttest1.cfm).

**Internalization Inhibition assays**

These assays were performed as above, except that before the addition of GBS to the ME180 cells, inhibitor (0.1 ml of 1–1000 \(\mu\)g ml\(^{-1}\) of diluted in RPMI medium) was added to the wells. Inhibitors included the alpha C protein N-terminal region, the 1-repeat region, the complete 2- or 9-repeat alpha C proteins, or BSA. The 24-well plates were then centrifuged at 800 \(\times\) g for 10 min and incubated at 37\(^\circ\)C with 5% CO\(_2\) for 1 h. Per cent inhibition was calculated as \([\text{cfu of internalized GBS}}/\text{cfu of original inoculum}]\)/(\([\text{cfu of internalized A909 uninhibited}}/\text{cfu of original inoculum of A909 uninhibited}]\) \times 100.

**Transcytosis assay**

The method was adapted from Nizet et al. (1997) as follows: ME180 cells were grown in 0.5 ml of RPMI medium on a 12-mm-diameter transwell-COL (3.0 \(\mu\)m pore size, Costar) membrane suspended in wells of a 12-well plate containing 1.5 ml of similar medium. The cells were incubated at 37\(^\circ\)C with 5% CO\(_2\) until confluent. The medium was replaced with fresh RPMI medium containing 0.4 mM CaCl\(_2\) to allow tight junction formation and incubated at 37\(^\circ\)C with 5% CO\(_2\) until the transmembrane resistance stabilized (approximately 3 days). The GBS strains A909 and JL2053 and \(E.\) coli strain DH5\(_\alpha\) were grown to mid-log phase \((A_{600} = 0.3)\) in 5 ml of THB, washed three times in 5 ml of PBS and suspended to \(2 \times 10^6\) cfu ml\(^{-1}\) in RPMI medium containing 0.4 mM CaCl\(_2\). DH5\(_\alpha\) was included as a control for intact ME180 membranes. The medium in the top chamber was replaced with 0.5 ml of GBS (approximately \(10^6\) cfu). The plates were incubated at 37\(^\circ\)C with 5% CO\(_2\). At 1 h intervals for a total of 3 h, the transwell-COL membranes were moved to different wells containing 1.5 ml of fresh RPMI medium containing 0.4 mM CaCl\(_2\). The amount of GBS and \(E.\) coli that translocated across the polarized ME180 cells was determined by plating dilutions of the medium remaining in the wells on THA plates after each hourly interval. The electrical resistance across the ME180 membranes was measured at the end of the incubation periods to confirm the integrity of the monolayers.

**Fluorescent labelling of proteins**

AlexaFluor 488 Protein Labeling Kit (A-10235; Molecular Probes) was used to covalently link AlexaFluor 488 dye to BSA and to purified protein products of the full 9-repeat alpha C protein, isolated N-terminal region and 9-repeat region. In brief, 0.5 ml of protein at 2 mg ml\(^{-1}\) in PBS was incubated with 0.05 ml of 1 M NaHCO\(_3\) (pH 8.3) and one vial of AlexaFluor 488 dye at room temperature, stirring, for 1 h. Labelled protein was separated from free dye on a resin column provided by the manufacturer according to instructions. Calculations of the protein concentration and moles of label per mole of protein were based on measurements of the OD\(_{280}\) and OD\(_{494}\) of the eluate, according to manufacturer's instructions:

\[
\text{Labelled protein concentration} = \frac{A_{280} - (A_{494} \times 0.11)}{\text{dilution factor/extinction coefficient}}
\]

\[
\text{Extinction coefficient (Beers law)} = \frac{A_{280}}{\text{unlabelled protein conc.}} \times \text{path length in cm}
\]

Moles of dye per mole of protein = \(\frac{A_{494}}{\text{dilution factor}} \times 71,000\) [labelled protein conc. (M)]

The concentrations of labelled protein ranged from 7 \(\times\) 10\(^{-6}\) M to 3 \(\times\) 10\(^{-5}\) M. The molar ratio of dye to protein ranged from 1 to 4.

**Flow cytometry analysis including inhibition assays**

ME180 cells were grown to confluence (\(\sim 6.9 \times 10^4\) cells) in each chamber of an eight-chamber Falcon CultureSlide (Falcon) in 250 ml of RPMI 1640 medium containing 10% FCS (Gibco) and 1% penicillin/streptomycin (Gibco). Twenty-five microlitres of AlexaFluor 488-labelled protein (9-repeat alpha C, N-terminal region or BSA) was added to individual chambers for three different final protein concentrations of 1 \(\mu\)M, 0.1 \(\mu\)M and 0.01 \(\mu\)M. The proteins were allowed to adhere to ME180 at 37\(^\circ\)C for 2 h. The monolayers were washed three times with 0.25 ml of PBS, treated with 0.1 ml of 2% paraformaldehyde fixative in PBS overnight at 4\(^\circ\)C and washed again with 0.25 ml of PBS. The chamber walls were then removed from the glass slide; Fluorotec mounting medium (Accurate Chem., Westbury, NY) was added before placing cover slips. The slides were reviewed with fluorescent and confocal microscopy (Zeiss Axioskop and PCM 2000 hardware and C-imaging software) to assess for fluorescent labelling of the cells. All images were viewed at the same gain value.

**Alpha C protein mediates internalization of GBS**

ME180 cells were grown to confluence (\(\sim 1.2 \times 10^5\) cells) in six-well plates with 2 ml of RPMI 1640, 10% FCS (Gibco), 1% penicillin/streptomycin (Gibco). The day before the assay, the medium was replaced with 1 ml of fresh medium and the cells were incubated overnight at 37\(^\circ\)C with 5% CO\(_2\). The next day, 0.1 ml of AlexaFluor 488-labelled 9-repeat alpha C protein, N-terminal region, or BSA was added to the wells for a final concentration of 1 \(\mu\)M. For the inhibition studies, 0.1 ml of unlabelled alpha C protein N-terminus, 9-repeat region, or BSA was added as inhibitor for a final concentration of 30 \(\mu\)M, 3 \(\mu\)M, or 0.3 \(\mu\)M. The six-well plates were centrifuged at 800 \(g\) for 10 min and then incubated at 37\(^\circ\)C with 5% CO\(_2\) for 1.5 h. The medium was removed from the wells and the monolayers were washed three times with 1 ml of PBS to remove non-adherent proteins. 350 \(\mu\)l of trypsin-EDTA (0.25% trypsin, 1 mM EDTA-4Na, Gibco) was added to the wells and the plates were incubated for 10 min at 37\(^\circ\)C. Cells were dislodged by repeat pipetting and harvested by centrifugation at 50 g (650 r.p.m) for 8 min. Cells were washed with 1 ml of PBS and resuspended in 0.1 ml of 2% paraformaldehyde in PBS at 4\(^\circ\)C overnight. The following morning, samples were washed with 1 ml of PBS to remove the fixative, resuspended in 0.4 ml of PBS, filtered through a cell-strainer cap (Falcon) and analysed by flow cytometry (MoFlo, Cytomation). Data were plotted as cell count versus fluorescent

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intensity. The cell population of interest was determined by using the AlexaFluor 488-labelled BSA sample to define non-specific fluorescence and/or autofluorescence levels. Positive staining was defined as a fluorescent signal greater than that of 98.8% of the BSA-treated control cell population.

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