The group B streptococcal alpha C protein binds $\alpha_1\beta_1$ -integrin through a novel KTD motif that promotes internalization of GBS within human epithelial cells

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Group B *Streptococcus* (GBS) is the leading cause of bacterial pneumonia, sepsis and meningitis among neonates and a cause of morbidity among pregnant women and immunocompromised adults. GBS epithelial cell invasion is associated with expression of alpha C protein (ACP). Loss of ACP expression results in a decrease in GBS internalization and translocation across human cervical epithelial cells (ME180). Soluble ACP and its 170 amino acid N-terminal region (NtACP), but not the repeat protein RR', bind to ME180 cells and reduce internalization of wild-type GBS to levels obtained with an ACP-deficient isogenic mutant. In the current study, ACP colocalized with $\alpha_1\beta_1$ -integrin, resulting in integrin clustering as determined by laser scanning confocal microscopy. NtACP contains two structural domains, D1 and D2. D1 is structurally similar to fibronectin's integrin-binding region (FnIII10). D1's (KT)D146 motif is structurally similar to the FnIII10 (RG)D1495 integrin-binding motif, suggesting that ACP binds $\alpha_1\beta_1$ -integrin via the D1 domain. The (KT)D146A mutation within soluble NtACP reduced its ability to bind $\alpha_1\beta_1$ -integrin and inhibit GBS internalization within ME180 cells. Thus ACP binding to human epithelial cell integrins appears to contribute to GBS internalization within epithelial cells.

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INTRODUCTION

Group B *Streptococcus* (GBS; *Streptococcus agalactiae*) is the leading cause of neonatal bacterial invasive diseases (Arisoy *et al.*, 2003; Manning *et al.*, 2004). The organism colonizes the vagina, rectum and urethra of 10–35% of adults without causing disease. Neonates acquire GBS either *in utero* or during birth, aspirating GBS-containing fluid. GBS enters the lungs, binds to and invades the alveolar epithelial cells, and enters the blood, allowing the organisms to infect multiple organs. Invasive disease occurs within hours of birth, with 4–6% mortality (Baker & Edwards, 1995; Schrag *et al.*, 2000). In a survey in the USA, the incidence of early-onset disease (within the first week of life) declined by 65% (from 1.7 per 1000 live births to 0.6 per 1000) from 1993 to 1998, due to intrapartum antimicrobial prophylaxis (Schrag *et al.*, 2000)

and stabilized at 0.35 per 1000 in 2004. The incidence of late-onset disease (>1 week to 3 months of age) remained stable, with an average of 0.35 per 1000 live births during the same time period (CDC, 1997, 2005).

GBS was more recently found to be pathogenic in the elderly, and in adults with underlying medical conditions, including diabetes (Dahl *et al.*, 2003; Jackson *et al.*, 1995; Tyrrell *et al.*, 2000). Adult invasive GBS diseases result in bacteraemia, meningitis, skin and soft-tissue infection, and bone and joint infections. The rate of invasive diseases is significantly higher in neonates than in adults. However, the case fatality rates are greater in adults (Schuchat, 1999), with the incidence of GBS disease increasing with advanced age (Farley *et al.*, 1993). Despite the clinical importance of these diseases, little is known about the events that lead up to GBS invasion and its virulence factors. Several studies suggest that surface proteins play a major role in GBS binding to and invasion of human mucosal surfaces (Bulgakova *et al.*, 1986; Tamura *et al.*, 1994). We previously reported that the alpha C protein (ACP) is one such protein (Bolduc *et al.*, 2002).

ACP is the prototype of a family of surface proteins known as alpha-like proteins (Alps) found on 90% of GBS clinical

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Abbreviations: ACP, alpha C protein; GAG, glycosaminoglycan; GBS, group B *Streptococcus*; NtACP, N-terminal domain of ACP; SCPB, GBS C5a peptidase.

isolates, as well as other Gram-positive organisms (Lachenauer *et al.*, 2000; Shankar *et al.*, 1999; Stalhammar-Carlemalm *et al.*, 1999; Turner *et al.*, 2003). ACP consists of an N-terminal domain (NtACP; 170 amino acids) followed by a variable number of tandem repeats (82 amino acids each) and a C-terminal domain (45 amino acids) containing an LPXTG peptidoglycan-anchoring motif (Michel *et al.*, 1992). Nearly all strains of serotype Ia, II and most of Ib express ACP on their surface (Madoff *et al.*, 1991). In addition, some human serotype III, IV and NT strains and bovine V, VII and non-typable strains contain the ACP gene (*bca*) (Creti *et al.*, 2004).

The biological function(s) of the Alp family of proteins remains unclear (Lindahl *et al.*, 2005). Deleting *bca* attenuates the virulence of GBS in the neonatal mouse model by approximately sevenfold (Li *et al.*, 1997). The lack of ACP expression *in vitro* reduces the ability of GBS to invade ME180 cells, a human cervical carcinoma epithelial cell line (Bolduc *et al.*, 2002). ME180 cells resemble stratified ectocervical squamous epithelial cells and are an established cellular model for studying group A and B streptococcal binding and invasion (Soriani *et al.*, 2006; Stalhammar-Carlemalm *et al.*, 1999; Sykes *et al.*, 1970). The group A *Streptococcus* Alp R28 promotes binding to ME180 cells and is essentially identical to GBS Alp3 (Lachenauer *et al.*, 2000). Similarly, adherence of *Enterococcus faecalis*, lacking expression of the Alp Esp, to urinary epithelium is reduced (Shankar *et al.*, 2001). Together, these data provide strong evidence for the role of Alps in the adherence to and invasion of epithelial cells by Gram-positive micro-organisms.

We solved the tertiary structure of NtACP by X-ray crystallography (Auperin *et al.*, 2005). NtACP can be further divided into two structurally distinct domains, D1 and D2. D1, the more distal (amino-terminal) portion, consists of a beta sandwich with strong structural homology to fibronectin's integrin-binding region (FnIII10). D2 consists of three antiparallel alpha helix coils containing a portion of the glycosaminoglycan (GAG)-binding domain. A partial putative GAG-binding site was mapped within domain 2 (D2), adjacent to the repeat region, consistent with our data showing that NtACP binds to heparin and GAGs only when it is covalently associated with the adjacent repeat region (Baron *et al.*, 2004). However, exogenous soluble NtACP alone (without the repeat region) can still competitively bind to ME180 cells and reduce internalization and translocation of GBS (Bolduc *et al.*, 2002), suggesting that NtACP binds to another non-GAG receptor. The structural data also revealed a potential integrin-binding site located on the most distal amino-terminal portion of NtACP (domain 1; D1), opposite the GAG-binding site on D2. We sought to determine if ACP binds to integrins present on the surface of ME180 cells and, if so, to identify the specific integrin heterodimeric molecules. Secondly, we tested the hypothesis that the binding of ACP's D1 domain to integrin plays a role in GBS invasion.

METHODS

Bacterial strains and epithelial cell line. GBS type Ia/C (α +, β +) strain A909 and the ACP-deficient mutant JL2053 have been described (Li *et al.*, 1997). A909 was obtained from Dr Rebecca Lancefield (Lancefield *et al.*, 1975). JL2053 was obtained from Dr Jing Li. ME180 cells (HTB33) (Sykes *et al.*, 1970) were purchased from the American Type Culture Collection (Manassas, VA, USA).

Cloning and site-directed mutagenesis of ACP-specific proteins. The amino acid sequence of ACP is in the NCBI Protein Database under accession number AAA26848 (Auperin *et al.*, 2005; Michel *et al.*, 1992). The DNA sequence encoding the ACP (pCL1), NtACP (pDEK14), repeat region RR' (pET24RR') and D2R has been cloned previously (Auperin *et al.*, 2005; Bolduc *et al.*, 2002; Gravekamp *et al.*, 1996; Kling *et al.*, 1997). The aspartic acid residue (Asp¹⁴⁶) of the recombinant NtACP was substituted with an alanine residue (D146A) using the Quik-Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Forward primer 5'-CCA CAT GTA AAG ACT GCT GGA CAA ATT GAT-3' and reverse primer 5'-ATC AAT TTG TCC AGC AGT CTT TAC ATG TGG-3' and the pDEK14 template were used, resulting in the pGB3 construct. The underlined nucleotides indicate the mutated codon.

The DNA sequence encoding the D2 region (Ser¹⁶¹-Leu²²⁵) was PCR amplified from pDEK14 using forward primer 5'-C GCT AGC ACA ACC TTG AGG GAT AAG ATT GAA-3' and reverse primer 5'-G GAA TTC TTA CAA TAC TAA CAA TTT CTC TAA TTC ATT AAC CTC-3'. The 209 bp PCR product was cloned into pCR2.1-TOPO, transformed into Top10 cells (Invitrogen), excised with *NheI* and *EcoRI*, and recloned into pTrcHisA. The resulting construct is pGB4. The DNA sequence encoding the Inv497 protein was PCR amplified from pRI203 (Hamburger *et al.*, 1999) using forward primer 5'-C GCT AGC AGC GTC ACC GTT CAG CAG CC-3' and reverse primer 5'-C CGA ATT CTT ATA TTG ACA GCG C-3'. The 1494 bp PCR product was cloned into pCR2.1-TOPO and transformed into TOP10 cells. The plasmid was isolated and digested with *NheI*, *EcoRI* and *SphI*. The *inv497* *NheI/EcoRI* fragment was gel purified and cloned into pTrcHisC. The resulting construct pGB5 was transformed into *Escherichia coli* strain BLR.

Protein expression and purification. Plasmids pET200/D-TOPO::D2-R, pDEK14 and pGB3 were transformed into *E. coli* BL21(DE3). Plasmids pGB4, pGB5 and pCL1 were transformed into *E. coli* BLR. Plasmid pET24RR' was transformed into BL21(DE3). Expression and purification of each protein was performed as previously described (Bolduc *et al.*, 2002). Briefly, protein expression was induced with 1 mM IPTG. The cells were harvested, lysed and the lysates loaded onto an 18 ml Fractogel (M) (Novagen) column charged with 50 mM NiSO₄. Fractions containing recombinant protein were pooled and dialysed in 20 mM HEPES, pH 7.2.

ME180-binding assay. ME180 cells were grown to confluence in 96-well titre plates (Falcon Microtest 96 353072) in RPMI 1640 with L-glutamine containing 10% Newborn Bovine Serum (26010-074; Invitrogen), 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹ (15140-122; Invitrogen) at 37 °C with 5% CO₂. Purified ACP, NtACP, NtACP D146A, D2-R and RR' were biotin-labelled using the EZ-Link Sulfo-NHS-Biotinylation kit (Pierce). The moles of biotin per mol protein were determined by the automatic HABA calculator available on the Pierce website (<http://www.piercenet.com>). The proteins were incubated with the cells for 4 h at 37 °C with 5% CO₂. Unbound proteins were removed by gently washing three times with Dulbecco's phosphate-buffered saline (PBS), pH 7.1 (Gibco) containing 0.1 mM CaCl₂ (PBS+CaCl₂). The cells were fixed with 2% paraformaldehyde in PBS+CaCl₂ and incubated in 0.5% BSA in PBS+CaCl₂ for 18 h at 4 °C. Bound proteins were detected with

avidin–peroxidase conjugate (1:100 000; Sigma) in 0.5 % BSA in PBS + CaCl₂ and developed with *o*-phenylenediamine tablets (Sigma P-6662) dissolved in phosphate/citrate buffer. The absorbance was read at 490 nm and 650 nm and recorded as ΔA . The molar ratios of biotin to ACP, NtACP, NTACP D146A, D2-R, and RR' were 2:1, 1:1, 1:1, 1:1 and 1:1, respectively. Therefore, the values obtained for ACP were divided by 2 to compare with the values obtained for the other proteins.

Inhibition assays were performed similarly with the exception that 0–500 μ M unlabelled NtACP, NtACP D146A and RR' was added to the wells containing 20 μ M biotinylated NtACP. The percentages of inhibition of bound biotinylated NtACP were calculated with the formula [(uninhibited ΔA – inhibited ΔA)/uninhibited ΔA] $\times$ 100. Statistical analysis was performed using the paired *t*-test (<http://www.graphpad.com/quickcalcs/ttest1.cfm>). A *P* value equal to or less than 0.050 is considered statistically significant.

$\alpha_1\beta_1$ -Integrin-binding assay. Purified human $\alpha_1\beta_1$ -integrin (5 μ g ml⁻¹; Chemicon International) in 50 mM carbonate/bis-carbonate buffer, pH 9.6, was allowed to bind to the wells of 96-well titre plates (NUNC flat-bottom MicroWell plate, MaxiSorp surface-treated) during an incubation of 2 h at 37 °C. The wells were blocked with 2 % BSA in 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 0.1 % Triton X-100 overnight at 4 °C. Antibodies to human α_1 - or β_1 -integrin subunits were added to the wells along with 0–20 μ M of NtACP, NtACP D146A mutant, RR' or BSA, in 2 % BSA in 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 0.1 % Triton X-100 to allow binding to the immobilized $\alpha_1\beta_1$ -integrin for 1 h at room temperature. Antibodies used were: 1:400 dilution of rabbit anti-human α_1 -integrin antibodies (AB1934; Chemicon International), 1:400 dilution of rabbit anti-human β_1 -integrin antibodies (AB1952; Chemicon International) and 1:500 dilution of mouse anti-human α_1 -integrin I-domain monoclonal antibody (MAB1973Z; Chemicon International). The wells were washed with 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 0.1 % Triton X-100 to remove unbound ACP and antibodies. Binding of rabbit polyclonal antibodies was detected by adding a 1:500 dilution of anti-rabbit IgG–peroxidase conjugated antibody (A6154; Sigma) followed by OPD substrate (P6662; Sigma). Absorbance was recorded at 490 nm and 650 nm. The results are reported as ΔA .

Confocal microscopy. ME180 cells were grown in 0.5 ml RPMI medium 1640 with L-glutamine, 10 % fetal calf serum, 25 mM HEPES, 0.4 M CaCl₂ on a 12 mm diameter transwell-COL (3.0 μ m pore size, Costar) membrane suspended in wells of a 12-well plate containing 1.5 ml of similar medium, as previously described (Bolduc *et al.*, 2002). ACP, BSA and Inv497 (Hamburger *et al.*, 1999) were labelled with AlexaFluor 568 using the AlexaFluor 568 Protein Labelling kit (Molecular Probes; A-10238). Aliquots of 0.25 ml of 1 μ M labelled proteins were added to the apical side of the cells for 4 h at 37 °C with 5 % CO₂. Unbound protein was removed by gently washing the cells three times with PBS. The cells were fixed by suspending the transwells in 2 % paraformaldehyde at 4 °C for 16 h. The membrane inserts were removed from the transwell and washed twice with PBS, cells facing up on a glass slide. The cells were permeated by incubating the membrane in 200 μ l of 0.1 % Triton X-100 in PBS for 10 min at 22 °C. The cells were washed once in PBS and incubated in 0.5 % BSA in PBS for 30 min at 22 °C to block non-specific binding. Primary antibodies to α -integrins (Chemicon International; ECM445) and β -integrins (Chemicon International; ECM440) were diluted 1:500 in 0.5 % BSA in PBS. MAB1951Z (mouse anti-human integrin β_1) was diluted in 0.5 % BSA in PBS containing 1 M CaCl₂, as its epitope binding is Ca²⁺-dependent; 200 μ l of the antibodies was added to the cells for 1 h at 22 °C. The cells were washed three times with PBS containing 1 M CaCl₂.

AlexaFluor 488 donkey anti-mouse IgG (H+L) (Molecular Probes; A-21202) or AlexaFluor 488 donkey anti-rabbit IgG (H+L) (Molecular Probes; A-21206) was diluted 1:200 in 0.5 % BSA in PBS containing 1 M CaCl₂ and added to the cells for 1 h at 22 °C. AlexaFluor 568 goat anti-rabbit IgG (H+L) (Molecular Probes; A-11011), diluted 1:200, was used to stain α -integrins when determining colocalization of α subunits with β_1 -integrin. Rabbit anti-integrin alpha-1 polyclonal antibody (Chemicon International; AB1934, 1:500 dil.) and AlexaFluor 660 goat anti-rabbit IgG (H+L) (Molecular Probes; A-21073, 1:500 dil.) were used to stain α_1 -integrin when determining colocalization of $\alpha_1\beta_1$ -integrin with ACP. The cells were washed three times in PBS containing 1 M CaCl₂. A drop of Fluorotec (Accurate Chemical) was added to the cells before they were covered with a cover glass. The edges were sealed with clear nail varnish.

Internalization assay. The assay was performed as previously described (Bolduc *et al.*, 2002). Briefly, ME180 cells were grown to confluence in 24-well culture plates containing 1 ml RPMI medium (RPMI 1640 with L-glutamine, 10 % fetal calf serum, 25 mM HEPES). On the day of the assay, the medium was removed from the wells and 0.4 ml fresh RPMI medium was added to the cells. Soluble proteins (0.1 ml of NtACP, NtACP D146A, D2, D2-R, RR' and BSA) were added to the cells 1 h before adding 0.5 ml of 2×10^6 c.f.u. ml⁻¹ of A909 and JL2503 (m.o.i. = 5 bacteria cell⁻¹). The cells were incubated at 37 °C with 5 % CO₂ for 2 h. The monolayers were washed three times with PBS before adding 1 ml RPMI medium containing 100 μ g gentamicin ml⁻¹ and 5 μ g penicillin G ml⁻¹ per well for 2 h at 37 °C with 5 % CO₂. The cells were then washed with PBS, lysed in 0.4 ml of 0.025 % Triton X-100, and serial dilutions of the epithelial lysates plated on THA plates. The percentage of internalized bacteria was calculated as [(c.f.u. per well after antibiotic treatment)/(c.f.u. originally added to the well)] $\times$ 100. Percentage inhibition was calculated as [(c.f.u. of internalized GBS)/(c.f.u. of original inoculum)]/[(c.f.u. of internalized GBS uninhibited)/(c.f.u. of original inoculum of GBS uninhibited)] $\times$ 100. Statistical analysis was performed using the unpaired *t*-test (<http://www.graphpad.com/quickcalcs/ttest1.cfm>). A *P* value equal to or less than 0.050 is considered statistically significant.

Translocation inhibition assay. The assay was performed as previously described with the additional step of preincubating the cell membranes with soluble protein before adding GBS (Bolduc *et al.*, 2002). Before GBS strain A909 or JL2053 was added to the cells, the medium was removed from the top chamber and replaced with 0.1 ml of 1, 5, 10, or 20 μ M of repeat protein (RR'), NtACP, ACP, or BSA in RPMI medium 1640 with L-glutamine, 10 % fetal calf serum, 25 mM HEPES, 0.4 M CaCl₂. The cells were incubated at 37 °C for 1 h. Without removing the soluble protein, 0.4 ml of approximately 2×10^5 c.f.u. ml⁻¹ of GBS strain A909 or JL2053 was added to the top chamber, and the cells were incubated for up to 4 h. *E. coli* DH5 α served as a non-invasive control.

RESULTS

The D1 KTD motif is involved in NtACP binding to ME180 cells

ACP, NtACP and D2-R (a construct containing D2 and the first adjacent repeat region) (Fig. 1a) were previously determined to bind qualitatively to ME180 by confocal microscopy and flow cytometry (Baron *et al.*, 2004; Bolduc *et al.*, 2002). In the current study, the proteins were biotin-labelled and quantitatively assessed for their ability to bind

Fig. 1. (a) ACP and its corresponding peptides. (b) ME180 binding assays. Varying concentrations of biotin-labelled ACP, NtACP, NtACP D146A, D2-R, and RR' were allowed to bind to confluent monolayers of ME180 cells. The presence of bound protein is indicated by the increase in $\Delta A_{490/650}$. The assay was performed twice with similar results. The values represent the mean $\pm$ SE of duplicate wells within one assay. (c) Ribbon representation of the structure of the N-terminal domain of ACP. β -Sheets are shown in blue; α - and 3_{10} -helices are in red. Side chains of residues R110, D118 and D146 (integrin triad) are indicated. The basic residues of the putative heparin-binding domain (K72, K90, R165, R172, N176, R185 and K196) are also indicated.

ME180 cells in an ELISA-based binding assay. The labelled proteins were allowed to interact with the cells for 4 h. The cells were then washed with saline to remove unbound protein, and the remaining proteins were detected with

avidin conjugated to horseradish peroxidase. ACP and NtACP bound similarly to ME180 cells, whereas NtACP D146A and D2-R demonstrated less binding. RR' did not bind (Fig. 1b), suggesting that the observed binding of ACP to ME180 cells is through the NtACP region.

NtACP consists of two structurally distinct domains, D1 and D2. We sought to locate NtACP's binding site(s) within one or both of these domains (Fig. 1c). Unable to express the isolated D1 region in a sufficient quantity, we chose to mutate D1 in an NtACP background and compare its ability to bind ME180 cells with that of wild-type NtACP. The integrin-binding activity of type III fibronectin (FnIII) requires the presence of the Arg¹⁴⁹³-Gly¹⁴⁹⁴-Asp¹⁴⁹⁵ (RGD) motif on FnIII module 10 (FnIII10) and Arg¹³⁷⁹ and Asp¹³⁷³ on FnIII module 9 (FnIII9) (Hamburger *et al.*, 1999). A structurally similar triad of charged residues required for integrin binding is present in *Yersinia pseudotuberculosis* Inv497. Inv497 is a non-RGD protein that competes with FnIII by binding to $\alpha_5\beta_1$ -integrin (Isberg *et al.*, 2000). NtACP's D1 region contains a K¹⁴⁴-T¹⁴⁵-D¹⁴⁶ (KTD) motif, located within a loop region that is structurally analogous to the loop containing the RGD integrin-binding motif in FnIII10. D1 also contains residues Arg¹¹⁰ and Asp¹¹⁸, forming a triangular arrangement of charged residues similar to those found in fibronectin and *Y. pseudotuberculosis* Inv497. If NtACP binds integrin, we hypothesize that a (KT)D146A substitution (NtACP D146A) may reduce its ability to bind ME180 cells.

Biotinylated NtACP along with either unlabelled NtACP or NtACP D146A was added to the cells for 4 h. We hypothesize that the labelling of NtACP with biotin may have increased the protein's ability to bind ME180 cells, since it required 25-fold more unlabelled NtACP to inhibit binding of biotin-labelled NtACP by 60 %. Nevertheless, NtACP D146A inhibited biotin-labelled NtACP binding significantly less than NtACP ($P=0.0152$ at 350 μM , $P=0.0211$ at 500 μM) (Fig. 2). Purified D2 was also tested for its ability to inhibit NtACP binding to ME180 cells; the inhibition was substantially less than that seen with NtACP ($P=0.0204$ at 250 μM , $P=0.0054$ at 500 μM). The data suggest that NtACP binds to an ME180 surface receptor via D1. The loss of inhibition due to a single mutation in the hypothetical integrin-binding KTD motif suggests that the receptor is an integrin heterodimer.

ACP binds $\alpha_1\beta_1$ -integrin

In order to identify the integrin heterodimer(s) that may bind ACP, we next characterized the integrin profile of ME180 cells. Individual α - and β -integrin subunits were probed with antibodies specific to the subunits, followed with AlexaFluor 488-labelled secondary antibodies, and examined by confocal microscopy. The cells stained with equally strong intensity for β_1 and β_2 subunits (Fig. 3a). Staining for β_3 , β_4 and β_5 subunits was near background levels and not detectable. ME180 cells also stained with

Fig. 2. ME180 inhibition binding assays. ME180 cells, grown to confluence in 96-well titre plates, were incubated with 20 μ M biotinylated NtACP plus varying concentrations (0–500 μ M) of unlabelled NtACP, NtACP D146A and D2. The mean percentage inhibition of bound biotinylated NtACP ($n=3-6$) and standard error (error bars) is graphed for each observed condition. The assay was performed three times.

strong positive intensity for α_1 , α_2 and α_3 subunits, but not for α_4 , α_5 and α_V subunits (Fig. 3b).

We sought to determine whether ACP colocalizes with the integrin subunits. ACP, Inv497 and BSA (all labelled with AlexaFluor 568) were added to a polarized, semipermeable layer of ME180 cells for 4 h and examined by confocal microscopy. Inv497 (the extracellular C-terminal 497 residues of invasins from *Y. pseudotuberculosis*) binds to $\alpha_3\beta_1$ -integrin and was included as a control (Van Nhieu & Isberg, 1991). Both ACP and Inv497 colocalized with β_1 but not with β_2 subunits (Fig. 4a, b). Interestingly, the cells with the most intense staining for ACP also demonstrated the highest fluorescent staining for the β_1 subunit, with little to no staining for the β_2 subunit. One of two possible explanations is that the interaction between ACP and ME180 cells results in a clustering of β_1 subunits. The other possibility is that, upon interaction with ACP, β_1 expression increases while β_2 expression decreases. AlexaFluor 568-labelled BSA served as a negative control (Fig. 4c). ACP also colocalized with the α_1 subunit (Fig. 4d) and not with α_2 or α_3 subunits. The α_1 and β_1 subunits colocalize with ACP on ME180 cells (Fig. 4e), identifying the $\alpha_1\beta_1$ -integrin heterodimer known as the very late antigen 1 (VLA1; CD49a/CD29; laminin and collagen receptor) (Hemler *et al.*, 1986).

Direct binding of NtACP, NtACP D146A, RR', D2-R (at 10 μ M) and BSA to $\alpha_1\beta_1$ -integrin was measured by ELISA. Purified $\alpha_1\beta_1$ -integrin was used to coat the wells of a 96-well microtitre plate. Mono- and polyclonal antibodies to the α_1 subunit were added to the wells containing various concentrations of the proteins. The proteins' abilities to inhibit binding of the antiserum were recorded as percentage inhibition. NtACP inhibited the α_1 subunit polyclonal antibodies from binding, while NtACP D146A showed significantly less inhibition ($P=0.0140$ at 5 μ M, $P=0.0042$ at 10 μ M and $P=0.0243$ at 20 μ M) (Fig. 5),

Fig. 3. ME180 α - and β -integrin subunit profile. (a) ME180 cells were probed with antibodies specific to β_1 -, β_2 -, β_3 -, β_4 - and β_5 -integrin subunits, followed by an AlexaFluor 488-labelled donkey anti-mouse IgG secondary monoclonal antibody, and analysed by confocal microscopy. β_1 and β_2 subunits are expressed on the surface of these cells with similar intensity and overall cell distribution. (b) ME180 cells were similarly probed with antibodies to the α_1 , α_2 , α_3 , α_4 , α_5 and α_V subunits. The α_1 , α_2 , and α_3 subunits were detected, resulting in a fluorescent intensity above background.

suggesting that NtACP binding to $\alpha_1\beta_1$ -integrin requires the intact KTD motif. RR', D2-R and BSA showed no significant difference in inhibition ($P>0.1$) from that observed with NtACP D146A. The monoclonal antibody MAB1973 recognizes an epitope located in the α_1 -integrin I domain (Fabbri *et al.*, 1996). It was included in the assay since it is known to inhibit binding of α_1 -integrin to laminin and collagen. MAB1973 did not affect ACP binding to $\alpha_1\beta_1$ -integrin. Similar assays were performed using polyclonal antibodies to the β_1 subunits, but no inhibition was observed (data not shown). NtACP may bind to an epitope(s) on β_1 that is not detectable with the polyclonal antibodies used, or it may bind only to the α_1 subunit.

Fig. 4. Colocalization of ACP and Inv497 with β_1 -integrin subunit. (a) ACP, (b) Inv497 and (c) BSA were labelled with AlexaFluor 568 (red) and allowed to interact with ME180 cells grown to confluence on transwells. β_1 - and β_2 -integrin subunits were detected with an anti-mouse antibody conjugated to AlexaFluor 488 (green). Colocalization of ACP and Inv497 with β_1 -integrin on the surface of ME180 cells results in yellow fluorescence. (d) ACP labelled with AlexaFluor568 (red) was allowed to interact with ME180 cells grown to confluence on transwells. α_1 -Integrin was probed with anti-mouse IgG antibodies conjugated to AlexaFluor 488 stained green. Colocalization of ACP with α_1 -integrin subunit was detected in yellow. (e) ACP labelled with AlexaFluor 568 (red) was allowed to interact with ME180 cells grown to confluence on transwells. α_1 -Integrin was probed with anti-rabbit IgG antibodies conjugated to AlexaFluor 660 (blue). β_1 -Integrin was probed with anti-mouse IgG antibodies conjugated to AlexaFluor 488 (green). Colocalization of $\alpha_1\beta_1$ -integrin with ACP was detected in white.

NtACP mediates internalization of GBS via the D1 region

We previously demonstrated that soluble recombinant ACP and NtACP, when added to ME180 cells, bind to the cells and reduce the level of internalized A909 to that achieved with the ACP-deficient isogenic GBS mutant JL2053. RR' does not affect internalization of either A909 or JL2053 (Bolduc *et al.*, 2002). In addition, structural data suggest that NtACP may bind integrin via a KTD motif in the D1 domain (Auperin *et al.*, 2005). Based on these results and the data presented above, we hypothesize that soluble NtACP may compete for binding to host cell $\alpha_1\beta_1$ -integrin, thus preventing GBS-associated ACP from binding and inhibiting its contribution to the internalization of GBS.

Therefore, in the current study we re-examined the internalization of A909 and JL2053 within ME180 cells in the presence of soluble NtACP D146A, D2 and D2-R to isolate the region within NtACP needed for maximal internalization.

Confluent ME180 cells were incubated with the soluble proteins for 2 h before GBS was added and the cells were allowed to internalize the bacteria. Both NtACP and NtACP D146A reduced A909 internalization, but did not affect the internalization of JL2053 (Fig. 6a). Pretreating cells with 20 μ M NtACP reduced the internalization of A909 by 82 % (5.55 times less) compared to that observed with non-treated cells. The single mutation within the KTD motif significantly attenuated NtACP's ability to inhibit

Fig. 5. $\alpha_1\beta_1$ -Integrin-binding assay. Varying amounts of NtACP (◆), NtACP D146A (■), RR' (▲), D2-R (10 μ M only; ○) and BSA (●) were added to 96-well plates coated with $\alpha_1\beta_1$ -integrin along with a 1:400 dilution of rabbit anti-human α_1 -integrin antibodies. Binding of the rabbit polyclonal antibody to the integrin was detected with anti-rabbit IgG–peroxidase conjugated antibody. The absorbance was recorded at 490 nm and 650 nm. The mean percentage inhibition ($n=3$) and standard error (error bars; not shown where smaller than symbol) for each protein concentration was calculated and interpreted as the ability to interact with $\alpha_1\beta_1$ -integrin. The assay was performed twice.

A909 internalization in a dose-dependent manner. Cells treated with 20 μ M NtACP D146A internalized 61 % less GBS than the non-treated cells.

A close structural homology exists between D1 and FnIII10 (Auperin *et al.*, 2005). In addition to FnIII10's RGD integrin-binding motif, FnIII requires two charged residues (Arg¹³⁷⁹ and Asp¹³⁷³) located on module 9 (FnIII9) for maximal integrin binding (Hamburger *et al.*, 1999). D1 residues (Arg¹¹⁰ and Asp¹¹⁸) occupy analogous positions in relation to the KTD motif, suggesting that these residues may also play a direct role in binding ACP to $\alpha_1\beta_1$ -integrin. Therefore, mutations within all three residues KTD¹⁴⁶, Arg¹¹⁰ and Asp¹¹⁸ may be required to completely prevent the competitive binding of exogenous NtACP and the subsequent entry of wild-type GBS into epithelial cells. The data support the hypothesis that the D1 domain of GBS-associated ACP is required for full GBS internalization into ME180 cells.

The addition of D2 (Fig. 6b) and D2-R (Fig. 6c) resulted in an increase in the percentage of internalized A909 (147.2 ± 7.8 and 158.3 ± 27.3 %, respectively). A 30.3 % increase in internalized ACP-deficient mutant JL2053 was also observed following the addition of D2-R to the cells. The causes for these observed increases are not understood. We previously demonstrated that GAGs bind D2-R (Auperin *et al.*, 2005). It is possible that D2-R binding to GAGs expressed on the surface of ME180 cells acts as a bridge to stabilize GBS binding and its subsequent internalization within ME180 cells. Alternatively, it is possible that the D2 domain stimulates the cells via an

Fig. 6. Internalization inhibition assays. ME180 cells grown in a 24-well plate format were pretreated with D2, D2-R, NtACP, NtACP D146A (treated), or no protein (untreated) before GBS strain A909 and mutant JL2053 were added. The percentage internalized GBS per well was calculated on the basis of the initial inoculum. The values are the mean of duplicate wells and the error bars represent the range. Unpaired *t*-test analyses between untreated and treated ME180 cells were performed [$**P \leq 0.05$, $*P = 0.05-0.1$, and NS=not significant ($P > 0.1$)]. Each assay was performed twice.

unknown mechanism resulting in the observed increase of GBS internalization.

DISCUSSION

GBS colonizes the large intestine of adults, leading to a possible secondary colonization of the vagina and urethra,

with an estimated average duration of colonization between 8 and 14 weeks (Picard & Bergeron, 2004). Higher frequency is seen among sexually active individuals (Manning *et al.*, 2004; Meyn *et al.*, 2002). GBS colonizing these mucosal sites can traverse the mucosal barriers, resulting in invasive disease. GBS adhesion is required for colonization and subsequent invasion. Several GBS surface proteins bind host extracellular matrix components and serum proteins (Beckmann *et al.*, 2002; Gutekunst *et al.*, 2004; Schubert *et al.*, 2002; Spellerberg *et al.*, 1999). ACP, a virulence factor and prototype for the Alp family of proteins, is the first surface protein identified to bind integral host cell-surface receptors, GAGs and $\alpha_1\beta_1$ -integrin. Structural analysis of the GBS C5a peptidase (SCPB) suggests that it too may bind integrins via the RGD motif (Brown *et al.*, 2005), but this has not been demonstrated experimentally.

GBS invasion of epithelial membranes involves internalization within membrane-bound vacuoles and paracellular translocation (Soriani *et al.*, 2006; Valentin-Weigand *et al.*, 1997). GBS internalization, but not translocation, requires actin polymerization and Rho GTPase activity (Baron *et al.*, 2004; Soriani *et al.*, 2006). Capsular expression also attenuates GBS internalization but not translocation (Gibson *et al.*, 1993; Soriani *et al.*, 2006). The different requirements for the two biological events suggest that they may be independent of each other. In contrast, expression of ACP appears to play a role during both events.

We initially reported that ACP binds heparin and epithelial cell-associated GAGs (Baron *et al.*, 2004). NtACP and the repeat region separately do not bind heparin. However, the D2-R peptide consisting of NtACP's D2 domain covalently associated with a single repeat binds heparin, suggesting that the heparin- and host GAG-binding domain is located within the junction between the D2 domain and the adjacent repeat. The NtACP tertiary structure maps a portion of the putative heparin-binding domain to BR2, a positively charged cluster within the D2 domain, adjacent to the first repeat region (Auperin *et al.*, 2005). We now report that ACP binds to a second cell-surface receptor, $\alpha_1\beta_1$ -integrin, via the D1 domain located on the opposite side of the molecule from the putative GAG-binding domain. The $\alpha_1\beta_1$ -integrin is one of four collagen-binding I-domain-containing integrins (Gullberg & Lundgren-Akerlund, 2002). It preferentially binds to collagen IV but also binds collagen I and laminin via the I domain (Calderwood *et al.*, 1997; Riikonen *et al.*, 1995). The monoclonal antibody MAB1973 recognizes an epitope specific to the human α_1 -integrin I domain and inhibits binding of activated human lymphocytes to these extracellular matrix components (Fabbri *et al.*, 1996). MAB1973 does not affect ACP binding to $\alpha_1\beta_1$ -integrin. However, the presence of a polyclonal antibody to α_1 -integrin does reduce ACP binding to $\alpha_1\beta_1$ -integrin by 42 %, suggesting that ACP binding to α_1 -integrin differs from that of collagen and laminin. One possible explanation is that ACP binds to α_1 -integrin via a unique non-I-domain interaction.

Proteins of other micro-organisms have similarly been reported to bind GAGs and integrin as co-receptors. For example, fibronectin acts as a bridge binding OpA, a *Neisseria gonorrhoeae* outer-membrane protein, to $\alpha_5\beta_1$ -integrin and is essential for *N. gonorrhoeae* internalization (van Putten *et al.*, 1998). GAG forms a complex with fibronectin and integrin for maximal internalization. The *Borrelia burgdorferi* surface lipoprotein BBK32 also binds fibronectin and GAG (Fischer *et al.*, 2006). ACP differs such that it binds directly to the integrin molecule without the use of a bridge. Pretreating ME180 cells with sodium chlorate, an inhibitor of sulfate incorporation, or with heparinase inhibits binding of ACP to the cells by 84–98 % (Baron *et al.*, 2004). We currently report that a single mutation within the KTD motif (D146A), present in the D1 domain, reduces NtACP binding to $\alpha_1\beta_1$ and ME180 by 20–25 %. Therefore, we sought to determine the contribution of integrin binding to GBS internalization within host cells.

Mutagenesis of NtACP, generating a KTD146A single mutation within the putative KTD integrin-binding motif, reduced its ability to inhibit GBS internalization. In similar assays where ME180 cells were preincubated with D2-R, containing the putative GAG-binding domain, internalization was not diminished, but instead increased. This effect does not require the expression of ACP on GBS. The RR' peptide had no measurable effect on GBS internalization. The data suggest that binding to both GAG and integrin may be needed for maximal binding of ACP to ME180 cells. However, the direct interaction between D1 of ACP and $\alpha_1\beta_1$ -integrin plays a key role in GBS internalization.

Structural analysis of the D1 domain, in particular the region containing the putative integrin-binding loop and KTD motif, shares a strong structural homology with the FnIII10's integrin-binding region. Amino acid sequence alignment of Alps indicates that KTD is highly conserved (Auperin *et al.*, 2005; Creti *et al.*, 2004), suggesting biological importance. The RGD motif present on FnIII10 interacts directly with integrin and is the canonical integrin-binding motif. Sixteen other motifs, not including KTD, were identified on short disintegrins (Sanz *et al.*, 2006). Each motif binds to a specific integrin heterodimer. The first two residues of the triads dictate the specificity of the α subunit (Sanz *et al.*, 2006). The motif KTS is expressed by obtustatin and viperistatin and binds $\alpha_1\beta_1$, in a I-domain-independent manner, similar to ACP (Marcinkiewicz *et al.*, 2003). We show that ACP interacts with the α_1 subunit of $\alpha_1\beta_1$ -integrins. Also, the KT(D146A) substitution reduces the binding of NtACP to $\alpha_1\beta_1$ -integrins. ACP did not alter binding of a polyclonal antibody specific to the β_1 subunit in similar experiments (data not shown). ACP may alter binding of a different source of poly- or monoclonal antibodies to β_1 -integrin. Therefore, we cannot rule out the possibility that ACP also binds to the β_1 subunit.

The $\alpha_1\beta_1$ -integrin is mainly expressed by mesenchymal cells and activated T lymphocytes and monocytes (Ben-Horin & Bank, 2004; Duband *et al.*, 1992; Hemler, 1990; Rubio *et al.*,

1995). However, it is also detected on epithelial and endothelial cells (Atsuta *et al.*, 1997; Miettinen *et al.*, 1993). Integrins in general can be involved in cell migration, tissue morphogenesis and immune response. The $\alpha_1\beta_1$ -integrin plays a role in chronic inflammation, fibrosis and angiogenesis (Cosgrove *et al.*, 2000; de Fougerolles *et al.*, 2000; Senger *et al.*, 1997). It remains to be determined what biological effects GBS may have on the host, when binding to $\alpha_1\beta_1$. The current study suggests that ACP binding to $\alpha_1\beta_1$ -integrin leads to the clustering of β_1 -integrin subunits. Prior to adding ACP to the cells, β_1 and β_2 subunits are equally dispersed on the surface of the ME180 cells. Following ACP treatment, the distribution of the subunits is altered. The two subunits segregate, resulting in the colocalization of the β_1 subunits with ACP. Clustering activates integrins, which form focal adhesions, and in turn activate a cascade of downstream intracellular signal pathways by a process known as 'outside-in' signalling. The cytoplasmic tails of the integrin subunits interact with cytoplasmic proteins, causing cytoskeletal rearrangements, phosphorylation and/or induction of gene expression (Loster *et al.*, 2001). Active $\alpha_1\beta_1$ -integrins may phosphorylate and activate focal adhesion kinase (FAK), resulting in the phosphorylation of other proteins necessary in signal transduction. FAK is capable of binding to the cytoplasmic tail of the α_1 subunit of $\alpha_1\beta_1$ -integrin.

ACP may also bind other integrins. The effect could broaden GBS tissue tropism and/or effects on cellular pathways. ME180 cells express β_2 -integrin subunits, to which ACP does not appear to bind. However, GBS can induce tumour necrosis factor alpha (TNF- α) release from monocytes and macrophages, enhancing CD11b/CD18 ($\alpha_M\beta_2$ -integrin)-dependent cell activation (Levy *et al.*, 2003). Therefore, further studies of β_2 -integrin may reveal its possible relation to GBS disease. Structural analysis of SCPB suggests that it binds integrin via one or more RGD motifs (Brown *et al.*, 2005), which would give GBS the ability to bind other integrin heterodimers and possibly activate multiple signal transduction pathways.

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