The *Streptococcus agalactiae* complement interfering protein combines multiple complement-inhibitory mechanisms by interacting with both C4 and C3 ligands

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**ABSTRACT:** Group B *Streptococcus* (GBS) colonizes the human lower intestinal and genital tracts and constitutes a major threat to neonates from pregnant carrier mothers and to adults with underlying morbidity. The pathogen expresses cell-surface virulence factors that enable cell adhesion and penetration and that counteract innate and adaptive immune responses. Among these, the complement interfering protein (CIP) was recently described for its capacity to interact with the human C4b ligand and to interfere with the classical- and lectin-complement pathways. In the present study, we provide evidence that CIP can also interact with C3, C3b, and C3d. Immunoassay-based competition experiments showed that binding of CIP to C3d interferes with the interaction between C3d and the complement receptor 2/cluster of differentiation 21 (CR2/CD21) receptor on B cells. By B-cell intracellular signaling assays, CIP was shown to down-regulate CR2/CD21-dependent B-cell activation. The CIP domain involved in C3d binding was mapped via hydrogen deuterium exchange–mass spectrometry. The data obtained reveal a new role for this GBS polypeptide at the interface between the innate and adaptive immune responses, adding a new member to the growing list of virulence factors secreted by gram-positive pathogens that incorporate multiple immunomodulatory functions.—Giussani, S., Pietrocola, G., Donnarumma, D., Norais, N., Speziale, P., Fabbrini, M., Margarit, I. The *Streptococcus agalactiae* complement interfering protein combines multiple complement-inhibitory mechanisms by interacting with both C4 and C3 ligands. *FASEB J.* 33, 4448–4457 (2019). www.fasebj.org

**KEY WORDS:** GBS · neonatal infection · B cells · virulence factor

*Streptococcus agalactiae* [group B *Streptococcus* (GBS)] is a gram-positive commensal microorganism normally present in the human gastrointestinal and genital tract. GBS is also an opportunistic pathogen that can cause life-threatening sepsis and meningitis in infants, systemic infections in the elderly, and bovine mastitis (1–3). Peripartum antibiotic prophylaxis has reduced infections occurring in the first week of life but has no effect on late-onset disease, and a vaccine is not yet available (4).

GBS is endowed with a number of virulence factors that facilitate interactions with multiple targets to penetrate host barriers and to counteract innate immune responses. Complement effectors serve as first line of defense against GBS infection, promoting phagocytic killing of these bacteria by neutrophils and macrophages (5). Therefore, the pathogen has evolved several mechanisms to inhibit complement activation to colonize and invade its host. GBS is surrounded by a thick, polysaccharidic capsule, containing sialic acid (6–8), which masks the bacterial surface and interferes with complement deposition (9). Antibodies directed to each of the 10 existing polysaccharide variants can block that effect and mediate bacterial serotype-specific phagocytic killing, making the GBS capsule a promising vaccine target. In addition to the capsular shield, GBS expresses cell wall–anchored proteins that manipulate complement attack by binding host regulators or by degrading its effectors, which have also been described as potential protective vaccine candidates. Among these are the Alp family protein members (6, 10)
and the streptococcal histidine triad (SHT) protein (11) that
bind factor H to accelerate the decay of the alternative
pathway C3 convertase, the BibA protein that interacts
with the classical and lectin pathway regulator C4 binding
protein (12) and ScpB, which exerts a proteolytic effect on
the anaphylatoxin C5a (13, 14).

In a recent study focusing on GBS-secreted virulence
factors that could potentially interfere with human
complement, we identified a 153-residues polypeptide
that was named complement interfering protein (CIP)
(15). The protein showed partial homology to the well-
studied Staphylococcus aureus complement modulator
extracellular adherence protein (Eap) (16, 17). Similar to
Eap, the secreted CIP binds to the bacterial surface via
an unknown ligand and interacts with C4 and C4b com-
plement effectors. CIP was shown to prevent the for-
mation of the C4b2a convertase, a common step in the
classical and lectin-complement pathways, to modulate
the formation/deposition of C3b on the GBS surface and
to prevent phagocytic killing of the microorganism.

CIP also shows partial homology to the Staphylococcus
aureus–secreted proteins extracellular fibrinogen-binding
protein Efb (18), extracellular fibrinogen-binding homol-
ogous protein (Ehp) (19), and staphylococcal-binding
IgG (Sbi) (20, 21) (10
–
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aureus–secreted proteins extracellular fibrinogen-binding
protein Efb (18), extracellular fibrinogen-binding homol-
ogous protein (Ehp) (19), and staphylococcal-binding
IgG (Sbi) (20, 21) (10–13% identity and 15–20% simi-
arity) (15). These 3 proteins are incapable of binding C4
ligands, but can interact with the C3 central component
of complement and its fragments C3b, iC3b, C3dg, and
C3d (18–23). Functional and structural studies have
revealed that binding of Efb, Ehp, and Sbi to C3b in-
duces conformational changes in this complement ef-
ector, which blocks its interaction with factor B,
thereby impairing the formation of the alternative
pathway C3 convertase C3bBb complex (16, 19, 23).
Further, binding of Efb and Ehp to the C3d ligand can
affect the interaction of this molecule with the comple-
ment receptor 2/cluster of differentiation 21 (CR2/
CD21) member of the B-cell surface coreceptor complex
implicated in the maturation of B cells when encoun-
tering specific antigens (22, 24).

The present study was aimed at investigating the
binding capacity of CIP toward C3 and its derived C3b
and C3d fragments by biophysical methods and its ability
to interfere with B-cell activation by inhibiting the in-
teraction between the C3d complement ligand and its
CR2/CD21 receptor.

MATERIALS AND METHODS

Cell cultures and media

Raji cells (American Type Culture Collection, Manassas,
VA, USA) were cultured in RPMI-1640 (GlutaMax; Thermo
Fisher Scientific, Waltham, MA, USA) supplemented with
10% fetal calf serum (GE Healthcare, Waukesha, WI, USA)
and Pen/Strep (MilliporeSigma, Burlington, MA, USA).
B cells were isolated from human peripheral blood mono-
nuclear cells (PBMCs; MAT Biotech, Leiden, The Nether-
lands) using the Human B Cell Purification Kit II, follow-
ing manufacturer’s instructions (Miltenyi Biotec, Bergisch
Gladbach, Germany).

Far-Western blot and ELISA analysis of CIP
interaction with complement factors

Affinity-purified, His-tagged CIP was subjected to 12.5% SDS-
PAGE (5 μg/lane), electroblotted onto a nitrocellulose mem-
brane, and incubated with 1% (v/v) normal or C3-depleted
human serum (MilliporeSigma). The membrane was probed
with goat anti-C3 serum (Complement Technology, Tyler, TX,
USA) and horseradish peroxidase (HRP)-conjugated mouse anti-
goat antibodies (Agilent Technologies, Santa Clara, CA, USA).

C3 and its C3b and C3d fragments (Merck, Darmstadt,
Germany) were loaded onto a 12.5% SDS-PAGE gel and electro-
blotted. The membrane was overlaid with CIP diluted to 1 μg/ml
in PBS–milk 3%–Tween 20 (0.05%), followed by primary rabbit
anti-CIP antisera and then goat HRP-conjugated anti-rabbit
antibodies (1:1000).

Nunc MaxiSorp microtiter plates (Thermo Fisher Scientific)
were coated with 100 ng of C3, C3b, C3d, C1q, and factor I
and incubated overnight at 4°C in 50-mM carbonate buffer (pH 9.5).
The wells were washed 2 times with PBS supplemented with
0.1% (v/v) Tween 20 (PBST), blocked with 2% (w/v) bovine
serum albumin (BSA) in PBST for 1 h at 22°C, and probed with
increasing concentrations of CIP, followed by incubation with
rabbit anti-CIP antibody (1:1000), and then with a HRP-
conjugated goat anti-rabbit IgG (1:1000). The signal was
revealed by HRP enzymatic activity. To calculate the relative
affinity association constant (Kd) for CIP for C3, C3b, and
C3d human ligands, the data were fitted using the following
equation:

$A = A_{max} [L] / [1 + K_d [L]]$

where [L] is the molar concentration of the ligand. The reported
dissociation constants (Kd) were calculated as reciprocals of the
Kd values. The assays were performed ≥2 times for each protein,
and the Kd values obtained were re-
producible in all cases.

In competitive ELISA experiments with immobilized C3b
and C4b, Nunc MaxiSorp Microtiter plates were incubated
overnight at 4°C with 100 ng of each protein in 50 mM carbonate buffer (pH
9.5). The plates were washed as previously described and probed
with 0.6 μM CIP preincubated or not with equimolar amounts of
C3b or C4b. The binding was revealed with rabbit anti-CIP
antibody (1:1000), followed by a HRP-conjugated goat anti-rabbit
IgG (1:1000).

For ELISA detection of C3d/CIP and C3d/CD21 complexes
under increasing ionic strength conditions, 250 ng of C3d
were surface coated onto microtiter plates, and CIP or CD21 were
diluted in a buffer containing increasing NaCl concentrations and
added to the wells. The complex formation was revealed through
rabbit anti-CIP or anti-CD21 serum (1:1000) and goat anti-rabbit
HRP.

Surface plasmon resonance analysis of
CIP–C3d interaction

The affinity of the interaction between CIP and C3d was evalu-
ated with a BIACore X100 instrument (GE Healthcare).
Human C3d was covalently immobilized on a dextran matrix CM5
sensor chip surface with a C3d solution (30 μg/ml in 50 mM sodium
acetate buffer, pH 5) diluted 1:1 with N-hydroxysuccinimide and
N-ethyl-N’-(3-dimethylaminopropyl)carbodiimide hydrochloride.
The excess of active groups on the dextran matrix was blocked with
1 M ethanolamine (pH 8.5) diluted 1:1 with NaCl concentrations and
added to the wells. The complex formation was revealed through
rabbit anti-CIP or anti-CD21 serum (1:1000) and goat anti-rabbit
HRP.
dissociation constant $K_d$ were estimated with a 1:1 interaction model (Langmuir model) by nonlinear fitting, using BIACore Evaluation 1.0 software (GE Healthcare).

**Competitive ELISA**

Microtiter wells were coated with 100 ng of soluble CR2/CD21 (Sino Biological, Beijing, China) and incubated overnight at 4°C in 50 mM carbonate buffer (pH 9.5). The wells were washed 3 times with PBST, blocked with 2% (w/v) BSA in PBST for 1 h at 22°C, and then probed with C3d (100 ng/well) preincubated with serial dilutions of CIP in PBS, followed by incubation with anti-C3 goat pAb (1:2000; Complement Technology) and then HRP-conjugated rabbit anti-goat IgG (1:1000; Agilent Technologies). Binding of the secondary antibody was revealed by HRP enzymatic activity.

**Flow cytometry analysis of C3d interaction with CR2/CD21 on B cells**

Biotinylated C3d (bC3d) was prepared using a Biotin Type A Conjugation Kit (Abcam, Cambridge, MA, USA), dialyzed to eliminate the excess of free biotin and incubated with streptavidin (SA; MilliporeSigma) for 30 min at 37°C. The bC3d-SA complex was subsequently incubated for 30 min at 37°C with His-tag–purified CIP at 1, 3, 6, and 9 μM to obtain CIP–bC3d-SA. The GBS-secreted, recombinant fibrinogen-binding protein 3 (Fib3) was used as a negative control at 9 μM (25). The bC3d-SA or CIP–bC3d-SA or Fib–bC3d-SA complexes were then added to Raji cells or B cells purified from human PBMCs (5 × 10^5/sample), and the mixture was left 15 min at 37°C and washed with PBS. Bound C3d was then stained with a mouse anti-C3d mAb (prediluted 1:100; Thermo Fisher Scientific) and revealed with a phycoerythrin-labeled anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Both incubations were conducted for 10 min at 37°C, and after each incubation, 2 washes in PBS–fetal calf serum 1% (v/v) were performed. Cells were fixed with Cytofix (BD, Franklin Lakes, NJ, USA) for 20 min at 4°C and analyzed using a Canto II Flow Cytometer with FACSDiva Software (BD). The geometric mean of the peaks was calculated through the FlowJo software (Tree Star, Ashland, OR, USA). The signals obtained from the samples with no CIP added were considered as 100% of C3d bound to CR2/CD21 on Raji cells and were used to normalize the signals obtained from the CIP-containing complex. The graph shows the residual C3d binding derived from the mean fluorescence intensity values of the peaks analyzed with the FlowJo software.

**Calcium mobilization on Raji cells**

Raji B cells (1 × 10^6/ml) were incubated with Fura Red (Molecular Probes, Thermo Fisher Scientific) in PBS with 2 mM EDTA at 37°C for 40 min and washed twice with the same buffer. The stimulus was formed by coincubating 0.1 μM C3d-biotin, 1 μM SA, and 0.2 μM biotinylated anti-human-IgM for 30 min at 37°C. To assess the inhibition of intracellular signaling by CIP, increasing CIP concentrations (0.5, 5, and 20 μM) were preincubated with biotinylated C3d before stimulus formation. Fib3 (20 μM) was used as negative control. Fluorescence detection was performed on 500 μl of cells (5 × 10^5/sample) using a LSRII Flow Cytometer (BD Biosciences). After establishing a baseline for 60 s, Raji cells were stimulated by the addition of the preformed stimuli (100 μl). Changes in Fura Red fluorescence emission were recorded using the LSRII. Data were analyzed through the FlowJo software and plotted as function of time and Fura Red emission ratio.

**Hydrogen deuterium exchange-mass spectrometry**

Sample preparation, digestion, and separation for hydrogen deuterium exchange-mass spectrometry (HDx-MS) analysis were performed as previously described (26). Briefly, the CIP–C3d complex was formed with 45 pmol of both CIP and C3d and incubated for 30 min at room temperature and then for 10 min on ice. The deuteration was initiated by diluting the sample with deuterated PBS and performed on ice. At different times during deuteration, samples were removed for quenching and dissociation of the protein–protein complex and immediately frozen in liquid nitrogen. A control experiment without C3d was performed using the same conditions. Labeled samples were rapidly thawed to 0°C and injected into a nanoAcquity ultraperformance liquid chromatographic system with HDX technology (Waters, Milford, MA, USA). Samples were digested online with a Poroszyme Immobilized Pepsin Cartridge (Thermo Fisher Scientific), and the generated peptides were trapped, concentrated, desalted, and separated on a reverse-phase Acquity Ultraprecision Liquid Chromatography Ethylene Bridged Hybrid C18, 1.7 μm, 1.0 × 100-mm column (Waters). Mass spectra were acquired in resolution mode (m/z 100–2000) on a Waters Synapt-G2 mass spectrometer equipped with a standard electrospray ionization source. The identity of each peptide was confirmed by mass spectrometry elevated energy, as previously reported (26). Data were processed with a Protein Lynx Global Server 2.5 (Waters), and each fragmentation spectrum was inspected manually to confirm the assignment. The DynamAX software (Waters) was used to select the peptides considered for the analysis and to extract the centroid mass of each of them and for each charge state as a function of the labeling time. Only the peptides present in ≥4 repeated digestions of the unlabeled proteins were considered for the analysis.

**Sequence alignment and statistical analysis**

Sequences of Efb-C and Sbi were downloaded from the UniProt Knowledgebase (UniProt Consortium, Munich, Germany), and sequence alignment was performed with the Geneious software (Biomatters, Auckland, New Zealand) setting BLOSUM62 Matrix to calculate sequence identities and similarities.

Statistical analysis was performed with Prism software (GraphPad, La Jolla, CA, USA).

**RESULTS**

**Interaction of CIP with C3 and its C3b and C3d fragments**

We first investigated whether CIP showed a binding capacity toward C3 present in human serum by Far-Western blot assays. A His-tagged, recombinant form of CIP was highly purified from *Escherichia coli*, analyzed by SDS-PAGE (Fig. 1A), and electroblotted onto nitrocellulose membranes. The membranes were incubated with normal or C3-depleted human serum before immunostaining with an anti-C3 pAb (Fig. 1B, lanes 1 and 2, respectively). A single protein band with an apparent MW of 15 kDa in agreement with the monomeric form of CIP was revealed on the membrane incubated with normal serum, whereas no signal was detected in the absence of C3, evidencing a specific interaction between CIP and C3.

In a second set of experiments, purified C3 and its C3b and C3d fragments were separated by electrophoresis under reducing conditions, electroblotted onto a membrane...
that was incubated with soluble, recombinant CIP, and then immune-stained with a CIP pAb (Fig. 1C, lanes 1–3). The data confirmed that the CIP protein interacted with the α chain of C3 (expected MW 110 kDa) and revealed an interaction with the α’ chain of its active form C3b (101 kDa) and with the C3d (35 kDa) degradation fragment, whereas no staining was detected for the band corresponding to the MW of C3α, C3βα’, and C3β are indicated on the right.

A dose-dependent and saturable interaction between CIP and C3, C3b, or C3d ligands was confirmed by ELISA. Surface-coated C3, C3b, and C3d were overlaid with increasing concentrations of purified CIP, which was followed by incubation with anti-CIP mouse serum and an HRP-conjugated secondary antibody (Fig. 2). Apparent K_d values (means ± sd) of 84.83 ± 0.009, 82.42 ± 0.0157, and 75.96 ± 0.0084 nM were estimated for the binding of CIP with C3, C3b, and C3d, respectively. These affinity values were comparable to those previously obtained for the interaction between CIP and C4b (15).

We sought to investigate by competitive ELISA whether CIP binding to its C4b and C3b ligands could occur at different sites on the GBS protein. Surface-coated C3b or C4b was overlaid with purified CIP in the absence or presence of equimolar concentrations of soluble C4b or C3b, washed with PBST, and incubated with anti-CIP mouse serum and HRP-conjugated secondary antibody. As shown in Fig. 3, soluble C3b inhibited binding of CIP to immobilized C3b, but not to C4b. Further, soluble C4b inhibited binding of CIP to immobilized C4b, but not to C3b. The above data suggested that the double-binding capacity of CIP toward C4 and C3 ligands relied on independent interaction sites.

**Biochemical characterization of the interaction between CIP and C3d**

The stoichiometry of the interaction between CIP and the C3d fragment of C3 was investigated by size-exclusion chromatography. When C3d and CIP were loaded separately onto the column, 2 elution peaks corresponding to the 35 and 15 kDa monomeric forms of CIP and C3d, respectively, were detected. Coincubation of CIP with C3d yielded, instead, a single peak of ~50 kDa, in agreement with the theoretical MW of the C3d–CIP complex, suggesting a binding ratio of 1:1 between the 2 proteins (Fig. 4A).

SPR experiments, in which purified C3d was immobilized onto the surface of a dextran chip and CIP was added in concentrations ranging from 0.039 to 5 μM, confirmed a concentration-dependent interaction closely fitting to a Langmuir 1:1 kinetic model (Fig. 4B). The GBS protein bound to C3d with an apparent K_d of 79 ± 0.62 nM (K_{on} 1.08 ± 0.25 × 10^4 M^{-1}s^{-1}; K_{off} 8.5 ± 0.46 × 10^{-4} s^{-1}), in agreement with the affinity measured by ELISA.

CIP is a highly positively charged protein with 20 Lys and 8 Arg out of 153 aa residues and a calculated isoelectric point of 9.62. The CR2–CD21 receptor is also rich in positively charged residues, and its interaction with C3d is known to be ionic strength dependent (27). The effect of ionic strength on the interaction between CIP and C3d was investigated by ELISA. As shown in Fig. 5, binding of surface-coated C3d to both soluble CR2–CD21 and CIP was inhibited by increasing NaCl concentrations, suggesting an electrostatic contribution to CIP interaction with C3d.

Recent structural studies revealed that C3d-binding staphylococcal proteins are folded in a 3-helix-bundle conformation (19, 23, 28). The circular dichroism spectrum of CIP in the far-UV indicated a prevalence of α-helix (46%), with a maximum positive molar ellipticity at ~7800° cm^2/dmol, a maximum negative molar ellipticity at around ~4500° cm^2/dmol, and the presence of β sheets (36%) and random coil (18%) structures (Supplemental Fig. S1).

**Mapping of the CIP peptide involved in C3d binding**

The C3d binding region of the CIP was investigated by HDx-MS. In this type of experiment, the interface between the binding partners can occlude solvent accessibility, thereby reducing the deuterium-exchange rate of the
backbone amide hydrogens. After pepsin digestion of the protein of interest, the resulting peptides were compared for their masses. A consistent mass shift of 1 Da was considered the threshold for a significant exchange of 1 deuterium atom (28, 29).

The CIP was incubated alone or in presence of C3d in a deuterated solution for different periods. Eleven CIP peptides, corresponding to 76% coverage of the mature protein, were generated from the subsequent pepsin digestion (Fig. 6A). Deuterium incorporation in the recovered peptides was monitored by mass spectrometry. As shown in Fig. 6B, a consistent difference of 1 Da of deuterium uptake was detected for fragments 96–127. No differences in exchange ratio were detected for any of the remaining peptides, including those partially overlapping with the region 96–127, and starting from aa 96 up to 121. The data suggested that the CIP amino acids involved in the C3d interaction were located in the stretch between residues 122 and 127 that contains an Arg residue (R123).

**Soluble CIP interferes with the interaction between C3d and CR2/CD21**

As previously reported, CIP inhibits the formation of the C4b2a classical and lectin C3 convertase, but not the C3bBb alternative pathway convertase (15).

We hypothesized that the interaction between CIP and C3d could interfere with the formation of the C3d–CR2/CD21 complex and reduce the kinetics of antibody-mediated B-cell intracellular signaling, as already observed for the staphylococcal Efb (22).

To investigate the capacity of CIP to interfere with the C3d–CR2/CD21 interaction, we performed a competitive ELISA experiment in which soluble C3d was preincubated with increasing concentrations of CIP, and the solution was overlaid on microtiter plates coated with CR2/CD21. Binding of C3d to the CR2/CD21 receptor was revealed with an anti-C3 pAb and secondary antibody incubation. The GBS protein Fib3 (25) was used as negative control. As shown in Fig. 7, CIP inhibited the C3d–CD21 interaction in a dose-dependent manner, whereas no effect was observed for Fib3 tested at the highest concentration.

Figure 3. Competitive inhibition assessment of CIP interaction with C3b and C4b. A) Results of an experiment with 100 ng of C3b was coated on a 96-well plate and overlaid with 0.6 μM of CIP preincubated with equimolar amounts of C3b or C4b, followed by incubation with anti-CIP IgG pAb and then an anti-mouse HRP-conjugated antibody. B) Similar experiment in which 100 ng of C4b was immobilized and overlaid with 0.6 μM of CIP preincubated with equimolar amounts of C4b or C3b.

Figure 4. Analysis of the interaction between CIP and C3d by size-exclusion chromatography and SPR. A) CIP and C3d were loaded separately or mixed in an equimolar concentration onto a Superdex column; the MW of eluted peaks was determined from a calibration curve. B) Two-fold linear dilution series (0.0390–5 μM) of CIP was injected over the C3d surface (250 response units) of a CM5 sensor chip. The sensograms obtained were normalized vs. the response obtained when the recombinant GBS protein was flowed over uncoated chips. Each sensogram was evaluated with BIA 3.0 software provided with the system. Shown is 1 representative of 3 experiments in which the start and the end of injection are indicated by arrows.
CIP inhibits the formation of the C3d–CR2/CD21 complex on B cells

We subsequently investigated whether CIP could inhibit the interaction between soluble C3d and CR2/CD21 present on the B-cell surface. It has been previously reported that C3d multimers can bind to CR2/CD21 on the surface of the Raji-immortalized B-cell line (22, 30). Flow cytometry experiments confirmed binding of biotinylated C3d preincubated with SA (C3d–biotin–SA) to Raji cells. Increasing concentrations of CIP were preincubated with the C3d–biotin–SA complex before adding the mixture to the cells, followed by flow cytometry analysis. As shown in Fig. 8A, the presence of CIP interfered with C3d binding to the B cells in a dose-dependent manner. Conversely, the same inhibitory effect was not detected when using the highest concentration of the negative control Fib3. CIP inhibition of C3d binding was also confirmed in a similar experiment with B cells enriched from human PBMCs, as evidenced by a dose-dependent shift in fluorescence in the corresponding flow cytometry histograms (Fig. 8B).

CIP inhibits B-cell intracellular signaling

The implications of CIP–C3d interaction on B-cell intracellular signaling were investigated in experiments in which C3d–biotin–SA and biotinylated anti-human IgM (BioLegend, San Diego, CA, USA) were combined to trigger intracellular calcium increase, as depicted in the diagram shown in Fig. 9A. Before the addition of stimuli, Raji B cells were treated with Fura Red, a cell-permeant fluorophore that changes its emission wavelength when bound to calcium. Changes in cellular fluorescence at different times were recorded by flow cytometry, and the acquisition of fluorescent cells, incubated with biotin–anti-human IgM only, was used as substimulatory baseline. To assess the CIP inhibition effect, intracellular signaling was triggered in the absence or in presence of increasing concentrations of the protein or in the presence of the highest concentration of Fib3 as a negative control. As shown in Fig. 9B, CIP decreased intracellular calcium release in a dose-dependent manner with maximum inhibition at 20 μM of CIP. Conversely, fluorescence was not affected by the presence of Fib3 (Fig. 9C).

DISCUSSION

The GBS CIP protein was first identified as a virulence factor secreted by GBS, showing a capacity to bind the complement proteins C4 and C4b and to block the deposition of C3b on the bacterial surface via the lectin and classical pathways (15). The present study identified, as new ligands for CIP, the C3 central complement component and its thioester containing effector fragments C3b and C3d and revealed new complement evasion mechanisms for this GBS protein.

By sequence homology comparisons with staphylococcal-secreted virulence factors, CIP appeared as a protein chimera showing partial similarity to the C4-binding protein Eap from S. aureus in its N-terminal region and to the C3b/C3d binding proteins Efb and Sbi in their C-terminal regions (15) (Supplemental Fig. S2). The CIP C4 and C3 binding sites appeared to be independent, based on competition experiments.

The interaction between Efb or Sbi and C3 induces conformational changes in C3b that abolish the C3bBb convertase function blocking downstream-activation...
events (20, 28). Moreover, Sbi was shown to form a tripartite complex with C3b and factor H that contributes to its inhibitory effect toward the complement alternative pathway (20). A similar role of the GBS CIP protein in preventing the formation/activation of the C3bBb convertase could have been expected based on its C3b-binding capacity but was excluded in our previous investigations (15). The molecular basis for the different modulatory capacity toward the alternative pathway between CIP and the Efb and Sbi staphylococcal proteins deserves further investigations. Here, we focused our attention on the interaction with C3d, the last degradation fragment of C3, which possibly contains the complete CIP binding site. A saturable, univalent interaction between CIP and C3d was demonstrated by coupling ligand and receptor in solid phase supports and by size-exclusion chromatography. SPR experiments confirmed an intrinsic affinity between CIP and C3d in the order of 0.1 μM, slightly higher than the $K_d$ measured for the staphylococcal Sbi (23) and less than the one reported for Efb (31).

Besides having a key role in innate immune surveillance, the complement system is implicated in the engagement of adaptive responses (32), and several in vivo studies support a role for CRs in the acquisition of target antigens (33). In particular, the C3d/C3dg physiologic degradation products of C3 enhance B-cell signaling by simultaneously binding the antigen–B-cell receptor complex and the coreceptor complex formed by CR2/CD21, CD19, and CD81. This receptor coligation has a profound molecular adjuvant effect that lowers the threshold of antigen required for B-cell activation by >1000-fold (34). The C3d-dependent crosslink also directs B cells toward their T-cell boundary, where they further differentiate through clonal expansion, somatic hypermutation, affinity maturation, and Ig class-switch recombination (35). Furthermore, the retention of C3d-opsonized antigens through CR2/CD21 on follicular dendritic cells in the lymph node is essential for the generation of high-affinity antibodies and memory B cells (36).

Here, we show that binding of CIP to C3d prevents the interaction of this protein with the CR2/CD21 B-cell receptor both in vitro and ex vivo and that this interference effect results in decreased intracellular signaling, as measured by lower levels of calcium release. Therefore, the data point toward a new role for the CIP protein in

![Figure 7. ELISA competitive experiments showing that CIP inhibits the formation of the C3d–CR2/CD21 complex.](image)

![Figure 8. Flow cytometry analysis of C3d interaction with B cells in presence or absence of CIP.](image)
counteracting adaptive immunity by modulating the activity of the C3d complement effector and may represent a new example of the coevolution between the GBS microorganism and its human host.

The CIP concentrations used in our experiments were in the range of ~0.1–10 μM, and equimolar amounts of CIP and C3d were used for analyzing the interaction between the 2 proteins by size-exclusion chromatography. An approximate quantification of CIP by dot blot in the supernatant of a GBS neonatal strain grown in vitro to stationary phase in parallel to recombinant CIP, indicated concentrations of ~0.3–0.5 μM (data not shown). This value could represent an underestimation because CIP is able to bind to the bacterial surface (15) not allowing a reliable quantification of the secreted form. We previously observed that CIP was differentially expressed in different growth media, and its expression might also be regulated during infection, making it difficult to draw definite conclusions on the physiologic relevance of concentrations measured from an in vitro culture.

Deep genomics studies have indicated that lateral transfer and recombination are strongly implicated in the evolution of GBS. In particular, phage insertions generate interstrain diversity and provide the pathogen with a number of virulence factors that facilitate its survival in the host (37). Of note, the gene coding for CIP is located in a hot spot phage insertion region of ~20 kbp (15), which also contains the bspC locus implicated in biofilm formation (38, 39). Interestingly, group A Streptococcus isolates that belong to serotypes associated with maternal–fetal urogenital infections contain a similar phage insertion that also encodes a secreted protein 46% identical to CIP (15), suggesting interspecies lateral transfer.

Cocrystal structural analysis highlighted essential salt bridges and hydrogen bond interactions between a convex acidic pocket from C3d and positively charged residues in the surface patches of CR2/CD21 SCR (short consensus repeat) 1 and 2 domains (27, 40). A similar type of interaction between C3d and cation residues present on α helix 2 of the staphylococcal Efb and Sbi was demonstrated by cocrystallization and mutagenesis experiments (23, 28). Secondary structure predictions confirmed by circular dichroism analysis indicate that the C-terminal region of CIP also presents an α-helical structure. Interestingly, HDx-MS led to the identification of a CIP peptide interacting with C3d that contains an arginine (R123) separated by 6 residues from an asparagine (N130). The 2 residues perfectly matched the R131 and N138 present on α helix 2 of the staphylococcal Efb and Efb-C and the R231 and N238 of Sbi. Unfortunately, the CIP peptide containing N130 was not recovered by MS possibly because of the proximity of pepsin-sensitive residues. Two recombinant variants of CIP, the first containing a substitution of R123 with an alanine (A) residue and the second a double-mutant R123 to A plus N130 to A, were expressed in E. coli to experimentally confirm their role in C3d binding. Despite high expression levels, the 2 mutant proteins were poorly soluble in aqueous buffer and quickly precipitated. Future cocrystallization experiments will be instrumental to confirm the amino acid residues directly involved in the interactions between CIP and C3d.

The gastrointestinal and urogenital GBS colonization sites are rich in mucosal-associated lymphoid tissues containing many B cells, in which CIP could exert its immunomodulation effect (41).

In addition to having an important role as the ligand of CR2/CD21, C3d also binds the CR3 (CD11b/CD18) on dendritic cells and macrophages via its integrin I domain (42). A possible additional effect of CIP not investigated here could be the prevention of the interaction between C3d and CR3 on antigen-presenting cells, limiting the transport of GBS antigens to the lymph nodes and their presentation to resting B cells.

To investigate the effect of CIP on GBS virulence in vivo, we tried to obtain an isogenic cip mutant in a GBS neonatal strain but were unsuccessful. Previous experiments had
shown that GBS survival in human blood was enhanced after preincubation of the bacteria with recombinant CIP (15). It is tempting to speculate that the observed antiphagocytic effect of CIP could rely on its capacity to bind both C4b and C3. Indeed, in addition to modulating the formation of C3b via the classical/lectin pathways, as previously reported, the CIP protein could prevent binding of complement-labeled bacteria to CR3 and CR1/CD35 receptors in neutrophils.

Finally, it has been postulated that differentiation of autoreactive B cells could also involve coreceptor engagement of C3d along with B-cell receptor binding to self-antigens (32). If that hypothesis is confirmed, proteins capable of interfering with the C3d-CR2/CD21 interaction, such as CIP or their derived peptides, could represent valuable therapeutic tools for the modulation of aberrant B-cell responses to combat antibody-mediated autoimmune disorders.

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AUTHOR CONTRIBUTIONS

S. Giussani, G. Pietrocola, N. Norais, P. Speziale, M. Fabbrini, and I. Margarit designed the research; S. Giussani, G. Pietrocola, and D. Donnarumma performed the research; S. Giussani, G. Pietrocola, D. Donnarumma, N. Norais, P. Speziale, M. Fabbrini, and I. Margarit analyzed the data; S. Giussani and I. Margarit drafted the manuscript; and all authors had full access to the data, were involved in revising the paper critically for intellectual content, and approved the manuscript before it was submitted by the corresponding author.

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