Binding of Complement Inhibitor C4b-binding Protein to a Highly Virulent Streptococcus pyogenes M1 Strain Is Mediated by Protein H and Enhances Adhesion to and Invasion of Endothelial Cells*

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Background: Pathogens such as Streptococcus pyogenes acquire protection by binding complement inhibitors to their surface.

Results: Domain A of protein H binds to C4BP CCP1–2, increasing invasiveness.

Conclusion: Protein H but not M1 protein is the major virulence factor mediating invasion and preventing C3b deposition.

Significance: Understanding molecular details of host pathogen interactions is crucial for development of novel therapeutics.

Streptococcus pyogenes AP1, a strain of the highly virulent M1 serotype, uses exclusively protein H to bind the complement inhibitor C4b-binding protein (C4BP). We found a strong correlation between the ability of AP1 and its isogenic mutants lacking protein H to inhibit opsonization with complement C3b and binding of C4BP. C4BP bound to immobilized protein H or AP1 bacteria retained its cofactor activity for degradation of 125I-C4b. Furthermore, C4b deposited from serum onto AP1 bacterial surfaces was processed into C4c/C4d fragments, which did not occur on strains unable to bind C4BP. Recombinant C4BP mutants, which (i) lack certain CCP domains or (ii) have mutations in single aa as well as (iii) mutants with additional aa between different CCP domains were used to determine that the binding is mainly mediated by a patch of positively charged amino acid residues at the interface of domains CCP1 and CCP2. Using recombinant protein H fragments, we narrowed down the binding site to the N-terminal domain A. With a peptide microarray, we identified one single 18-amino acid-long peptide comprising residues 92–109, which specifically bound C4BP. Biacore was used to determine $K_D = 6 \times 10^{-7}$ m between protein H and a single subunit of C4BP. C4BP binding also correlated with elevated levels of adhesion and invasion to endothelial cells. Taken together, we identified the molecular basis of C4BP-protein H interaction and found that it is not only important for decreased opsonization but also for invasion of endothelial cells by S. pyogenes.

Streptococcus pyogenes, also known as group A streptococcus is a β-hemolytic Gram-positive bacterium and considered to be one of the most important bacterial pathogens worldwide (1). S. pyogenes can cause a variety of diseases, from superficial skin to life threatening systemic infections (2, 3). The bacteria evolved a distinct set of secreted and surface bound virulence factors, which enable S. pyogenes to effectively infect the human host. The probably best characterized virulence factors are the members of the M protein family. Because M proteins are unique to each strain, they are used to classify the different S. pyogenes serotypes. Currently, there have been >200 different M proteins typed (4). These proteins can bind a variety of host proteins such as complement inhibitor factor H, C4b-binding protein (C4BP),3 CD46 as well as fibrinogen, albumin, and immunoglobulins (5–8). It is believed that covered with those proteins, S. pyogenes can evade the immune system by avoiding complement-mediated opsonization and uptake by professional phagocytes.

Complement is a powerful weapon protecting the host from invading pathogens by opsonizing microorganisms, activating the immune system, recruiting phagocytes, and eventually directly lysing certain pathogens. This system must be tightly regulated not to harm the host itself, which is guaranteed by a set of soluble and membrane-bound inhibitors of complement (9). Several pathogens however acquired the ability to bind exactly these inhibitors to their surface to evade complement recognition and subsequent elimination (10).

One important molecule, which S. pyogenes recruits to its surface, is complement inhibitor C4BP. C4BP is a plasma glycoprotein of 570 kDa involved in the regulation of the classical and lectin complement pathways (11). It is made of seven identical α-chains and one unique β-chain (12). All α-chains consist of eight complement control protein (CCP) domains, each comprised of around 60 aa (13). The β-chain consists of three

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3 The abbreviations used are: C4BP, C4b-binding protein; aa, amino acid(s); CCP, complement control protein; NHS, normal human serum; HUVEC, human umbilical vein endothelial cell.
CCP domains and is connected with the α-chains through disulfide bridges in a central core (14). C4BP accelerates the decay of the C3 convertase (C4bC2a) as well as acting as a cofactor for the cleavage of C4b by factor I (15). Thus, recruited to a surface, C4BP inhibits classical pathway complement activation and subsequent opsonization followed by lysis. Unfortunately this does not only occur on host cells, but also on pathogens, such as \textit{S. pyogenes}, which bind C4BP through virulence factors of the M protein family.

One member of the family of M proteins is protein H, which is encoded by a gene directly adjacent to the M1 protein gene (16). Protein H was identified only in \textit{S. pyogenes} M1 strains and probably evolved from gene duplication (17). Interestingly, protein H but not M1 protein was described to bind complement inhibitors (18). In contrast to M proteins, the binding of protein H especially to C4BP is not well characterized. In this study, we analyzed the molecular basis of the interaction between protein H and C4BP in detail as well as its functional consequences.

**EXPERIMENTAL PROCEDURES**

**Proteins and Antibodies**—M1 protein, protein H, and its fragments were expressed in \textit{Escherichia coli} and purified on a human IgG column as described previously (8, 19, 20). C4BP (21), C4BP mutants lacking individual CCP domains (22), aa insertion mutants (22), as well as point mutants M14Q, D15N, R22H, K24E, L34R, R39Q, K63Q, R64Q, R66Q, H67Q, K79Q, A12V/M14V, T28Q/T47Q, T43D/T45M, K126Q/K128Q, E53Q/V55T/N57T, and Q109A/D110N/R11Q/D112S have been described and characterized previously (22–26). Insertion mutants (22), as well as point mutants M14Q, D15N, R22H, K24E, L34R, R39Q, K63Q, R64Q, R66Q, H67Q, K79Q, A12V/M14V, T28Q/T47Q, T43D/T45M, K126Q/K128Q, E53Q/V55T/N57T, and Q109A/D110N/R11Q/D112S have been described and characterized previously (22–26).

**Bacteria, Cells, and Culture Conditions**—\textit{S. pyogenes} strains AP1 (\textit{S. pyogenes} strain 40/58 serotype M1, WHO Collaborating Centre for Reference and Research on Streptococci, Prague, Czech Republic), MC25 (28), BM27.6 (19), and BM71 (29) were inoculated and grown in Todd-Hewitt broth overnight at 37 °C and 5% CO2 without shaking. Cultures of isogenic mutants were supplemented with either 150 μg/ml kanamycin (MC25), 1 μg/ml erythromycin (BM27.6), or 5 μg/ml tetracycline (BM71). Overnight cultures were diluted to \( A_600 = 0.1 \) in fresh Todd-Hewitt broth and incubated at 37 °C and 5 % CO2 without shaking until they reached exponential growth at \( A_600 = 0.3 \) to 0.4. Prior to use, bacteria were harvested, washed with 1× PBS, and diluted in desired medium. Human umbilical vein endothelial cells (HUVECs) were obtained from Invitrogen and cultivated in M200 media supplemented with low serum growth supplement (Invitrogen) at 37 °C and 5% CO2 for a maximum of 5 passages (30).

**Characterization of Protein H and C4BP Interaction**
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on a 10–15% SDS-PAGE. Analysis of C4b degradation was performed as described above.

Surface Plasmon Resonance (Biacore)—The interaction between protein H or C4met and C4BP single α-chains was analyzed using surface plasmon resonance (Biacore 2000; Biacore, GE Healthcare). The individual flow cells of a CM5 sensor chip were activated, each with 20 μl of a mixture of 0.2 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 0.05 M N-hydroxysulfosuccinimide at a flow rate of 5 μl/min, after which C4BP diluted to 50 μg/ml in 10 mM sodium acetate buffer, pH 4.0, was injected over one flow cell to reach 4000 resonance units. Not reacted groups were blocked with 20 μl of 1 M ethanolamine, pH 8.5. A negative control was prepared by activating and subsequently blocking the surface of flow cell one. The binding kinetics was studied for various concentrations of purified protein H and C4met in 25 mM Hepes, pH 7.5, supplemented with 150 mM NaCl, 3 mM EDTA, and 0.005% Tween 20. Protein solutions were injected for 300 s during the association phase at a constant flow rate of 30 μl/min and at room temperature. Each sample was injected first over the negative control surface and then over immobilized C4BP. Signal from the control surface was subtracted. The dissociation was followed for 200 s at the same flow rate. In all experiments, two 10-μl injections of 2 M NaCl, 10 mM HCl were used to remove bound ligands during a regeneration step. The concentration series were analyzed at least twice. The BiaEvaluation software (version 3.0, Biacore) was used to analyze obtained sensorgrams. Response units obtained at plateau of sensorgrams were plotted against concentrations of injected protein and used for calculation of equilibrium affinity constants ($K_a$) using steady state affinity model.

Peptide Microarray—PepSpots peptide microarray on cellulos membrane was purchased from JPT Peptide Technologies. The membrane-spotted peptides were 14 consecutive 18-mers of domain A from protein H (aa 42–122) with an overlap of 13-aa each. The membrane was washed three times with wash buffer and then incubated with blocking solution for 2 h at room temperature. 10000 kcpm of $^{125}$I-labeled C4BP were added to the membrane and incubated with shaking for 1 h at 37 °C to allow binding of C4BP to the spotted peptides. After intense washing, bound $^{125}$I-C4BP was visualized and quantified using a PhosphorImager (Fuji, GE Healthcare).

Flow Cytometry Analysis of Surface Deposited Complement Proteins—Harvested bacteria from *S. pyogenes* strains AP1, MC25, BM27.6, and BMJ71 were incubated with increasing amounts of normal human serum for 1 h at 37 °C, 5% CO$_2$. Subsequently, bacteria were washed three times with 1× PBS and stained for C4BP (MK104, coupled to Dylight 633, Pierce), C3 (F(ab)$_2$ fragments coupled to Dylight 633), C4c or C4d, respectively. Unconjugated primary antibodies were detected with secondary antibodies coupled to PE. Amount of surface bound complement was measured using a Cyflow space flow cytometer (Partec).

Host Cell Adherence and Invasion Assay—HUVECs were seeded at a density of 3 × 10° cells per well. Confluent monolayers of HUVECs were washed thoroughly and infected for 3 h with *S. pyogenes* in 500 μl of medium at 37 °C using a multiplicity of infection of 25. Prior to infection, bacteria were incubated for 30 min with plasma-purified C4BP (25 μg) in a total volume of 100 μl PBS at 37 °C. Post-infection, cells were washed three times with PBS to remove unbound bacteria. The total number of adherent and intracellular recovered bacteria was monitored after detachment and lysis of cells with saponin (1% w/v) (Sigma) and plating the bacteria on blood agar. The number of intracellularly survived bacteria was quantified by utilizing the antibiotic protection assay (31). Briefly, *S. pyogenes* was preincubated with or without C4BP prior to the infection of HUVECs. After 3 h, the infected cells were washed and further incubated for 1 h with cell culture medium containing 100 mg ml$^{-1}$ gentamicin (Sigma) and 100 units ml$^{-1}$ penicillin G (Sigma) at 37 °C and 5% CO$_2$ to kill extracellular and non-adherent bacteria. Invasive and viable bacteria were recovered from the intracellular compartments of the host cells by a saponin-mediated host cell lysis, and the total number of invasive bacteria was monitored after plating sample aliquots on blood agar plates.

Ethics Statement—NHS was prepared from venous blood of healthy volunteers and according to the recommendations of the local ethical committee in Lund (permit 418/2008). Written informed consent was obtained, and all investigations were conducted according to the principles of the Declaration of Helsinki.

Statistics—Statistical analysis was performed using GraphPad Prism software (version 5.0f). To test for significance, a two-way analysis of variance with Bonferroni’s post test was performed. $p < 0.05$ was considered to be significant.

RESULTS

Protein H Protects *S. pyogenes* from Opsonization and Promotes Endothelial Adherence and Invasion—First, we confirmed that protein H but not M1 protein is important for *S. pyogenes* ability to bind C4BP. Therefore, we utilized *S. pyogenes* AP1 and the isogenic mutants MC25, lacking M1 protein; BM27.6, lacking protein H; and BMJ71, lacking the mga regulon (missing protein H and M1 protein, streptococcal inhibitor of complement and C5a-peptidase). BM27.6 was described and confirmed as a specific protein H deletion still harboring an intact M1 gene. However, mass spectrometry analysis of surface proteins revealed that this mutant features only ~10% of M1 protein compared with the wild type strain (personal communication Dr. J. Malmström, Lund University). We incubated these bacteria in increasing amounts of NHS for 1 h at 37 °C. Subsequently, we analyzed binding of C4BP and deposition of C3b using flow cytometry. As expected, the wild-type AP1 strain and the MC25 strain, lacking M1 protein, bound significant amounts of C4BP from NHS (Fig. 1A). On the contrary, both isogenic mutants lacking protein H, BM27.6, and BMJ71 did not bind C4BP. Interestingly, these two mutants had large amounts of C3b deposited on their surface, whereas AP1 and MC25 showed significantly reduced amounts of C3b deposition (Fig. 1B). We then tested whether C4BP bound to protein H was still able to support degradation of C4b. Immobilized protein H was incubated with C4BP, and after repeated washing steps, factor I and $^{125}$I-C4b were added. As expected, bound C4BP acted as factor I cofactor in degradation of C4b (Fig. 1C, top). No degradation of C4b was observed on plates coated with BSA, which did not bind C4BP. Additionally, in the absence of
either C4BP or factor I C4b was intact. We confirmed these findings by analyzing C4b degradation directly on the surface of S. pyogenes AP1 and BM27.6. Only C4BP bound to AP1 supported degradation of C4b (Fig. 1C, bottom). Because BM27.6 binds solely trace amounts of C4BP, we could observe only very little C4b degradation using that strain. We further confirmed our findings in more physiological setting by incubating S. pyogenes AP1 and BM27.6 in 10% NHS for 1 h at 37 °C and subsequent flow cytometry analysis for C4c and C4d (Fig. 1D). Intact and active C4b is degraded into C4c and C4d fragments by factor I, upon which the C4c fragment is released, whereas C4d remains bound to bacterial surface. Thus, we analyzed the ratio of the mean fluorescence intensities of C4d:C4c on AP1 and BM27.6. On AP1, the ratio of C4d:C4c was 2.07 ± 0.31, indicating that a considerable amount of surface bound C4b was cleaved and inactivated. On BM27.6, the ratio between C4d and C4c was 1.01 ± 0.09, confirming that the C4c fragment was not removed and C4b was fully active. Taken together, protein H indeed is an important virulence factor in AP1 mediating C4BP binding and hence preventing cleavage of C4b and further opsonization with C3b.

Furthermore, we analyzed the influence of C4BP on adherence of S. pyogenes to HUVECs. Therefore, we incubated AP1 and its isogenic mutant BM27.6 with C4BP followed by addition of HUVECs. After 3 h at 37 °C, the bacteria were plated on blood agar plates and counted (Fig. 1E). To assess invasion, extracellular bacteria were killed using gentamicin and penicillin (Fig. 1F). S. pyogenes strains featuring protein H show strong C4BP binding but decreased deposition of C3b as well as increased adherence to and invasion of endothelial cells. Results from at least three independent experiments are shown. In C and D, representative experiments are shown. Error bars indicate S.D. values. Statistical significance is shown: *, p < 0.05 or ****, p < 0.0001 by two-way analysis of variance. ns, not significant; neg con, negative control; pos con, positive control.
tion to HUVECs at a multiplicity of infection of 25. We found that protein H mediated binding of C4BP is important for adherence. AP1 displayed a 4.7-fold increase in adherence to endothelial cells in the presence of C4BP (Fig. 1C). As expected, BM27.6, lacking protein H and thus unable to bind C4BP showed no difference in binding to endothelial cells in the presence or absence of C4BP. Interestingly, invasion of AP1 was increased by more than 11 times in the presence of C4BP. Again, BM27.6 had no significant increase in invasion to HUVECs (Fig. 1D). Taken together, protein H-mediated binding of C4BP increases adherence as well as invasion to endothelial cells.

**C4BP Binds Strongly to Protein H but Not to M1 Protein**—Because we observed that protein H mediates C4BP binding, we aimed to characterize the interaction in more detail. Immobilized recombinant protein H and M1 protein were incubated with increasing amounts of plasma-purified C4BP specifically bound to protein H but barely to M1 protein and not to BSA (A). As expected, immobilized C4BP (1 μg/ml) also bound 125I-labeled protein H in a dose-dependent manner (B). Binding of 125I-labeled C4BP (50 kcpm) to immobilized protein H was analyzed under increasing amounts of either NaCl (C) or ethylene glycol (D), revealing a partly ionic and hydrophobic interaction. Using Biacore, binding of C4BP to immobilized C4met (E) or protein H (F) was analyzed. The binding is illustrated as response units obtained at plateau of sensorgrams (Req) plotted against concentrations of injected protein and as the sensorgrams obtained for several concentrations of injected C4BP (inserted graph). Results from three independent experiments performed in duplicates are shown in A–D. Error bars indicate S.D. values. RU, resonance units.
coated C4BP was nearly 100 times lower than using soluble \(^{125}\)I-C4BP and immobilized protein H, which is due to the polymeric nature of C4BP and avidity effects. Therefore, we decided to coat protein H and use soluble \(^{125}\)I-C4BP for all further experiments.

The Binding of Protein H to C4BP Is Partially of Ionic Nature—The binding of C4BP to M proteins is largely hydrophobic (26). Based on the structural similarity, we expected similar behavior for protein H and C4BP. To our surprise, we found that increasing ionic strength reduced the binding of C4BP to protein H. In fact, we detected a decrease of binding by 95% when increasing NaCl concentration from 100 mM to 1 M (Fig. 2C). In this assay, we used C4b as a positive control because it has been previously reported that the C4BP-C4b interaction is strictly of ionic nature. As expected, we could detect a 95% decrease in binding of C4b to C4BP already at a salt concentration < 500 mM, which is less than half of the concentration necessary to achieve similar effects on protein H and C4BP. To estimate the influence of hydrophobic interactions between C4BP and protein H, we incubated \(^{125}\)I-C4BP with immobilized protein H in the presence of increasing amounts of ethylene glycol. We found that a concentration of 40% ethylene glycol already reduced > 85% of the binding between the two proteins (Fig. 2D). Taken together, the interaction between C4BP and protein H seems to depend on both electrostatic as well as hydrophobic interactions. The affinity of interaction between protein H or C4met and single C4BP \(\alpha\)-chain was estimated using Biacore (Fig. 2, E and F). The obtained sensorgrams yielded \(K_D = 6 \times 10^{-7}\) M for protein H-C4BP and \(K_D = 8.7 \times 10^{-8}\) M for C4met-C4BP interactions. These are affinities of single purified molecules, and the affinity of these interactions is greatly enhanced by avidity effects when native, polymeric C4BP binds to clustered C4b or protein H on bacterial surface. Importantly, our kinetic data are in good agreement with the observed fact that C4BP bound to protein H can still use the same binding site on another \(\alpha\)-chain to bind C4b and inhibit complement.

Mapping of the Binding Site for Protein H on C4BP—First, we performed blocking assays using monoclonal antibodies against different CCP domains located on the \(\alpha\)-chain of C4BP (Fig. 3A). Soluble \(^{125}\)I-C4BP was incubated with increasing amounts of the different anti-C4BP antibodies prior to the incubation on immobilized protein H. Only MK104, directed against the CCP1 domain (Fig. 3A) blocked the interaction (Fig. 3B). Interestingly, another antibody against CCP1, MK102 could not block binding, which was also the case for antibodies directed against other CCP domains. An isotype control for MK104 showed no effect on the binding. Because CCP1 seemed to be involved, we hypothesized that heparin binding to CCP1–2 or C4b binding to CCP1–3 might interfere with pro-
tein H binding as well. We performed a cross-competition assay, adding soluble C4BP, C4b, heparin, or BSA in increasing amounts together with $^{125}$I-C4BP. As expected, soluble C4BP was able to completely compete out $^{125}$I-C4BP-protein H binding at already nanomolar concentrations (Fig. 3). C4b also competed with the binding, although to a lower degree, indicating a strong interaction between C4BP and protein H. Addition of 100 nM C4b reduced binding of protein H by 50%. Interestingly, neither BSA nor heparin were able to interfere with C4BP-protein H binding (Fig. 3). All other mutants were able to block binding of C4BP and protein H. Ala-Ala insertion between CCP1 and CCP2 abrogated C4BP binding completely, whereas insertions between CCP2-CCP3 and CCP3-CCP4 only had little or no influence at all (D). Results from three independent experiments performed in duplicates are shown. Error bars indicate S.D. values. rec. wt, recombinant wild type.

Characterization of Protein H and C4BP Interaction

**FIGURE 4. Protein H binds specifically to CCP1-CCP2 domain on C4BP.** $^{125}$I-labeled C4BP (25 kcpm) was incubated with increasing amounts of indicated C4BP mutants on immobilized protein H (0.5 µg/ml). A, a schematic representation of C4BP mutants used to compete for C4BP-protein H binding. Increasing amounts of C4BP mutants lacking different CCP domains were tested to compete out binding of $^{125}$I-C4BP to immobilized protein H (B and C). C4BP ΔCCP1 could not, and ΔCCP2 could only partially inhibit the binding. All other mutants were able to block binding of C4BP and protein H. Ala-Ala insertion between CCP1 and CCP2 abrogated C4BP binding completely, whereas insertions between CCP2-CCP3 and CCP3-CCP4 only had little or no influence at all (D). Results from three independent experiments performed in duplicates are shown. Error bars indicate S.D. values. rec. wt, recombinant wild type.
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C4BP. The interface between CCP1–2 seems to be important for the interaction, although a certain influence of CCP2 and CCP3 cannot be excluded.

Encouraged by these results, we tried to identify key aa on C4BP CCP1 and CCP2 crucial for the interaction between C4BP and protein H. Altogether, we tested 17 different C4BP mutants: 11 single, four double, one triple, and one quadruple mutant. From these 17 mutants, three were completely unable to block $^{125}$I-C4BP-protein H interaction, namely R64Q, R66Q, and H67Q (Fig. 5A). These findings suggest that the three aa are individually pivotal for binding of the two proteins. Another set of four mutants, R39Q, K63Q, K79Q, and Q109A/D110N/R111Q/D112S could at least partially interfere with the interaction between C4BP and protein H but still showed significantly less inhibition compared with the recombinant wild type C4BP (Fig. 5B). Obviously, these aa influence the binding but to a much lesser extent than the previous set of tested mutants. The remaining 10 mutants, A12V/M14V, M14Q, D15N, R22H, K24E, T28Q/T47Q, L34R, T43D/T45M, E53Q/V55T/N57T, and K126Q/K128Q did completely block the $^{125}$I-C4BP-protein H interaction, at similar or even significantly lower concentrations compared with the recombinant wild type C4BP (Fig. 5, C and D), indicating that those aa did not play a crucial role for the interaction. We highlighted all tested aa in a solution structure of C4BP CCP1–2 (Fig. 5E, lower model is a 180° turn through the $y$ axis). All aa involved in the binding of C4BP to protein H cluster mainly on one side of C4BP at the level between the domains CCP1 and CCP2 (Fig. 5E, aa in red and orange). Taken together, we can assume that the crucial binding site for protein H on C4BP is partially located between CCP1 and CCP2 and comprises at least aa Arg-64, Arg-66, and His-67.

Identifying the Region on Protein H Responsible for the Interaction with C4BP—We generated recombinant fragments of protein H by deleting consecutive domains starting from the COOH terminus. All recombinant fragments contained domain A (Fig. 6A). Each protein migrated at the predicted molecular size and showed no or only negligible contamination (Fig. 6B). The full-length protein H as well as the different fragments were coated in similar molar concentrations prior to incubation with increasing amounts of soluble $^{125}$I-C4BP. All tested fragments bound C4BP and showed no significant difference to the full-length protein H. Thus, domain A on protein H mediates C4BP binding. To map the binding site on protein H in more detail, we used a peptide microarray. The peptide microarray consisted of 14 consecutive peptides covering domain A on protein H. Every peptide is 18-aa-long and has an overlap of 13 aa from the previous peptide (Fig. 6D). The analysis showed, that exclusively the peptide comprising aa 92–109 bound significant amounts of C4BP (Fig. 6E). To summarize, the binding to C4BP is mediated by aa 92–109 in domain A of protein H.

**DISCUSSION**

The aim of this study was to characterize the interactions between protein H and C4BP and to compare it with the available data on M proteins. We addressed those questions by using C4BP mutants, which lacked individual CCP domains or had two additional alanines inserted within the interface between the first CCP domains. Also, we used C4BP mutants having single amino acid mutations in altogether 26 different positions. To identify the binding site on protein H, we generated five consecutive truncations of protein H starting from the COOH terminus as well as a peptide microarray. Taken together, our data identified a cluster of positively charged amino acids on interface of CCP-2 of C4BP as mediator of the binding to the C-terminal part of domain A in protein H.

C4BP consists of seven identical $\alpha$-chains and a unique $\beta$-chain (12). The identified binding site for protein H is localized to the NH$_2$ terminus of the $\alpha$-chain, which implies that every C4BP molecule comprises of seven identical binding sites. For the binding to C4b, it is known that at the same time up to four molecules can be bound by a single C4BP molecule (32), which supports our finding that C4BP can bind protein H and degrade C4b at the same time. Although, the affinity between C4BP and C4b is rather low, C4BP exhibits a high avidity to C4b due to the polymeric structure of C4BP and the multiple binding sites per molecule. A similar mechanism explains significantly higher binding of soluble C4BP to immobilized protein H than vice versa. The polymeric nature of C4BP also has functional implications because the same binding site can be used for binding to the bacterial surface as to inhibit complement.

Protein H and Arp4 are members of the M protein family (33). Most, if not all family members share a closely related coiled-coil structure (33) and in their interaction C4BP, probably also a similar binding site. Hence, we expected a likewise behavior, not only regarding binding sites, but also in terms of salt sensitivity of the binding to C4BP. To our surprise, we found that the binding of protein H to C4BP is indeed far more sensitive to salt than the binding of M protein Arp4 to C4BP (26). Both proteins are $\alpha$-helical and share a coiled-coil structure. However, the presence of 800 mM NaCl reduced C4BP binding to protein H to <10% of the initial binding, whereas under the same conditions, Arp4 still bound 75% of available C4BP (26). Obviously, Arp4 binding to C4BP depends more on hydrophobic interactions than protein H. In combination with the hydrophobicity analysis using ethylene glycol, protein H seems to depend on a combined effect of hydrophobic and ionic interaction.

We previously showed that M proteins are capable of displacing C4BP-bound C4b, however their binding site seems to be overlapping, but not identical (26). Consistently, we found that binding of C4BP to protein H can be inhibited with an excess of C4b. Indeed, C4b and protein H seem to bind to the same key amino acids Arg-64 and Arg-66 of C4BP.

Interestingly, we did not detect any effect of heparin on the protein H-C4BP binding. Heparin shares a common binding site with C4b on C4BP and can block C4b-C4BP interaction (25). Thus, it is possible that heparin and protein H have only slightly overlapping binding sites and hence could be bound at the same time. More likely, the interaction of protein H with C4BP is of much higher affinity, so that heparin cannot affect protein H-C4BP binding.

Interestingly, the described binding site on C4BP is not exclusive for protein H, but seems to bind a variety of different bacterial structures besides C4b and heparin (22). In addition to the here described protein H, also other S. pyogenes M proteins...
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A

B

C

D

E

CCP 2

H67Q
R66Q
Q109A
G1125
K63Q
D110N
R22H
R111Q

CCP 1

K79Q
L34R
M14Q
N57T
V55T
E53Q
R39Q
K63Q
Q109A
D110N/R111Q/D112S

CCP 2

D110N
K79Q
L34R
M14Q
N57T
V55T
E53Q

CCP 1

R39Q
A12V/M14V
L34R
K24E
E53Q/V55T/N57T

0°

180°
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(34), Neisseria gonorrhoeae porins (35), and Bordetella pertussis (36) bind to a positively charged cluster at the interface of CCP1–2 on C4BP. Obviously, these pathogens exploit the natural binding site for C4b, probably the most fundamental and natural interaction partner of C4BP. Other pathogens are also known to bind C4BP, including Moraxella catarrhalis (37), Borrelia recurrentis (38), Candida albicans (39), and Haemophilus influenzae (40). These pathogens also specifically target CCP1 and CCP2. Recruiting C4BP seems to be a general protection mechanism for human pathogens and could increase their pathogenic potential. C4BP accelerates the decay of the C3 convertase and acts as a cofactor for C4b cleavage (15, 41). This, however, does not only occur on the human cells but also on pathogens, which acquire inhibitors to evade complement detection mechanism for human pathogens and could increase their pathogenic potential. C4BP accelerates the decay of the C3 convertase and acts as a cofactor for C4b cleavage (15, 41). This, however, does not only occur on the human cells but also on pathogens, which acquire inhibitors to evade complement attacks (24).

Responsible for the binding of C4BP is a NH2-terminal hypervariable region in the M proteins. A sequence comparison between three different M proteins and protein H showed five conserved amino acids in the analyzed C4BP-binding proteins, however, these five amino acids were also present in an M protein incapable of binding C4BP (18). Therefore, it does not appear that binding to C4BP relies on a distinct peptide sequence. More interestingly, although several M proteins do bind C4BP, immunological cross-reactivity between the different M proteins could not be detected (42). Obviously, the binding of C4BP is conserved while at the same time, antibody recognition is prevented. Because sequence identities are not present in different M proteins, similar structural folding seem to be responsible for the conserved C4BP binding (43). It has been hypothesized, that C4BP binding might be mainly mediated through the amino acid back bone structure (44), whereas antibodies favor binding through amino acid side chains (45). Based on the combined data one would rather favor a structural motif over a consensus sequence responsible for C4BP binding.

Besides binding various host proteins, the M protein is also responsible for antiphagocytic properties as well as for adhesion and invasion (46). It has been shown that S. pyogenes can invade a variety of different host cell types such as epithelial

**FIGURE 6.** The COOH-terminal part of protein H domain A is responsible for the interaction with C4BP. Five different truncations of protein H, as indicated in A were expressed in E. coli and subsequently purified. The purity and size of full-length protein H as well as the corresponding fragments were analyzed by SDS-PAGE and subsequent Coomassie staining (B). Increasing amounts of 125I-labeled C4BP were incubated with different immobilized protein H fragments (each molar equivalent to 3 C4BP, increasing amounts of indicated C4BP mutants were incubated together with 125I-labeled C4BP (25 kcpm) on immobilized protein H (0.5 g/ml full-length protein H) (C), showing that the binding site is located within domain A. A peptide microarray comprising 14 consecutive 18-aa-long peptides (each with a 13-aa overlap with the previous peptide) from protein H domain A (aa 41–122, depicted in D) was incubated with 125I-labeled C4BP (10,000 kcpm) to identify the binding motif on protein H (E). C4BP binding was located in peptide 11 at the COOH-terminal part of domain A. In C, results from three independent experiments performed in technical duplicates are shown. Error bars indicate S.D. values.
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cells, keratinocytes, or endothelial cells (47–49). Interestingly, so far, C4BP has not been linked with invasion or adherence of \textit{S. pyogenes} to host cells. We found a direct effect of \textit{S. pyogenes}-surface bound C4BP on adhesion as well as invasion to HUVECs. It might be possible that C4BP directly acts as a bridging molecule and links bacteria to the human cells, as it has been reported for liver cells taking up adenoviruses via C4BP (50). However, even though interaction of C4BP with some proteoglycans has been reported, the exact mechanism of C4BP-mediated adhesion is yet not clear.

The data presented here demonstrate the molecular details of the binding of protein H and C4BP. It is of utmost importance to understand how \textit{S. pyogenes} is capable of undermining the immune system and establishing fatal systemic infections. Our data on the molecular details might help in the design of novel therapeutic agents and treating severe \textit{S. pyogenes} infections successfully.

REFERENCES

1. Carapetis, J. R., Steer, A. C., Mulholland, E. K., and Weber, M. (2005) The global burden of group A streptococcal diseases. \textit{Lancet Infect. Dis.} 5, 685–694
2. Nowak, R. (1994) Flesh-eating bacteria: not new, but still worrisome. \textit{Science} 264, 1665
3. Bisno, A. L., and Stevens, D. L. (1996) Streptococcal infections of skin and soft tissues. \textit{N. Engl. J. Med.} 334, 240–245
4. Steer, A. C., Law, I., Matatolou, L., Beall, B. W., and Carapetis, J. R. (2009) Global emm type distribution of group A streptococci: systematic review and implications for vaccine development. \textit{Lancet Infect. Dis.} 9, 611–616
5. Horstmann, R. D., Sievertsen, H. J., Knobloch, J., and Fischetti, V. A. (1988) Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. \textit{Proc. Natl. Acad. Sci. U.S.A.} 85, 1657–1661
6. Thern, A., Stenberg, L., Dahlbäck, B., and Lindahl, G. (1995) Ig-binding surface proteins of \textit{Streptococcus pyogenes} also bind human C4b-binding protein (C4BP), a regulatory component of the complement system. \textit{J. Immunol.} 154, 375–386
7. Whitnack, E., and Beachey, E. H. (1982) Antipossonic activity of fibrinogen bound to M protein on the surface of group A streptococci. \textit{J. Clin. Invest.} 69, 1042–1045
8. Frick, J. M., Akesson, P., Cooney, J., Sjöbring, U., Schmidt, K. H., Gomi, H., Hattori, S., Tagawa, C., Kishimoto, F., and Björck, L. (1994) Protein H—a surface protein of \textit{Streptococcus pyogenes} with separate binding sites for IgG and albumin. \textit{Mol. Microbiol.} 12, 143–151
9. Ricklin, D., Hajiashengallis, G., Yang, K., and Lambris, J. D. (2010) Complement: a key system for immune surveillance and homeostasis. \textit{Nat. Immunol.} 11, 785–797
10. Blom, A. M., Hallström, T., and Riesbeck, K. (2009) Complement evasion strategies of pathogens—acquisition of inhibitors and beyond. \textit{Mol. Immunol.} 46, 2808–2817
11. Blom, A. M., Villoutreix, B. O., and Dahlbäck, B. (2004) Functions of human complement inhibitor C4b-binding protein in relation to its structure. \textit{Arch. Immunol. Ther. Exp.} 52, 83–95
12. Scharfstein, I., Fereirea, A., Gigli, I., and Nussenzweig, V. (1978) Human C4-binding protein. I. Isolation and characterization. \textit{J. Exp. Med.} 148, 207–222
13. Norman, D. G., Barlow, P. N., Baron, M., Day, A. J., Sim, R. B., and Campbell, I. D. (1991) Three-dimensional structure of a control complement control protein module in solution. \textit{J. Mol. Biol.} 219, 717–725
14. Kask, L., Hillarp, A., Ramesh, B., Dahlbäck, B., and Blom, A. M. (2002) Structural requirements for the intracellular subunit polymerization of the complement inhibitor C4b-binding protein. \textit{Biochemistry} 41, 9349–9357
15. Fujita, T., and Nussenzweig, V. (1979) The role of C4-binding protein and β1H in proteolysis of C4b and C3b. \textit{J. Exp. Med.} 150, 267–276
ular mechanism of serum resistance of *Neisseria gonorrhoeae*. *J. Exp. Med.* 193, 281–295
36. Berggård, K., Lindahl, G., Dahlbäck, B., and Blom, A. M. (2001) Bordetella pertussis binds to human C4b-binding protein (C4BP) at a site similar to that used by the natural ligand C4b. *Eur. J. Immunol.* 31, 2771–2780
37. Nordström, T., Blom, A. M., Forsgren, A., and Riesbeck, K. (2004) The emerging pathogen *Moraxella catarrhalis* interacts with complement inhibitor C4b binding protein through ubiquitous surface proteins A1 and A2. *J. Immunol.* 173, 4598–4606
38. Meri, T., Cutler, S. J., Blom, A. M., Meri, S., and Jokiranta, T. S. (2006) Relapsing fever spirochetes *Borrelia recurrentis* and *B. duttonii* acquire complement regulators C4b-binding protein and factor H. *Infect. Immun.* 74, 4157–4163
39. Meri, T., Blom, A. M., Hartmann, A., Lenk, D., Meri, S., and Zipfel, P. F. (2004) The hyphal and yeast forms of *Candida albicans* bind the complement regulator C4b-binding protein. *Infect. Immun.* 72, 6633–6641
40. Hallström, T., Jarva, H., Riesbeck, K., and Blom, A. M. (2007) Interaction with C4b-binding protein contributes to nontypeable *Haemophilus influenzae* serum resistance. *J. Immunol.* 178, 6359–6366
41. Gigli, I., Fujita, T., and Nussenzweig, V. (1979) Modulation of the classical pathway C3 convertase by plasma proteins C4 binding protein and C3b inactivator. *Proc. Natl. Acad. Sci. U.S.A.* 76, 6596–6600
42. Morfeldt, E., Berggård, K., Persson, J., Drakenberg, T., Lindahl, G. (2001) Isolated hypervariable regions derived from streptococcal M proteins specifically bind human C4b-binding protein: implications for antigenic variation. *J. Immunol.* 167, 3870–3877
43. André, I., Persson, J., Blom, A. M., Nilsson, H., Drakenberg, T., Lindahl, G., and Linse, S. (2006) Streptococcal M protein: structural studies of the hypervariable region, free and bound to human C4BP. *Biochemistry* 45, 4559–4568
44. Persson, J., Beall, B., Linse, S., and Lindahl, G. (2006) Extreme sequence divergence but conserved ligand-binding specificity in *Streptococcus pyogenes* M protein. *PLoS Pathog.* 2, e47
45. Davies, D. R., Padlan, E. A., and Sheriff, S. (1990) Antibody-antigen complexes. *Annu. Rev. Biochem.* 59, 439–473
46. Courtney, H. S., Hasty, D. L., and Dale, J. B. (2002) Molecular mechanisms of adhesion, colonization, and invasion of group A streptococci. *Ann Med.* 34, 77–87
47. Greco, R., De Martino, L., Donnarumma, G., Conte, M. P., Seganti, L., and Valenti, P. (1995) Invasion of cultured human cells by *Streptococcus pyogenes*. *Res. Microbiol.* 146, 551–560
48. LaPenta, D., Rubens, C., Chi, E., and Cleary, P. P. (1994) Group A streptococci efficiently invade human respiratory epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* 91, 12115–12119
49. Schrager, H. M., Rheinwald, J. G., and Wessels, M. R. (1996) Hyaluronic acid capsule and the role of streptococcal entry into keratinocytes in invasive skin infection. *J. Clin. Invest.* 98, 1954–1958
50. Shayakhmetov, D. M., Gaggero, A., Ni, S., Li, Z. Y., and Lieber, A. (2005) Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. *J. Virol.* 79, 7478–7491