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 ^{125}I-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 it self, 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

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 *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 *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 *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). C4met were prepared by treatment of purified C4 with 100 mM methylamine, pH 8.0, at 37 °C for 1 h to hydrolyze the internal thioester bond. C4met still retains the anaphylatoxin domain but resembles C4b in overall conformation and functional properties. C4BP, C4b, and protein H were ^{125}I-labeled using Na^{125}I (PerkinElmer Life Science) and Iodobeads (Pierce) according to the manufacturer's instructions. The following antibodies were used for detection, blocking, and binding assays: α-C4BP MK104, MK102, MK96 and MK67 (27), α-C3d (A0063, Dako), α-C4c (A211, Quidel), α-C4d (A213, Quidel), and mouse isotype IgG1 and IgG2a (ImmunoTools). As secondary antibody F(ab')$_2$ donkey α-mouse IgG-PE (12-4012-87, EBioscience) was used.

Bacteria, Cells, and Culture Conditions—*S. pyogenes* strains AP1 (*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 BMJ71 (29) were inoculated and grown in Todd-Hewitt broth overnight at 37 °C and 5% CO$_2$ 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 (BMJ71). Overnight cultures were diluted to $A_{600} = 0.1$ in fresh Todd-Hewitt broth and incubated at 37 °C and 5% CO$_2$ without shaking until they reached exponential growth at $A_{600} = 0.3$–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% CO$_2$ for a maximum of 5 passages (30).

Binding of ^{125}I-C4BP and ^{125}I-protein H to Purified Proteins—Purified proteins (protein H, protein H fragments, C4BP, M1, or BSA) were diluted to indicated concentrations in PBS and immobilized in microtiter plates (MaxiSorp BreakApart, Nunc) at 4 °C overnight. The plates were washed three times with wash buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20), and subsequently, nonspecific binding sites were blocked with 3% fish gelatin (Norland Products) in wash buffer. ^{125}I-labeled protein H or C4BP, respectively, diluted in binding PBS (1× PBS supplemented with 0.1% Tween 20 and 0.1% BSA) were added in increasing amounts. After incubation overnight at 4 °C and washing, radioactivity in the wells was detected using a Wizard^2 γ counter (PerkinElmer Life Science). All further binding assays are based on this protocol.

Influence of Salt and Ethylene Glycol on C4BP-protein H Binding—50 kcpm of ^{125}I-C4BP were diluted in binding PBS with either salt concentration ranging from 100 mM to 2 M NaCl or ethylene glycol concentrations up to 75% prior to incubation with immobilized protein H.

Antibody Blocking Assays—Increasing amounts of monoclonal antibodies MK104, MK102, MK96, MK67 (0.05–50 ng) were incubated with 50 kcpm of ^{125}I-C4BP for 30 min at 37 °C prior to the incubation with immobilized protein H. The antibodies were a kind gift of Prof. Dahlbäck (Lund University).

Competition Assays with C4BP Ligands—Increasing amounts of either C4met (0.1 ng to 1 μg), C4BP (0.1 ng to 1 μg), heparin (12.2 μg to 12.5 mg), or BSA (0.1 to 12.6 mg) were incubated with 25 kcpm of ^{125}I-C4BP for 30 min at 37 °C prior to the incubation with immobilized protein H.

Competition Assays with C4BP Mutants—Increasing amounts of C4BP mutants (4.3 fM to 4.4 nM) were incubated with 25 kcpm of ^{125}I-C4BP for 30 min at 37 °C prior to the incubation with immobilized protein H.

C4b Degradation Assays—C4b degradation was either analyzed bound on *S. pyogenes* or in solution using purified proteins. For the C4b degradation on plate, 10 μg/ml protein H or BSA in PBS were immobilized in microtiter plates (MaxiSorp, Nunc) at 4 °C overnight. The plate was washed three times with wash buffer, and subsequently nonspecific binding sites were blocked with 3% fish gelatin in wash buffer. As indicated, wells were either left untreated or incubated with 20 μg C4BP/well for 2 h at 4 °C to allow binding. After three washing steps, trace amounts of ^{125}I-C4b were added to all wells and in addition indicated wells received 500 ng factor I. The plate was incubated at 37 °C for 5 h. Reaction was stopped by adding Laemmli loading buffer and heat inactivation at 95 °C for 5 min prior to loading on a 10–15% SDS-PAGE. Degradation was analyzed by placing the gel on a phosphorimaging screen for 5 days prior to analysis in a Typhoon FLA 9500 (GE Healthcare). To analyze C4b degradation on *S. pyogenes*, harvested bacteria (1×10^8 CFU) were incubated with 100 μg of C4BP for 2 h at room temperature or left untreated. After three times washing with wash buffer, bacteria were incubated with trace amounts of ^{125}I-C4b in the presence or absence of 500 ng of factor I for 18 h at 37 °C. Reaction was stopped by adding Laemmli loading buffer and heat inactivation at 95 °C for 5 min prior to loading

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, 100 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_D) using steady state affinity model.

Peptide Microarray—PepSpots peptide microarray on cellulose 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 3×10^4 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. 1*A*). 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. 1*B*). 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. 1*C*, *top*). No degradation of C4b was observed on plates coated with BSA, which did not bind C4BP. Additionally, in the absence of
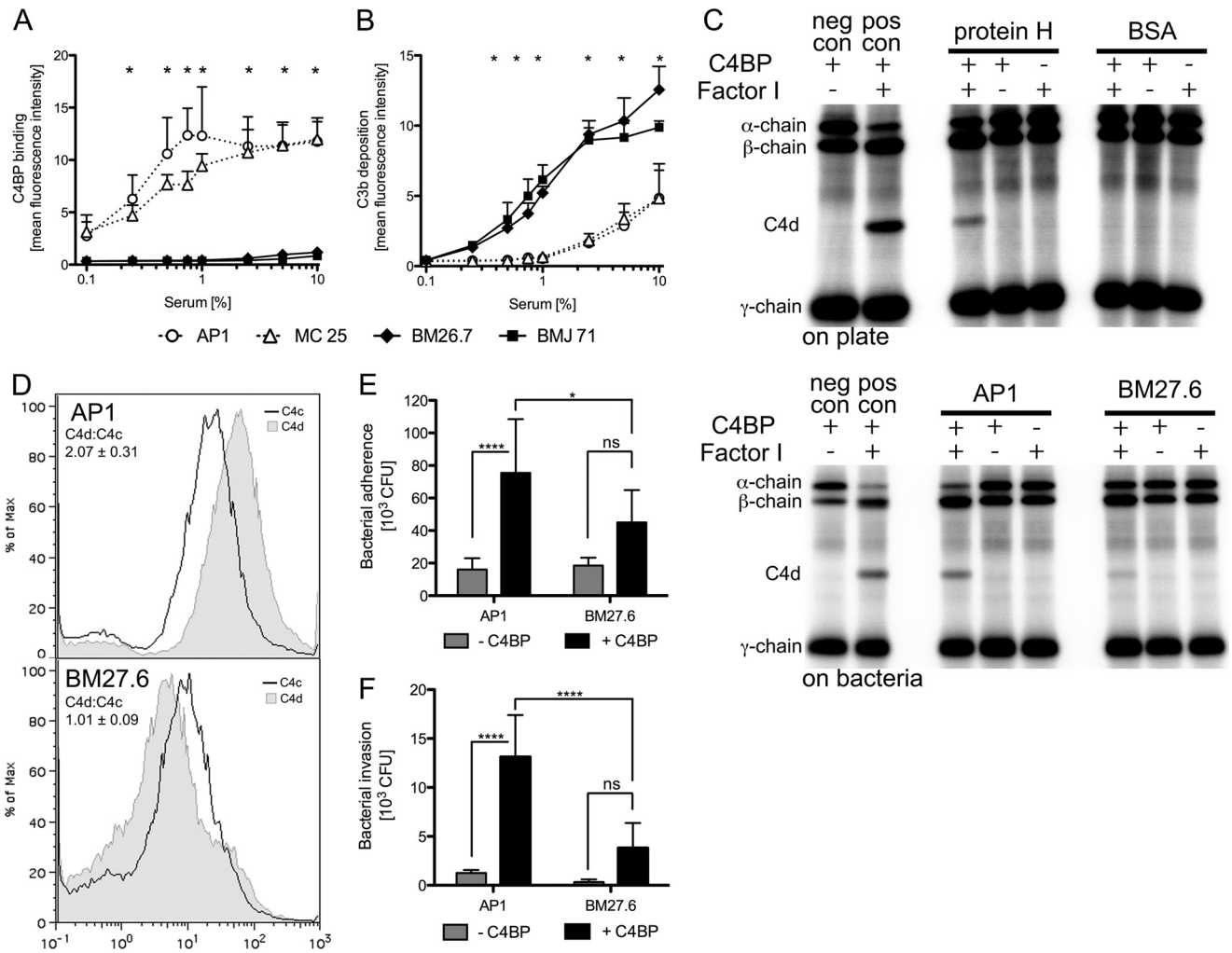
FIGURE 1. **Protein H binds C4BP thus degrading C4b and reducing C3b deposition on *S. pyogenes* as well as increasing adherence to and invasion of endothelial cells.** For analysis of complement activation and opsonization *S. pyogenes* strains AP1, MC25, BM27.6, and BMJ71 were incubated in increasing concentrations of NHS for 1 h at 37 °C. Subsequently, bacteria were stained with the respective antibodies and subjected to flow cytometry analysis to asses C4BP binding (*A*) and C3b deposition on the bacterial surface (*B*). C4BP bound to protein H serves as factor I cofactor in degradation of C4b (*C*). Immobilized protein H was incubated with 20 μg C4BP, washed thoroughly, and then incubated with Factor I and ^{125}I-C4b as indicated. As control, soluble purified proteins were used. Subsequently, samples were separated by SDS-PAGE. Because BSA cannot bind C4BP, even in the presence of factor I, C4b degradation could not be observed (*C, top*). *S. pyogenes* AP1 and BM27.6 were incubated with 100 μg of C4BP, washed thoroughly, and then incubated with factor I and ^{125}I-C4b as indicated. Only AP1 binding C4BP was able to support degradation of C4b (*C, lower panel*) as indicated by the appearance of the C4d band. BM27.6 unable to bind C4BP could nearly not degrade C4b. *S. pyogenes* AP1 and BM27.6 were preincubated with 10% NHS and subsequently stained with antibodies for C4d and C4d. Flow cytometry analysis revealed that AP1 had decreased levels C4c compared with C4d, indicating that C4c was released by cleavage. BM27.6 had comparable values of C4c and C4d judged by mean fluorescence intensities (*D*) confirming that cleavage did not occur. *S. pyogenes* AP1 and BM27.6 were preincubated with 25 μg of C4BP for 30 min and surplus C4BP was removed. Bacteria were then incubated with HUVECs at an multiplicity of infection of 25 at 37 °C for 3 h to let *S. pyogenes* adhere. After removing all unbound *S. pyogenes*, remaining bacteria were plated on blood agar plates and counted (*E*). To assess invasion, prior to plating, extracellular bacteria were killed using gentamicin and penicillin (*F*). *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.

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. 1*C, 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. 1*D*). 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 addi-
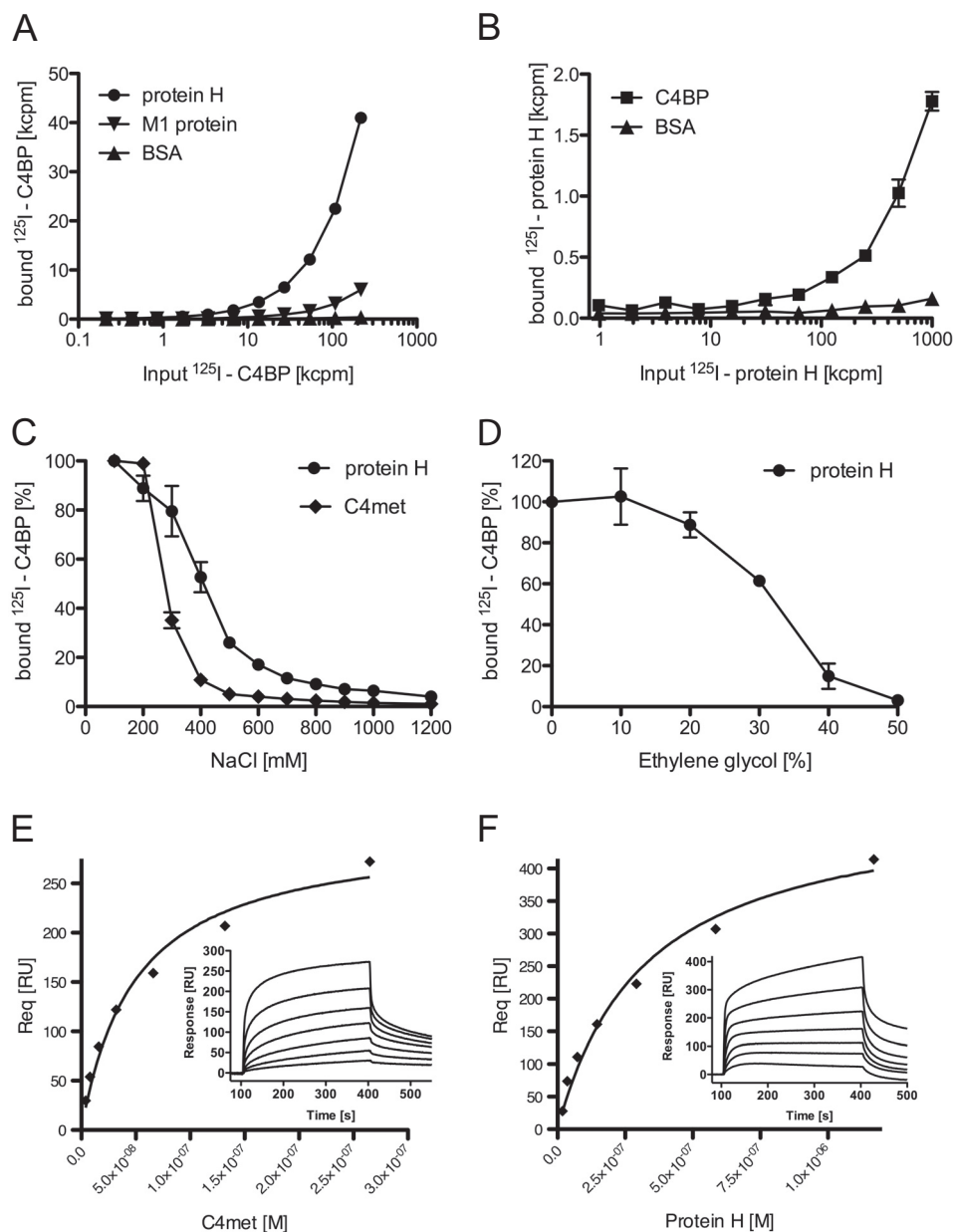
FIGURE 2. **Protein H binds C4BP in a partially ionic interaction.** Increasing amounts of ^{125}I-labeled C4BP were incubated with immobilized protein H (3 μg/ml), M1 protein (3 μg/ml), or BSA (10 μg/ml). C4BP specifically bound to protein H but barely to M1 protein and not to BSA (*A*). As expected, immobilized C4BP (1 μg/ml) also bound ^{125}I-labeled protein H in a dose-dependent manner (*B*). Binding of ^{125}I-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.

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. 1*C*). 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. 1*D*). 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 and ^{125}I-labeled C4BP. ^{125}I-C4BP bound to protein H in a dose-dependent manner (Fig. 2*A*) but not to BSA-coated wells, serving as a negative control. In good agreement with previous studies, ^{125}I-C4BP barely bound to M1 protein. Furthermore, soluble ^{125}I-protein H also bound to C4BP in a dose-dependent manner (Fig. 2*B*), whereas binding to coated BSA could not be observed. However, the detectable radioactivity of bound ^{125}I-protein H on
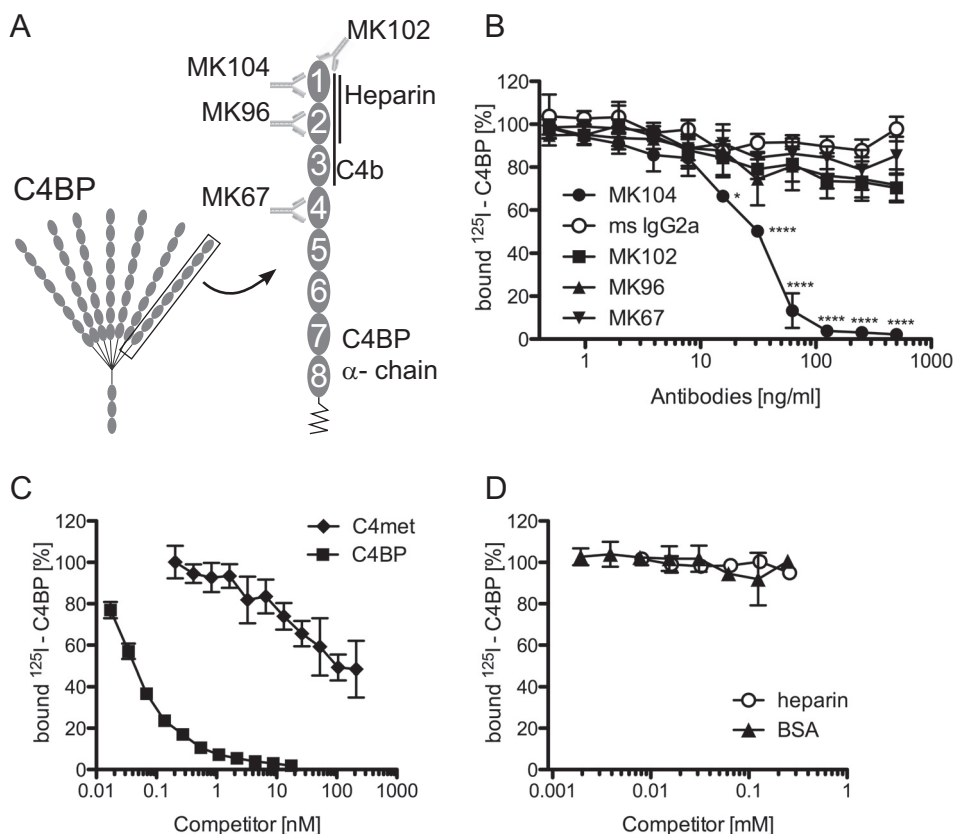
FIGURE 3. **Protein H binding to C4BP can be inhibited with C4met or mAb against CCP1 but not with heparin.** Schematic representation of C4BP and the involved CCP domains in binding of C4met, heparin, and different monoclonal antibodies on the C4BP α-chain (*A*). ^{125}I-labeled C4BP (50 kcpm) was incubated for 30 min at 37 °C with increasing amounts of indicated antibodies prior to binding on immobilized protein H (3 μg/ml) (*B*). Exclusively, MK104, binding to C4BP CCP1, blocked the interaction. Binding of ^{125}I-labeled C4BP with immobilized protein H could be competed out by addition of C4BP and C4met to the fluid phase (*C*), but not with BSA or heparin (*D*). Results from three independent experiments are shown. *Error bars* indicate S.D. values. Statistical significance is shown as: *, $p < 0.05$ or ****, $p < 0.0001$ by two-way analysis of variance.

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. 2*C*). 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. 2*D*). 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 α-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 α-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 α-chain of C4BP (Fig. 3*A*). 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. 3*A*) blocked the interaction (Fig. 3*B*). 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-
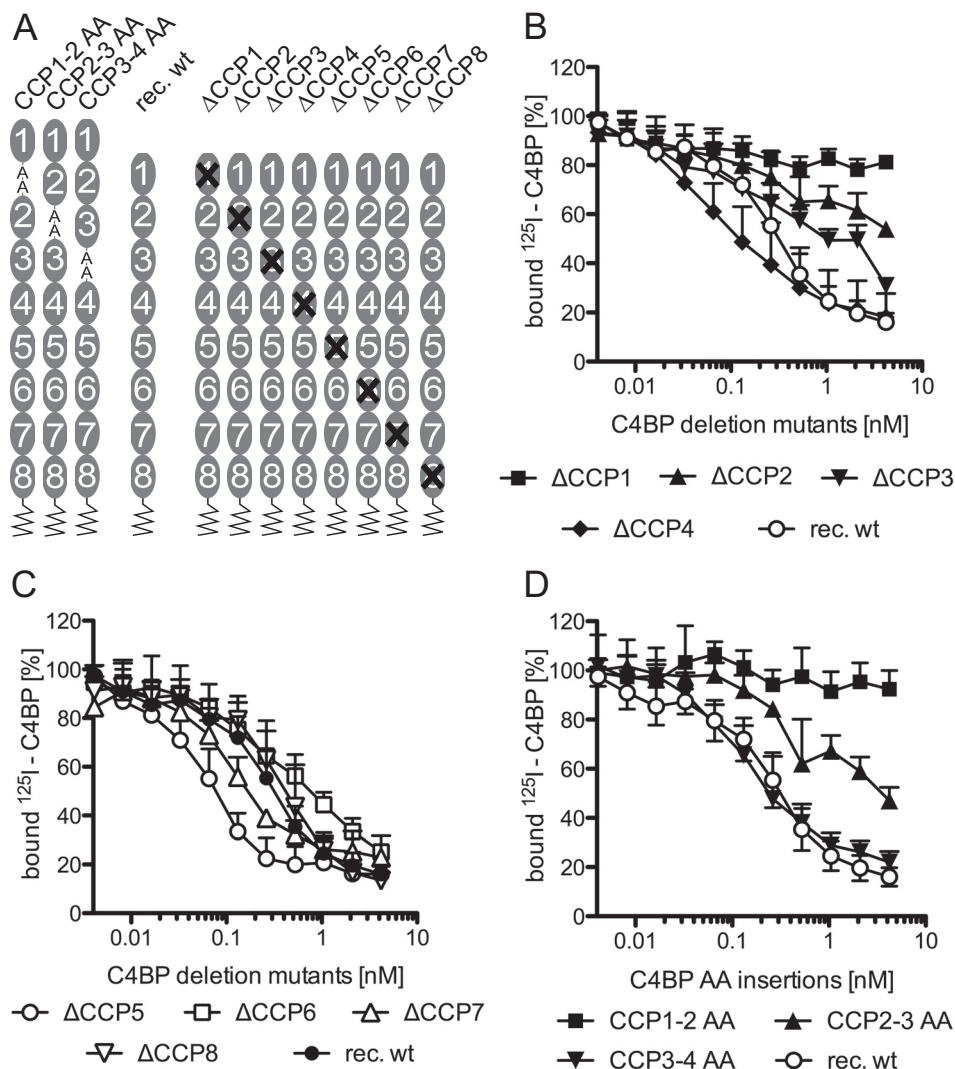
FIGURE 4. **Protein H binds specifically to CCP1-CCP2 domain on C4BP.** ^{125}I-labeled C4BP (25 kcpm) was incubated with increasing amounts of indicated C4BP mutants on immobilized protein H (0.5 μg/ml). *A*, a schematic representation of C4BP mutants used to compete for C4BP-protein H binding. Increasing 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, respectively (*D*). Results from three independent experiments performed in duplicates are shown. *Error bars* indicate S.D. values. rec. wt, recombinant wild type.

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. 3C). 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. 3D). This suggests that protein H and C4b might share at least partially a binding site while having different affinities to C4BP.

To further investigate the binding site, we performed competition assays using recombinant mutants of C4BP, which either lack one of the eight CCP domains or had two additional alanines inserted between CCP1–2, CCP2–3, and CCP3–4, respectively (Fig. 4A). In good agreement with the previously performed antibody blocking experiment, the ΔCCP1 mutant

failed to compete (Fig. 4B). Surprisingly, ΔCCP2 could block only 40% of the binding between ^{125}I-C4BP and protein H, indicating a certain involvement in the binding. All other deletion mutants blocked ~70–90% of the binding of protein H to C4BP (Fig. 4, *B* and *C*), similar to recombinant wild type C4BP. Because ΔCCP2, in addition to ΔCCP1, exhibited an effect on binding, we speculated that the binding site might span over two neighboring CCP domains. Therefore we performed a cross competition assay with the C4BP insertion mutants, which contain two additional alanines between the different CCP domains. In fact, the CCP1–2 AA mutant did not block ^{125}I-C4BP-protein H binding (Fig. 4D), confirming our hypothesis that the interface between CCP1–2 is involved in the interaction of the two proteins. The other two mutants containing the Ala-Ala motif between CCP2–3 and CCP3–4 blocked 53 and 78% of the binding at the highest indicated concentration. In summary, these experiments suggest that in addition to CCP1, CCP2 is also involved in the binding of protein H to

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. 5*A*). 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. 5*B*). 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. 5*E*, 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. 5*E*, 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. 6*A*). Each protein migrated at the predicted molecular size and showed no or only negligible contamination (Fig. 6*B*). 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. 6*D*). The analysis showed, that exclusively the peptide comprising aa 92–109 bound significant amounts of C4BP (Fig. 6*E*). 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 α-chains and a unique β-chain (12). The identified binding site for protein H is localized to the NH$_2$ terminus of the α-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 α-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
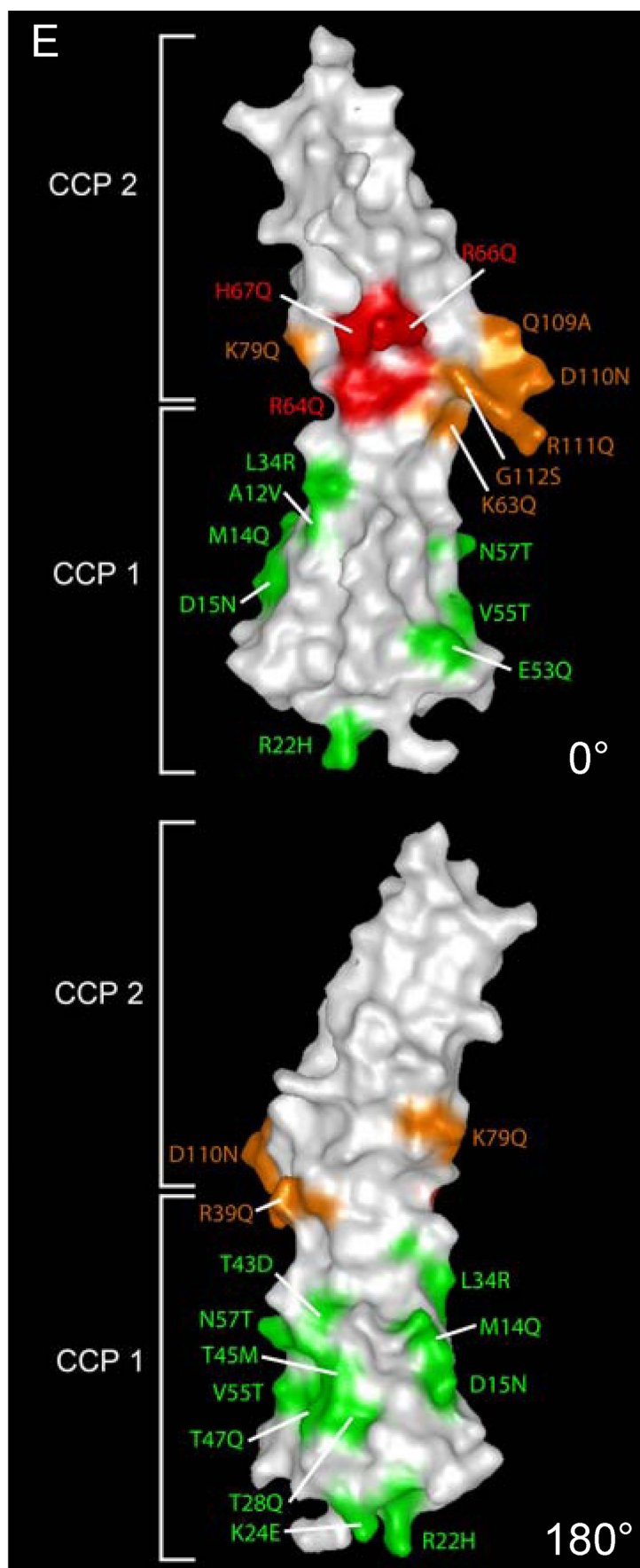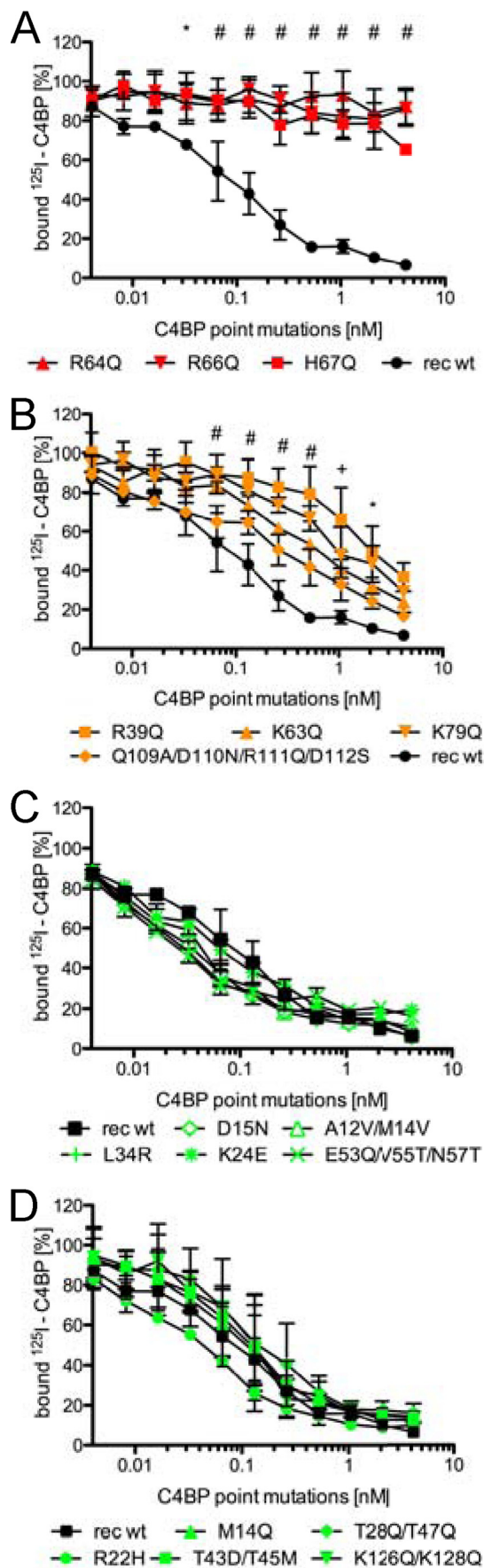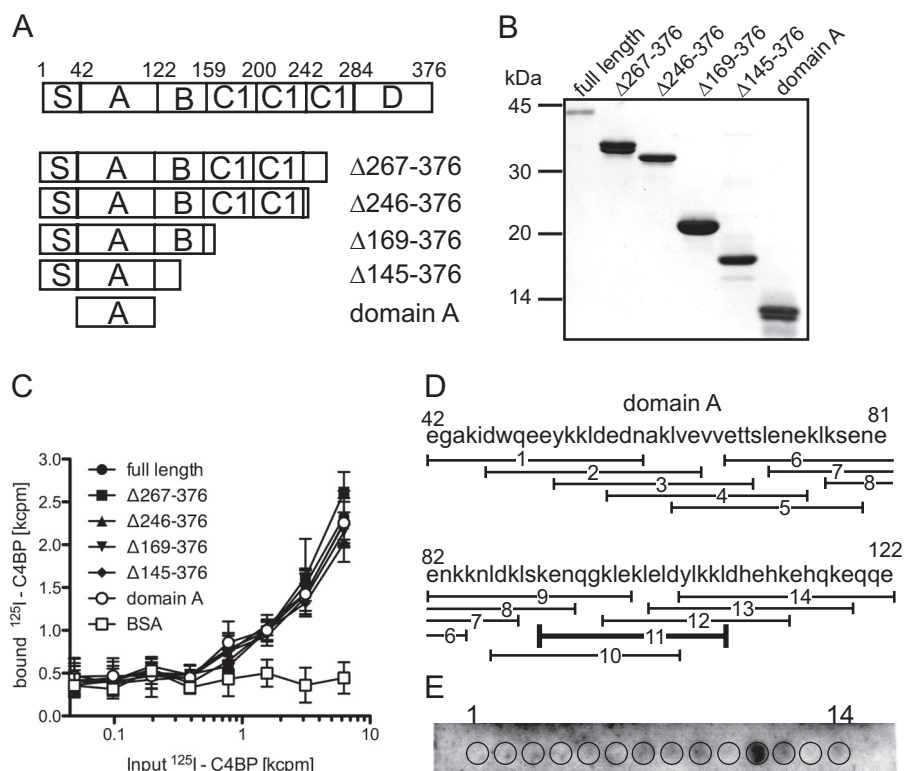
FIGURE 6. **The COOH-terminal part of protein H domain A is responsible for the interaction with C4BP.** Five different truncations of protein H, as indicated in *A* were expressed in *E. coli* and subsequently purified. The purity and size of full-length protein H as well as the corresponding fragments were analyzed by SDS-PAGE and subsequent Coomassie staining (*B*). Increasing amounts of ^{125}I-labeled C4BP were incubated with different immobilized protein H fragments (each molar equivalent to 3 μg/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 ^{125}I-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.

(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 attacks (24).

Responsible for the binding of C4BP is a NH_2-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 antiphagocytotic 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 5. **Amino acids at the interface of C4BP CCP1 and CCP2 form the binding site for protein H binding.** To compete for the binding of protein H on C4BP, increasing amounts of indicated C4BP mutants were incubated together with ^{125}I-labeled C4BP (25 kcpm) on immobilized protein H (0.5 μg/ml). Some single amino acid mutants could not interfere with the binding of ^{125}I-C4BP to protein H (*A*). Point mutations in the vicinity of the putative binding site exhibited only a minor blocking effect on the binding (*B*), whereas the rest of the mutations inhibited the interaction as efficient as the recombinant wild type C4BP (*C* and *D*). In the solution structure (*E*, lower 180° turn around the *y* axis; Protein Data Bank code 2A55 (34)) of C4BP CCP1–2 aa pivotal for the binding of protein H are marked in *red*, whereas *orange* indicates residues influencing the binding to a minor extent. *Green* mutations do not interfere with binding or even enhance the interaction. Results from at least three independent experiments are shown. *Error bars* indicate S.D. values. Statistical significance of all curves *versus* recombinant WT C4BP tested by two-way analysis of variance with Bonferronis post test (*, $p < 0.05$; +, $p < 0.001$; and #, $p < 0.0001$ for all curves of indicated point). rec, recombinant.

cells, keratinocytes, or endothelial cells (47–49). Interestingly, so far, C4BP has not been linked with invasion or adherence of *S. pyogenes* to host cells. We found a direct effect of *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 *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 *S. pyogenes* infections successfully.

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