Localization by Site-Directed Mutagenesis of the Site in Human Complement Factor H That Binds to *Streptococcus pyogenes* M Protein

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M-protein receptors located on *Streptococcus pyogenes* cells are known to bind human plasma protein factor H. Human factor H is composed of 20 short consensus repeat (SCR) domains containing approximately 60 amino acids each. Factor H controls the activation of the alternative pathway of complement in plasma. We have scanned the entire human factor H molecule by site-directed deletion mutagenesis, expressed the recombinant proteins in insect cells using the baculovirus system, and measured the binding of different purified mutant proteins to three strains of *S. pyogenes*. These studies have revealed that recombinant factor H lacking SCR domains 6 to 10 does not bind to wild-type M⁺ *S. pyogenes* JRS4. Experiments performed with *S. pyogenes* JRS251, in which both C-repeat domains of M protein were deleted, demonstrated that all of the factor H mutant proteins bound weakly to these cells except those lacking the SCR region from domains 6 to 10. Neither human factor H nor any of the recombinant proteins bound to the M[−] strain JRS145. Our results indicate that the only binding site on human factor H that interacts with streptococcus M protein is located in SCR domains 6 to 10 of factor H and that regions of M protein outside the C-repeat domains are involved in binding factor H.

Streptococcus pyogenes, a gram-positive bacterium, is an important human pathogenic organism that causes diseases such as impetigo, erysipelas, cellulitis, and necrotizing fasciitis in skin and is the most common cause of strep throat or pharyngitis. Other, life-threatening diseases such as rheumatic fever and acute glomerulonephritis are also caused by *Streptococcus* infection. The exact mechanism by which *S. pyogenes* evades phagocytosis by polymorphonuclear leukocytes is not yet known. There are several reports that M protein protects the organism from phagocytosis by inhibiting activation of the complement system (6, 7, 9, 16–18). A review of the role of streptococcal M protein has been written by Fischetti (6). Horstmann et al. (9) have shown that M protein on the surface of bacteria selectively binds factor H. Factor H functions as a cofactor for the inactivation of C3b by factor I, inhibits the formation and function of C3/C5 convertase, and contributes to inhibition of C3b on the streptococcal surface (9, 15, 24, 25). Binding of the C3b deposited on streptococci to C3b receptors of neutrophils is a critical step for phagocytosis. Recently, Fischetti et al. (7) showed that a 14.6-kDa fragment of M protein binds human factor H, and they localized a binding site in this fragment to amino acids 256 to 292, using synthetic peptides. Perez-Casal et al. (17) have constructed a mutant form of *S. pyogenes* in which both C-repeat domains, containing the proposed binding site at amino acids 256 to 292, were deleted. This strain, JRS251, showed reduced binding of human complement factor H but retained its resistance to phagocytosis.

Factor H is a crucial regulator of the complement system and was the first member of what is now known to be a large family of proteins containing the unique short consensus re-

peat (SCR) structural motif (19). These domains are characterized by four invariant cysteines and many highly conserved amino acids folded in compact units containing approximately 60 amino acids each. Factor H is a 150-kDa glycoprotein composed entirely of 20 SCR domains (20). We have expressed full-length, biologically functional factor H using the baculovirus system (21). Using site-directed mutagenesis of various SCR domains of human factor H, we have generated a family of purified recombinant proteins lacking different regions of the molecule. In a recent study, these recombinant proteins were used to identify two new binding sites for C3b that are physically and functionally distinct from the previously known site for C3b located in the amino-terminal four SCR domains (22). To test directly which region of human complement factor H binds to *S. pyogenes*, we performed binding experiments with M⁺ strain JRS4 and radiolabeled wild-type and mutant forms of recombinant factor H (rH). We report here that the binding site for *S. pyogenes* on human complement factor H is located in SCR domains 6 through 10.

MATERIALS AND METHODS

Materials. Linearized Baculogold baculovirus DNA was purchased from PharMingen (San Diego, Calif.). The Bac-To-Bac baculovirus expression system, *Escherichia coli* DH10Bac, Grace's insect media, yeastolate extract, lactalbumin hydrolysate, sodium bicarbonate, and gentamicin were purchased from Life Technologies (Gaithersburg, Md.). *E. coli* strains NM 522 and Topp 10' as well as *Spodoptera frugiperda* (Sf9) and High Five cells were purchased from Invitrogen (San Diego, Calif.). Fetal calf serum was purchased from Atlanta Biologicals (Norcross, Ga.).

Bacteria. *S. pyogenes* strains JRS4, JRS251, and JRS145 used in this study were kindly provided by June R. Scott, Emory University Health Science Center, Atlanta, Ga. (17). The cells were grown in Todd-Hewitt medium supplemented with 0.2% yeast extract and were killed by incubation with mitomycin at 50 µg/ml (Bristol Laboratories, Princeton, N.J.) for 2 h as described previously (13). The cells were resuspended in Veronal-buffered saline containing 0.02% sodium azide at 10⁹ cells/ml.

Insect cell culture. The Sf9 cells and High Five cells were maintained in complete insect medium containing 10% fetal calf serum and 50 µg of gentamicin per ml at 27°C. Cells in suspension culture were maintained in spinner flasks at 80 rpm on a magnetic stirrer.

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FIG. 1. Schematic representation of the human factor H deletion mutants used in this study. rH and rH constructs lacking the indicated domains were generated by overlap extension PCR, expressed in insect cells using the baculovirus system, and purified by immunoaffinity chromatography as described previously (21, 22).

Site-directed mutagenesis. Site-specific deletions in human factor H cDNA (5) were created by overlap extension (8). In this procedure, three PCRs were performed for each mutant. In the initial two reactions, one of two mutagenic primers (22) and one of two external primers were used to amplify the primary PCR products. The two mutation-containing PCR products were used along with two external primers for overlap extension PCR using hot-start PCR. All PCR-derived products were sequenced completely for correct sequence determination. The final constructs were restricted with *Xba*I and ligated with *Xba*I-treated, dephosphorylated pBacPAK 8/9 (Clontech, Palo Alto, Calif.) or pFastBac1 (Life Technologies). The mutants constructed with the pBacPAK 8/9 vector were used for cotransfection with linearized Baculogold baculovirus DNA in Sf9 insect cells. The mutants constructed with the pFastBac1 vector were transferred into DH10Bac competent cells. Total DNA prepared from white colonies selected on plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), isopropyl- β -D-thiogalactopyranoside (IPTG), gentamicin, tetracycline, and kanamycin was used for transfection of Sf9 cells according to the manufacturers' procedures.

Purification of mutant proteins. Sf9 or High Five insect cells were infected with recombinant virus at 10 viruses/cell. The medium supernatant was collected 4 days after infection and applied to an anti-factor H-Sepharose immunoabsorbent column. The proteins were eluted and concentrated as described previously (22).

Proteins. Human complement factor H was purified from human plasma (15). All of the purified recombinant mutant proteins used in the present study were prepared as described previously (22). The proteins were radiolabeled with 125 I by the Iodogen technique (Pierce Chemical Co., Rockford, Ill.), and the specific activities ranged from 0.3 to 2.8 μ Ci/ μ g.

Binding of human factor H and its mutant proteins to *S. pyogenes*. Different numbers of JRS4, JRS251, and JRS145 cells were incubated with 10 ng of labeled human factor H, rH, or different mutant proteins, and the mixtures were incubated for 30 min in half-normal-ionic-strength GVB (Veronal-buffered saline containing 2.5 mM Veronal, 75 mM NaCl, and 0.01% NaN₃ [pH 7.4] and 0.05% gelatin) in a total volume of 60 μ l. The bound radiolabel and free radiolabel were separated by layering the mixtures on 20% sucrose in half-ionic-strength GVB, centrifuging for 3 min at 10,000 $\times$ g, and cutting the tube to separate the pellet and supernatant. The percentage of radiolabeled proteins specifically bound to the cells was calculated by dividing the radioactivity in the pellet by that in the pellet plus supernatant.

RESULTS

A schematic representation of the mutations introduced into the six different recombinant proteins used in this study is shown in Fig. 1. The mutants were generated by deleting whole domains starting with the first Cys of each domain and ending with the amino acid before the first Cys of the next expressed domain. These specific deletions were made by using overlap extension PCR as we have described previously (22). The different mutant proteins were expressed in insect cells using the baculovirus system and purified by immunoaffinity chromatography (21, 22).

Binding of labeled rH to JRS4, JRS251, and JRS145 cells. To determine the binding of rH to *S. pyogenes* cells, rH was radiolabeled with 125 I and incubated with the indicated cells for 30 min. The bound radiolabel and free radiolabel were separated by centrifugation through a sucrose gradient. A

quantitative comparison of the binding of rH to JRS4, JRS251, and JRS145 cells is shown in Fig. 2. There was three- to fourfold-lower binding of labeled rH to JRS251 cells, which lack both of the C repeats of M protein, than to JRS4 cells, which express wild-type M protein. The M⁻ strain JRS145 did not show any binding to rH.

Radiolabeled plasma-derived human factor H bound to JRS4 and JRS251 cells with six- to eightfold-less affinity than rH (results not shown). Although the percentage of plasma-derived factor H bound was lower, the ratio of binding to the cell lines was same as found for rH (Fig. 2), and binding to JRS145 cells was negligible. Enhanced binding of rH compared to that of plasma factor H has been reported previously and was attributed to a lower negative charge on rH due to the lack of sialic acid and perhaps due to less bulky carbohydrate groups on the rH proteins produced in insect cells (22).

Binding of different mutant proteins to JRS4 cells. All the mutant proteins used in this study were expressed by using the baculovirus system and purified by immunoaffinity chromatography on anti-H agarose columns. The 125 I-radiolabeled proteins were incubated with JRS4 cells, and the bound radiolabel and free radiolabel were separated by centrifugation as described for Fig. 2. As shown in Fig. 3, the binding of labeled rHΔ1-5, rHΔ11-15, rHΔ16-20, and rHΔ11-20 proteins to JRS4 cells did not change significantly compared to that of rH and was between 32 and 45%. In contrast, the binding of rHΔ6-10 to JRS4 cells was almost undetectable, i.e., less than 3%. A second recombinant protein in which the first 10 N-terminal domains were deleted (rHΔ1-10) also failed to bind to JRS4 cells (i.e., less than 3% binding). All the other mutant proteins containing SCR domains 6 to 10 of factor H exhibited binding quantitatively similar to that of wild-type rH.

FIG. 2. Different concentrations of JRS4 cells (containing complete wild-type M protein), JRS251 cells (in which the C repeats of M protein were deleted), and JRS145 cells (M⁻ strain) were incubated with 10 ng of radioiodinated rH in 60 μ l of half-ionic-strength GVB. After a 30-min incubation at 22°C, bound radiolabel and free radiolabel were separated by centrifugation of cells through 20% sucrose in half-ionic-strength GVB. The results are plotted after subtraction of the background (<1% without cells).

FIG. 3. Different concentrations of JRS4 cells containing wild-type M protein were incubated with 10 ng of radioiodinated rH, rHΔ1-5, rHΔ6-10, rHΔ11-15, rHΔ16-20, rHΔ1-10, or rHΔ11-20 in 60 μ l of half-ionic-strength GVB. After a 30-min incubation at 22°C, bound radiolabel and free radiolabel were separated by centrifugation of cells through 20% sucrose in half-ionic-strength GVB.

Binding of mutant rH to JRS251 cells. A strain of *S. pyogenes* in which the entire C-repeat region of the M protein had been deleted was examined for its ability to bind factor H. Although this region of M protein has been described previously (17) as the factor H binding site, these cells exhibited low but significant levels of binding to all of the recombinant proteins except those lacking SCR domains 6 to 10 (Fig. 4). Several mutant proteins bound better than the intact rH, but all bound to JRS251 cells with lower affinity than to JRS4 cells. The binding of rHΔ6-10 and rHΔ1-10 to JRS251 cells was nearly undetectable, at less than 3% (Fig. 4), as was observed with JRS4 cells (Fig. 3).

Binding of mutant rH to JRS145 cells. The M^- strain JRS145 was checked for binding to all mutants of factor H shown in Fig. 1. This strain did not bind to any of the proteins, i.e., binding was less than 0.4% with all the proteins.

DISCUSSION

Previous studies have shown that human complement factor H specifically binds to M protein on the cell surface of *S. pyogenes* (6, 7, 9, 17). These studies have also localized the binding site in the M protein in a region containing two C-repeat domains in the C-terminal half of the molecule. The corresponding site in factor H has been identified in the present study. As shown in Fig. 3, any deletion of SCR domains 6 to 10 (rHΔ6-10 or rHΔ1-10) caused a complete loss of binding to *S. pyogenes* JRS4, which bears the wild-type M protein. No significant loss of binding was observed upon deletion of the N-terminal 5 domains (rHΔ1-5) or of the C-terminal 10 domains (rHΔ11-15, rHΔ16-20, and rHΔ11-20) of the recombinant proteins. The fact that these deletions were the same size as or larger than the deletion in rHΔ6-10 suggests that

such deletions do not by themselves cause loss of specific binding. This reaffirms an underlying assumption of this study and many previous studies that SCR domains are stable, independently folding modules that may be interchanged with retention of function (1, 2, 4, 11, 12, 22, 23).

Previous work examined binding of factor H to JRS251, which lacks the C-repeat region (17). In this study (17), the binding of factor H was measured by enzyme-linked immunosorbent assay, and the low signal remaining with JRS251 compared to M^+ strains was considered background. Figure 2 shows that the wild-type rH molecule exhibited a similar level of residual binding to JRS251. However, Fig. 4 suggests that this binding was highly specific for the site located in the region of SCR domains 6 to 10 of factor H compared to the low binding observed with M^- JRS145 cells. Binding decreased to less than 3% with the two mutants lacking the SCR 6 to 10 region (rHΔ6-10 and rHΔ1-10). Binding of all other mutants to JRS251 was the same as or greater than that of full-length rH. The binding site on JRS251 must reside in regions of the M protein outside the C-repeat structure. Furthermore, residual binding of factor H to M protein lacking one (18) or both (17) C repeats would explain the persistent resistance of such strains to phagocytosis (17) and the loss of resistance upon complete removal of M protein (6, 16, 17). This conclusion would also be consistent with the observations of Horstmann et al. (10) that even in the presence of high saturating concentrations of fibrinogen there was significant residual binding of factor H to M^+ streptococci. Although those authors suggest that factor H may bind to the fibrinogen on the cell surface, it is equally possible that factor H binds to a second site on M protein distinct from the C-repeat region, as suggested by the data presented in this paper.

Based on previous studies (14, 21, 22) and on the data

FIG. 4. Demonstration of the specific binding of rH and all the mutants of rH containing SCR domains 6 to 10 to JRS251 cells, on which the M protein lacks both C repeats. Different concentrations of JRS251 cells were incubated with 10 ng of the indicated radioiodinated mutant proteins as described for Fig. 2.

FIG. 5. Localization in complement factor H of the binding site for *S. pyogenes* M protein and comparison with locations of other known functional sites in factor H.

presented here, Fig. 5 illustrates the current understanding of the locations of different biologically significant sites in human factor H. Three binding sites for C3b have recently been localized on factor H to the N-terminal SCR domains 1 to 14, SCR domains 6 to 10, and the C-terminal region from SCR domains 16 to 20 (22). Two polyanion binding sites were localized to the region near SCR domain 13 and to SCR domains 6 to 10 (3, 22). Functional differences were found among the C3b binding sites. C3b site 1 (Fig. 5) is the only site capable of binding to C3b and inducing cleavage and inactivation of C3b by factor I (15). While C3b on host cells or other non-complement-activating surfaces can bind to all three sites, C3b on activators of the alternative pathway of complement binds primarily through C3b site 2 (SCR domains 6 to 10) (22). This is the same region where streptococcal M protein binds to human factor H. Thus, the region from SCR domains 6 to 10 of factor H appears to exhibit multiple functions and is now the focus of more-detailed site-directed mutagenesis analysis.

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