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Identification of the Streptococcal M Protein Binding Site on Membrane Cofactor Protein (CD46)

Eleni Giannakis,* T. Sakari Jokiranta,* Rebecca J. Ormsby,* Thomas G. Duthy,* Dean A. Male,* Dale Christiansen,† Vince A. Fischetti,‡ Chris Bagley,§ Bruce E. Loveland,¶ and David L. Gordon²*

Adherence of group A streptococcus (GAS) to keratinocytes is mediated by an interaction between human CD46 (membrane cofactor protein) with streptococcal cell surface M protein. CD46 belongs to a family of proteins that contain structurally related short consensus repeat (SCR) domains and regulate the activation of the complement components C3b and/or C4b. CD46 possesses four SCR domains and the aim of this study was to characterize their interaction with M protein. Following confirmation of the M6 protein-dependent interaction between GAS and human keratinocytes, we demonstrated that M protein binds soluble recombinant CD46 protein and to a CD46 construct containing only SCRs 3 and 4. M6 protein did not bind to soluble recombinant CD46 chimeric proteins that had the third and/or fourth SCR domains replaced with the corresponding domains from another complement regulator, CD55 (decay-accelerating factor). Homology-based molecular modeling of CD46 SCRs 3 and 4 revealed a cluster of positively charged residues between the interface of these SCR domains similar to the verified M protein binding sites on the plasma complement regulators factor H and C4b-binding protein. The presence of excess M6 protein did not inhibit the cofactor activity of CD46 and the presence of excess C3b did not inhibit the ability of CD46 to bind M6 protein by ELISA. In conclusion, 1) adherence of M6 GAS to keratinocytes is M protein dependent and 2) a major M protein binding site is located within SCRs 3 and 4, probably at the interface of these two domains, at a site distinct from the C3b-binding and cofactor site of CD46. The Journal of Immunology, 2002, 168: 4585–4592.

Streptococcus pyogenes (group A streptococcus, GAS) is a causative agent of pharyngitis, skin infections, cellulitis, necrotizing fasciitis, and sepsis. Pharyngeal and skin infections can lead to the life-threatening postinfectious sequelae of rheumatic heart disease and glomerulonephritis, which cause widespread morbidity especially in developing countries and among Australia’s Aboriginal community (1–4).

GAS possess numerous virulence factors but their M protein is regarded as one of the most important (5). Streptococcal M proteins appear as long hairlike filaments on the bacterial surface and are composed of two α-helical chains which are predominantly arranged in a coiled-coil conformation (6, 7). All of the M proteins contain a conserved C-terminal region near the cell surface and a hypervariable N-terminal region which provides the basis for serological typing (5).

M protein is involved in the attachment of GAS to skin epithelial cells, which is a vital first step in the establishment of impetigo and cellulitis. The M protein receptor on keratinocytes is membrane cofactor protein (CD46) (8), which is a regulator of the complement system that functions as a cofactor for factor I-mediated inactivation of the opsonins C3b and C4b. M protein is also critical for protecting organisms from phagocytosis. In the absence of type-specific Abs, strains of GAS expressing M protein fail to activate either the classical or alternative pathways of complement. M-negative organisms, however, activate complement and are thus efficiently opsonized and phagocytosed (9–12). M6 and other M serotypes bind three plasma complement regulators: C4b-binding protein (C4BP) and the C3b-binding proteins, factor H (fH) and factor H-like protein 1 (fHL-1). These interactions are thought to contribute to the antiphagocytic properties of M protein by regulating complement activation on the GAS surface (13–15).

CD46, C4BP, fH, and fHL-1 belong to a family of complement regulatory proteins that share a structurally conserved motif termed the short consensus repeat (SCR). Each SCR is composed of approximately 60 aa, contains four invariant cysteines forming two intradomain disulfide bonds, and a linker region of three to eight residues joining the adjacent domains (16). CD46 possesses four SCR domains and, in addition, a region rich in serine, threonine, and proline, a region of 12 aa of unknown function, a transmembrane region, and a short cytoplasmic tail (17). C4BP contains α- and β-chain subunits of eight and three SCRs, respectively. The most common isoform of C4BP consists of seven α-chains and one β-chain linked by disulfide bonds (18). It has been demonstrated that the M protein binding site within C4BP resides in SCRs 1 and 2 of the α-chain (19, 20). fH is composed of 20 SCRs (21) and fHL-1, alternatively spliced variant of fH, consists of 17 SCRs (1–7, with four additional hydrophobic amino acids at the carboxyl terminus (22). Binding of M protein to fH and fHL-1 is mediated by SCR 7 (23–25) and recently an additional hydrophobic binding site at the carboxyl terminus of fHL-1 has also been suggested (26).

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Abbreviations used in this paper: GAS, group A streptococcus; C4BP, C4b-binding protein; fH, factor H; fHL-1, factor H-like protein 1; SCR, short consensus repeat.

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In this study, the adherence of M6 GAS to the human keratinocyte cell line HaCat was confirmed as being M protein dependent. A major M protein binding site was localized to SCRs 3 and 4 by use of CD46 SCR deletion and substitution mutants. A molecular model of SCRs 3 and 4 revealed similarity to the M protein binding sites identified on Hf SCR 7 (27) and C4BP a-chain SCRs 1 and 2 (28), allowing prediction of potential residues in CD46 involved in M protein binding. Furthermore, the M protein- and C3b-binding sites were identified as being distinct since CD46 cofactor activity was not inhibited by excess M protein, and the presence of excess C3b did not inhibit the ability of CD46 to bind M protein.

Materials and Methods

M6 protein and bacterial strains

M6 protein was purified from the periplasm of transformed Escherichia coli as previously described (29). The final preparation was shown to be homogenous based on Coomassie blue-stained SDS-polyacrylamide gels. Western blotting using a monoclonal anti-M6 protein Ab, and N-terminal amino acid sequence analysis. The GAS strains examined in this study included JRS4, an M6 protein-expressing organism, and JRS145, an M6 protein-deficient derivative of JRS4 (30, 31).

Cloning and expression of soluble CD46 and CD46/CD55 chimeras

Soluble recombinant CD46 protein and soluble chimeric proteins (isofrom STP BC), lacking the transmembrane and cytoplasmic sequence, were expressed in transfected human kidney fibroblasts (293 cell line) (32). Constructs encoding the chimeric proteins, in which the third and/or fourth SCR domains were replaced with the corresponding SCRs from CD55, were generated from cdNA constructs by splice-overlap extension PCR and mutagenesis to insert a stop codon before the transmembrane sequence (33). The proteins included soluble CD46_4, CD46-_CD55_4, CD46_3, CD55 and CD46_2 at 100 HaCat cells were examined and the number of bacteria bound per the means were calculated.

Western blotting

The identity of recombinant CD46 proteins was confirmed by Western blotting. Proteins were separated on a nonreducing 12.5% SDS-polyacrylamide gel and transferred to Hybond C+ nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were incubated with rabbit polyclonal anti-CD46 Ab (1:10,000 v/v), followed by HRP-conjugated anti-rabbit IgG (1:2000 v/v; Silenus, Hawthorn, Australia) and washed between incubation steps with 50 mM phosphate buffer. Labeled proteins were finally detected by ECL (Amersham Pharmacia Biotech).

Adherence assay

The binding of M6-positive GAS and M6-negative GAS to the human keratinocyte cell line HaCat was performed as previously described (35). Briefly, HaCat cells were cultured on glass coverslips. The M6-positive or M6-negative GAS were grown overnight at 37°C under 5% CO2 in brain-heart infusion medium (Oxoid, Basingstoke, U.K.), washed in PBS, and resuspended in serum-free DMEM (HyClone Laboratories, Logan, UT) at an A600 of 0.05 (~4 × 10^8 bacteria/ml). The HaCat-covered glass coverslips were incubated for 2 h with 500 μl of the bacterial suspension, and nonadherent organisms were removed by washing with PBS. The coverslips were immersed in ethanol, glued to microscope slides, Gram stained, and finally examined under oil immersion. Five random fields containing >100 HaCat cells were examined and the number of bacteria bound per HaCat cell was determined. Experiments were repeated six times in duplicate and the means were calculated.

ELISA analysis of the interaction between recombinant CD46 and M6 protein

Purified M6 protein (5 μg) and bovine albumin (5 μg; CSL, Victoria, Australia) were dried onto Hybond C+ nitrocellulose membranes. After blocking with 5% skim milk for 1 h, the membranes were sequentially incubated with recombinant CD46 (0.5 μg/ml) for 2 h, rabbit polyclonal anti-CD46 Ab (1:10,000 v/v) for 1 h, and then HRP-conjugated anti-rabbit IgG (1:2000 v/v) for 1 h. CD46 and Abs were all diluted in 1% skim milk in 50 mM phosphate buffer. Membranes were washed between incubation steps with 50 mM phosphate buffer and bound proteins were detected by ECL.

ELISA analysis of the interaction between CD46 and M protein

Purified M6 protein (1 μg) or bovine albumin (1 μg) in 100 μl of 100 mM bicarbonate buffer (pH 9.5) was coated overnight to wells of Maxisorb ELISA plates (Nunc, Roskilde, Denmark). Nonspecific sites were blocked with 200 μl of 5% skim milk for 1 h. Plates were then sequentially incubated with 100 μl of recombinant CD46 at 10 μg/ml for 2 h, rabbit polyclonal anti-CD46 Ab (1:4000 v/v) for 1 h, and HRP-conjugated anti-rabbit IgG (1:2000 v/v) for 1 h. CD46 and Abs were all diluted in 1% skim milk in 50 mM phosphate buffer. Wells were washed between each incubation with 50 mM phosphate buffer. Substrate was added and the A490 was determined. Experiments were repeated four times in duplicate and the means and SDs were calculated.

The possible effect of C3b on the CD46-M6 protein interaction was studied in a similar assay except that the binding of M6 protein (1 μg) to immobilized CD46 (1 μg) was determined in the presence or absence of 500 μg/ml of C3b (kindly supplied by Dr. T. Seya, Osaka, Japan). M6 protein was detected by sequential incubations with monoclonal anti-M6 protein Ab (10 μg/ml) for 1 h and HRP-conjugated anti-mouse IgG (1:1000 v/v) for 1 h. M6 protein and Abs were all diluted in 1% skim milk in 50 mM phosphate buffer.

ELISA analysis of the interaction between CD46 and GAS

M6-positive and M6-negative GAS were grown overnight in brain-heart infusion medium without agitation at 37°C under 5% CO2. Cells were harvested by centrifugation, washed twice with PBS, and resuspended in 100 mM bicarbonate buffer (pH 9.5) to achieve 10^9 bacteria/ml. Subsequently, 100 μl of each suspension was aliquoted into wells of an ELISA plate and dried overnight at 37°C. Wells were blocked with 5% skim milk, washed with 50 mM phosphate buffer, and then sequentially incubated with 100 μl of recombinant CD46 (2.5 μg/ml) for 1 h, rabbit polyclonal anti-CD46 Ab (1:4000 v/v) for 30 min, and HRP-conjugated protein A (1:2000 v/v; Amersham Pharmacia Biotech) for 30 min. CD46 and Abs were all diluted in 1% skim milk in 50 mM phosphate buffer. Wells were washed between each incubation with 50 mM phosphate buffer. Substrate was added and the A490 was determined. Experiments were repeated four times in duplicate and the means and SDs were calculated.

Purification of recombinant CD46 proteins

Recombinant CD46 proteins were purified on affinity columns by coupling either the mouse anti-CD46 mAb E4.3 (34) or the IgG fraction of polyclonal rabbit anti-CD46 antisera to cyanogen bromide-activated Sepharose (Amersham Pharmacia Biotech, Amersham, U.K.) using the manufacturer’s protocol. Supernatants (100–500 ml) from the transfected 293 cell line and P. pastoris expression cultures were passed over the affinity columns. After extensive washings with PBS, bound proteins were eluted, respectively, with diethyldiamine (34) or 3 M potassium thiocyanate and dialyzed immediately into 50 mM phosphate buffer. The P. pastoris eluates were concentrated in a stirred ultrafiltration cell (Amicon, Beverly, MA). The concentration of soluble CD46_3,4 was determined by absorbance at 280 nm using an extinction coefficient value of 0.92 (34). The specific concentration of the CD46/CD55 chimeras and the truncated CD46 SCR deletion mutants was determined by serial dilutions against a standard concentration of soluble CD46_3,4 in a dot blot assay or ELISA.
Molecular modeling of SCR domains 3 and 4 of CD46

Homology-based molecular modeling of CD46 SCRs 3 and 4 was performed using the experimentally determined tertiary structure of CD46 SCRs 1 and 2 as template (Brookhaven Protein Data Bank, PDB, entry 1ICL) (37) and the Insight II program package (Molecular Simulations, San Diego, CA) and Indigo 2 work station (Silicon Graphics, Mountain View, CA). Nonhomologous residues were replaced and a total of three loops was needed to equalize the number of amino acids in the model and the template. Loop 1 was constructed for residues 40–47 of the model (spliced from PDB entry 1AP8), loop 2 for residues 62–64 (from 1ACC), and loop 3 for residues 113–117 (from 1AZO). The interdomain angles were adapted from the CD46 SCRs 1 and 2 template structure. The preliminary model structure was subjected to energy minimizations by gradually relaxing the molecule: first, only hydrogens were allowed to move; second, only side chains were allowed to move; and third, all of the atoms were allowed to move, also allowing the interdomain angle to change. In all energy minimizations, a conjugate gradient algorithm with CFF91 force field was used until the maximal derivative was <4.2 J Å⁻¹. Subsequently, the structure was soaked in a waterbox of 2091 water molecules (box dimensions, 60 × 46 × 31 Å) to achieve an 8–6Å-thick layer of water around the model. The soaked model was again subjected to energy minimizations by gradually relaxing the structure. Thereafter, the assembly of the model and the water molecules was subjected to 10 picoseconds of molecular dynamics simulation at 100,000 and 50 picoseconds at 300,000 under periodic boundary conditions as described previously (38). A low potential energy structure was chosen and processed as described previously (39).

The achieved model of CD46 SCRs 3 and 4 was then compared with the previously published molecular models of two M protein binding human proteins, TH SCRs 6 and 7 (27, 40, 41) and C4BP α-chain SCRs 1 and 2 (20). In the comparison, the known M protein-binding residues of TH SCRs 6 and 7 and C4BP α-chain SCRs 1 and 2 and the corresponding positively charged residues on CD46 SCRs 3 and 4 model were highlighted. The illustrations were designed with WebLab ViewerPro 3.5 (Molecular Simulations). The model structure of CD46 SCRs 3 and 4 has been deposited in the Brookhaven PDB (entry 1HR4). The model structure of CD46 SCR domains from CD55 was kindly provided by Dr. A. Blom (Malmö, Sweden).

Cofactor assay

The cofactor activity of recombinant CD46 was assayed in 20 mM phosphate buffer (pH 6.0) in a final volume of 30 μl containing 0.25 μg of C3b, 1 μg of factor I (Calbiochem-Novabiochem, La Jolla, CA), 40 ng of soluble CD46, and either buffer or 2.5 μg of M6 protein (final concentration of 83.5 μg/ml). Reactions that did not contain factor I or soluble CD46 were used as negative controls. The reactions were incubated at 37°C for 3 h. Samples were separated under reducing conditions in a 7.5% polyacrylamide gel, transferred to a nitrocellulose membrane, and sequentially incubated with 5% skim milk for 30 min, goat anti-C3c Ab (1:1000 v/v, Silenus) for 30 min, and HRP-conjugated anti-goat IgG (1:2000 v/v, Calbiochem-Novabiochem) for 30 min. The membrane was washed between incubations with PBS and protein was detected by ECL.

Results

Adherence of M6 GAS to keratinocytes

To confirm the role of M protein in the adherence of M6 GAS to host cells, we examined the binding of M6-positive and M6-negative GAS strains to the human keratinocyte cell line HaCat. M6-positive organisms adhered to HaCat cells with an average of 60 ± 23 organisms/cell, whereas the M6-negative organisms failed to bind in significant numbers (average, 6 ± 2 organisms/cell). This indicates that adherence of M6 GAS to HaCat cells is M protein dependent.

Recombinant CD46 mutants

It has been previously shown that CD46 is the receptor on keratinocytes responsible for M protein binding (8). To map the M protein binding site, we used recombinant soluble CD46 and soluble CD46/CD55 chimeras, in which the transmembrane and cytoplasmic domains of CD46 were deleted, and for the chimeras, SCRs 3 and/or 4 of CD46 were replaced with the corresponding SCR domains from CD55 (CD461–4, CD462–4, CD461–3, and CD461–2, CD552–4). In addition, truncated CD46 SCR deletion mutants, CD462–4 and CD461–4, were constructed (Fig. 1). Each protein migrated on SDS-PAGE according to its predicted molecular mass, allowing for N- and O-linked glycosylation. The P. pastoris produced CD461–4 SCR deletion mutant migrated as two bands (25 and 30 kDa) under nonreducing conditions (Fig. 2), but as a single band under reducing conditions. This may be due to misfolding of a portion of the protein, resulting in aberrant migration of one band under nonreducing conditions.

Identification of the M protein binding site within CD46

The M protein binding site within CD46 was mapped by a number of different assays. We initially used soluble CD461–4 and the CD46/CD55 chimeras and determined their M protein-binding characteristics by dot blot. In this assay, M6 protein or albumin was immobilized onto nitrocellulose before incubation with the test proteins. Soluble CD461–4 bound to M6 protein but not to the negative control, albumin. The chimeras, CD461–2,CD552–4, CD461–3,CD552–4, and CD461–2,CD551–3, exhibited no consistent binding to M6 protein or albumin (Fig. 3). These preliminary data indicated that CD46 SCRs 3 and 4 are responsible for M protein binding.

To further confirm this finding and to assess whether CD46 SCRs 3 and 4 alone are sufficient for M protein binding, we subsequently created the truncated CD46 SCR deletion mutants, CD462–4 and CD461–4, and compared their M protein-binding capacity to that of soluble CD461–4, CD461–2,CD552–4, CD461–3, CD552–4, and CD461–2,CD551–3. Binding of the CD46 mutants to M protein was detected by ELISA, in which M6 protein or albumin was immobilized onto the wells of an ELISA tray before incubation with the CD46 mutants. Soluble CD461–4 bound to M6 protein but not to albumin. The chimeras, CD461–2,CD552–4, CD461–3, CD552–4, and CD461–2,CD551–3, did not bind either M6 protein or albumin. The truncated CD46 SCR deletion mutants, CD462–4 and CD461–4, bound to M protein, but not albumin (Fig. 4). These data...
indicate that CD46 SCRs 3 and 4 alone, in the absence of adjacent SCRs, can mediate M protein binding.

We then assessed whether the CD46 mutants could bind M6 protein on the surface of whole streptococci in a cell-ELISA. In this assay, M6-positive or M6-negative GAS strains were coated onto the ELISA tray and then incubated with the CD46 mutants. We initially demonstrated that soluble CD461–4 bound to M6-positive GAS but not M6-negative GAS, indicating that the GAS-CD46 interaction is M protein dependent. The truncated CD46 SCR deletion mutants, CD462–4 and CD463–4, also bound GAS in an M protein-dependent fashion but the chimeras failed to bind either M6-positive GAS or M6-negative GAS (Fig. 5).

Conserved residues among M protein-binding SCR domains

M protein binding sites have been mapped for C4BP (19, 20), fH (23–25), and in this report for CD46 to/9251-chain SCRs 1 and 2, SCRs 6 and 7, and SCRs 3 and 4, respectively. The sequence similarity between these M protein-binding SCR was determined by sequence alignments using the ClustalW alignment program (Genetics Computing Group, Madison, WI) with manual corrections to align structurally important cysteine residues. The overall sequence identity of the domains ranged from 35 to 46%. The highest similarity was between CD46 SCR 4 and fH SCR 7 (46%), followed by CD46 SCR 3 and fH SCR 7 with 41% identity. The identity and homology between C4BP/9251-chain SCRs 1 and 2 and fH SCRs 6 and 7 or CD46 SCRs 3 and 4 was lower (<35% and <39%, respectively).

In a previous study, we used site-directed mutagenesis to identify four residues within fH SCR 7 and the linker region between fH SCRs 6 and 7 (Arg369, Lys370, Arg386, and His387) to be critical for M protein binding (41). Blom et al., (20) used site-directed mutagenesis to identify four residues within C4BP/9251-chain SCRs 1 and 2 to be involved in M protein binding (Lys63, Arg64, Arg66, and His67). An amino acid sequence alignment of the M protein-binding domains of fH, C4BP, and CD46 revealed one common feature: two or three positively charged residues within or next to the linker region between the two domains. In addition, SCR 4 of CD46 contained two positively charged residues (Lys210, Lys211) in the same location as the M protein-binding residues on SCR 7 of fH (Arg404, Lys405). In the primary structures of fH SCRs 6 and 7 or CD46 SCRs 3 and 4, there were phenylalanine residues corresponding to the position of the M protein binding His67 of C4BP (Fig. 6).
Molecular modeling of CD46 SCR domains 3 and 4

To model the tertiary location of the positively charged residues that were suggested by the sequence alignments to be involved in M6 protein binding, we constructed a molecular model of CD46 SCRs 3 and 4. The experimentally determined tertiary structure of CD46 SCRs 1 and 2 was used as a template. The locations of the suggested M6 protein-binding residues and other positively charged residues were visualized using a structure that had been subjected to energy minimizations and a total of 60 picoseconds of molecular dynamics simulations. Four positively charged residues (Lys190, Lys193, Arg195, and Lys210) were found to form a cluster of positive charges at the interface of CD46 SCRs 3 and 4 (Fig. 7), similar to that observed at the interface of fH SCRs 6 and 7 (27) and C4BP α-chain SCRs 1 and 2 (20, 28). In all three M protein-binding complement regulators, the positively charged residues formed a semicircle, which contained one phenylalanine residue.

Sites on CD46 for C3b cofactor and M protein binding are distinct

The primary function of CD46 is cofactor activity for proteolytic cleavage of C3b and C4b, with SCR domains 3 and 4 shown to bind C3b (42). As SCRs 3 and 4 have been identified to bind both C3b and M protein, we assessed whether these ligand binding sites within SCRs 3 and 4 are distinct or overlapping. The effect of M

FIGURE 6. ClustalW multiple sequence alignment of CD46 SCRs 3 and 4, C4BP α-chain SCRs 1 and 2, and fH SCRs 6 and 7, with positively charged residues highlighted in black boxes. The four basic amino acids in the linker between fH SCRs 6 and 7 and in SCR 7, which are known to be involved in M protein binding are indicated with numbers in reference to the full-length mature fH protein (Arg369, Lys370, Arg386, Lys387). The corresponding or nearby positively charged residues in the sequence of CD46 are indicated (Lys190, Lys193, Arg195, Lys210, Lys211), as are the four residues in C4BP involved in M protein binding (Lys63, Arg64, Arg66, His67). Phenylalanine residues in fH, CD46, and C4BP which we propose may be involved in M protein binding are indicated in gray boxes.

FIGURE 7. Homology-based molecular model of CD46 SCRs 3 and 4 and its comparison with other M protein binding structures. The structure of CD46 SCRs 3 and 4 was acquired by homology-based molecular modeling using an x-ray crystallographic structure of CD46 SCRs 1 and 2 as the template (37). The preliminary model was subjected to energy minimizations and a total of 60 picoseconds of molecular dynamics simulations. The electrostatic surface potential of the CD46 SCR 3 and 4 model is shown in a front view (A), back view (B), a view from above (C), and a view from below (D). The positively charged residues of CD46 SCRs 3 and 4 that were identified from the amino acid sequence alignment (Fig. 6) to be potentially involved in M protein binding are highlighted (Lys190, Lys193, Arg195, Lys210) along with the closely located phenylalanine residue (Phe206). The CD46 structure (view from below) is compared with fH SCRs 6 and 7 (E) (27, 41) and C4BP α-chain SCRs 1 and 2 (F) (20) (view from below); the electrostatic surface potential of these structures is shown, as are the known M protein-binding residues and the closely located phenylalanine residues. Red denotes negatively charged surface areas and blue denotes positively charged surface areas.
protein binding on cofactor activity of CD46 was determined with a C3b fluid-phase cofactor assay in the presence of a 30-fold molar excess of M protein, using conditions in which limited C3b cleavage occurred. C3b is composed of an α-chain (107 kDa) and β-chain (75 kDa) and, as expected, in the presence of factor I and CD46 the α-chain was cleaved at two sites to generate fragments of 67, 42, and 40 kDa (Fig. 8). In the presence of excess M protein, no reduction of CD46 cofactor activity was detected, suggesting that the binding sites on CD46 for M protein and C3b are distinct (Fig. 8). To further confirm this finding, we performed an ELISA in which CD46 was immobilized to the wells of an ELISA tray and then incubated with M protein in the presence or absence of a 30-fold molar excess of C3b. No reduction in binding of M protein to CD46 in the presence of C3b was observed (mean binding of M6 in the presence of C3b was 103% relative to control).

Discussion

Adherence of pathogens to host cells is a critical first step in establishing colonization and infection. Multiple types of interactions can contribute to bacteria-host cell adherence and frequently this involves carbohydrate-lectin recognition that may determine bacterial tropism for a host. In other instances, an even more specific interaction may be responsible for adherence and colonization. In this study, we used an in vitro experimental model system adapted from Okada et al. (35) to examine the adherence of GAS to human keratinocytes. We demonstrated that a strain expressing M6 protein adheres to keratinocytes, while an M6 deficient strain is unable to bind. This is consistent with previous studies in which the adherence of M6 and M24 serotypes of GAS was also shown to be M protein dependent (8, 43). However, the role of different M proteins in adherence to keratinocytes seems to be variable since equivalent adherence of the M49, M2, and M18 GAS serogroups and their isogenic M protein-negative mutants has been reported (43, 44). Taken together, these data suggest that M protein is not a universal adhesion but mediates attachment of certain M protein-expressing GAS to keratinocytes.

The M6 protein receptor on keratinocytes has been reported to be CD46 (8), a widely expressed complement regulator (45, 46). A number of other pathogens have also been identified which utilize CD46 as a receptor to initiate infections. These include measles virus (47), which binds CD46 SCRs 1 and 2 (48), human herpesvirus 6 (49), Neisseria gonorrhoeae, and Neisseria meningitidis (50). The broad tissue expression of CD46 provides opportunities for wide tissue tropism of disease; however, it is not clear whether this is a factor in pathogen-host receptor selection.

To further examine the interaction between M protein and CD46, we used a number of CD46/CD55 chimeric SCR replacement mutants (CD46δ3−CD55δ4, CD46δ1−δ3,CD55δ4, and CD46δ1−δ4,CD55δ2) and examined their ability to interact with M protein. Replacement of either CD46 SCR 3 and/or 4 with the corresponding SCR domains of CD55 largely diminished binding to M6 protein, indicating that both of these SCR domains are essential for full binding. As previous mutagenesis studies have revealed that some SCRs involved in binding interactions require flanking SCRs to allow full functional activity (51), we therefore determined the minimum number of SCRs that are required for M protein recognition. Truncated CD46 SCR deletion mutants consisting of SCRs 2 and 4 and 3 and 4 were therefore constructed and both were shown to mediate binding, indicating that SCRs 3 and 4 contain a major binding site for M protein without a need for adjacent domains.

M proteins of GAS strains also bind other structurally related complement regulators including fH and C4BP via SCRs 6 and 7 and α-chain SCRs 1 and 2, respectively (19, 20, 23–25). Previously, molecular models of both fH SCRs 6 and 7 and C4BP α-chain SCRs 1 and 2 had been constructed to define M protein binding sites. Both models define a cluster of positively charged residues at the interface of the SCR domains, which by site-directed mutagenesis have been identified to be involved in M protein binding (20, 27, 41). His67 of C4BP has also been identified to be involved in M protein binding (20). To further delineate the residues of CD46 involved in M protein binding, we constructed a homology-based molecular model of CD46 SCRs 3 and 4 and compared it with the models of fH SCRs 6 and 7 and C4BP α-chain SCRs 1 and 2. The model of CD46 SCRs 3 and 4 similarly contained positively charged residues in an equivalent position (Fig. 7). Thus, it is probable that these residues are directly involved in the binding of M6 protein to CD46. Our finding that both the SCR 3 and SCR 4 domains of CD46 are needed for full M6 binding is in agreement with this hypothesis, since the chimeric CD46δ3−CD55δ4 protein lacks three of these four positively charged residues, whereas the CD46δ3−CD55δ4 chimera introduces a negatively charged (Glu91) residue between two positively charged residues (Lys196 and Lys199).

In addition to the positively charged semicircle found on all three models of the M protein-binding domains, we identified one phenylalanine residue within the semicircle of each model. To ascertain the role of these phenylalanine residues in M protein binding, mutagenesis studies are needed. Since His67 of C4BP has been shown to be necessary for efficient M protein binding by C4BP and since the M protein-C4BP interaction is relatively insensitive to high salt concentration (up to 1.5 M), it seems that hydrogen bonds and hydrophobic interactions are involved in this interaction (20). Combined with the fact that phenylalanine is the most frequently found residue involved in protein-protein interactions in general (52), it is relatively likely that the structurally conserved phenylalanine in C4BP, fH, and CD46 is involved in M protein binding.

Our predictions regarding the residues in CD46 involved in M protein binding do, however, require some caution. Although in general each single domain of a multidomain protein can be accurately modeled if good quality homologous templates are used, the prediction of the rotation and torsion angles between two SCR domains can be problematic. The possible rotation and torsion angles vary considerably in different SCR-containing proteins. In our CD46 SCRs 3 and 4 model, we have used a relatively long molecular dynamics simulation at 300,000 to allow possible movement of the SCR domains to find one possible conformation for the SCR pair. Our model shows one of several possible interdomain torsion and rotation angles and the distribution of surface charge

FIGURE 8. Cofactor activity of recombinant soluble CD46 for C3b cleavage in the presence of M6 protein. CD46-dependent C3b cleavage (lane 5, arrow, smaller α’-chain cleavage products of 67, 42, and 40 kDa) required the protease factor I (lane 3). In the presence of a 30-fold excess of M protein, cleavage of the α’-chain was unaffected (lane 6).

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associated with it. Additional experimental mutagenesis studies are required to confirm that the residues, Lys<sup>190</sup>, Lys<sup>193</sup>, Arg<sup>195</sup>, Phe<sup>196</sup>, and Lys<sup>210</sup> are involved in M protein binding.

In addition to M protein binding, SCRs 3 and 4 of CD46 are also involved in the C3b/C4b binding and cofactor activity (42). In this study, we demonstrate that 1) CD46 maintains its C3b cofactor activity in the presence of excess M protein and 2) excess C3b does not inhibit the ability of CD46 to bind M protein by ELISA, suggesting that the binding sites on CD46 for M protein and C3b are distinct. These results, however, need confirmation with specific mutagenesis studies but are similar to the binding with H1, in which cofactor and M protein binding sites are clearly distinct (24, 51). Assuming the fluid-phase assay utilizing soluble proteins predicts what occurs for cell surface receptor-ligand interaction, these results also suggest that wild-type CD46 would retain functional activity after binding to GAS via M6 protein. Thus, CD46 may not only act as a receptor on host cells for the establishment of streptococcal infections, but it also may be involved in protection of the organism from destruction by the host complement system.

The results suggesting the separate location of M protein and C3b binding sites on CD46 and our prediction of the CD46 residues involved in M protein binding (Lys<sup>190</sup>, Lys<sup>193</sup>, Arg<sup>195</sup>, Phe<sup>196</sup>, and Lys<sup>210</sup>) are in partial agreement with a recent study by Liszewski et al. (53). By alanine-scanning mutagenesis, it was shown that replacement of either Lys<sup>193</sup>, Arg<sup>195</sup>, Phe<sup>196</sup>, or Lys<sup>210</sup> did not abolish C3b binding but decreased binding to 37, 50, 35, and 68%, respectively. A Lys<sup>190</sup> mutant was not constructed. Of these residues, only the replacement of Lys<sup>193</sup> or Phe<sup>196</sup> with alanine abrogated the cofactor activity of CD46. However, the inhibition caused by alanine substitutions of Lys<sup>193</sup>, Arg<sup>195</sup>, or Phe<sup>196</sup> might be due to an altered orientation between the SCR 3 and 4 domains, since these residues are located at the hinge region. In contrast and, more importantly, other substitutions in CD46 SCRs 3 and 4 that were outside our predicted M protein site resulted in the complete loss of C3b binding and cofactor activity. To determine whether the suggested four positively charged residues and one hydrophobic residue on CD46 are in fact important for M protein binding, further analyses are needed with amino acid substitution constructs.

In summary, we have shown that the binding of CD46 to serotype M6 GAS requires M protein. We have also shown that CD46 SCRs 3 and 4 contain a major M protein binding site and the M protein/CD46 interaction 1) does not measurably inhibit proteolytic cleavage of C3b and 2) is not inhibited by C3b. Our results therefore suggest that CD46 not only acts as a receptor on host cells for M6 GAS adherence but might also function by regulating complement activation on GAS and have a direct pathogenic role. On the basis of sequence comparisons and molecular modeling, we additionally propose that Lys<sup>190</sup>, Lys<sup>193</sup>, Arg<sup>195</sup>, Phe<sup>196</sup>, and Lys<sup>210</sup> are involved in M protein binding. However, further studies are needed to locate the precise residues involved in the M protein-CD46 interaction.

References