Homologue Scanning Mutagenesis Reveals CD66 Receptor Residues Required for Neisserial Opa Protein Binding

By Martine P. Bos, Daniel Hogan, and Robert J. Belland

From the Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840-2999

Summary

The immunoglobulin-like family of CD66 antigens, present on human neutrophils and epithelial cells, are used as receptors for adhesins expressed by the pathogenic Neisseriae. Neisseria gonorrhoeae strain M511 can express 11 isoforms of these adhesins, called opacity-related (Opa) proteins. Each M511 Opa protein recognizes a distinct spectrum of CD66 receptors. CD66–Opa binding is mediated by the NH2-terminal domain of the receptor and occurs through protein–protein interactions. In this report, we have investigated the molecular basis for the binding between the CD66 and Opa protein families by mapping amino acids in CD66 receptors that determine Opa protein binding. We performed homologue scanning mutagenesis between CD66e, which binds multiple Opa variants, and CD66b, which binds none, and tested both loss-of-function by CD66e and gain-of-function by CD66b in solution assays and in assays involving full-length receptors expressed by epithelial cells. We found that three residues in the CD66e N-domain are required for maximal Opa protein receptor activity. Opa proteins that recognize the same spectrum of native CD66 molecules showed differential binding of receptors with submaximal activity, indicating that the binding characteristics of these Opa proteins are actually slightly different. These data provide a first step toward resolving the structural requirements for Opa–CD66 interaction.

Key words: Neisseria gonorrhoeae • carcinoembryonic antigen • bacterial adhesion • opacity protein • mutagenesis
to this list. To date, five members of the CD66 family (CD66a–e) have been studied for their interaction with gonococcal Opa proteins. We and others have found that this interaction is highly differential, i.e., some CD66 family members are recognized by four Opa variants while other CD66 receptors are recognized by none (CD66b), six (CD66a), or nine (CD66e) different Opa variants of N. gonorrhoeae strain M11 (10, 11, 17). We recently showed that the differential interaction observed with native receptors expressed on epithelial cells is mimicked by the binding pattern of recombinant CD66 N-domains produced in E. coli (18), indicating that the Opa–CD66 interaction is mediated by protein sequences in CD66 N-domains. It is remarkable that, despite extensive sequence similarity among the CD66a–e N-domains (71–90% amino acid sequence identity; see Fig. 1A), the N-domains are recognized by different groups of Opa proteins. In our effort to understand the differential Opa–CD66 interactions, we investigated the molecular basis for the binding between the Opa and CD66 protein families. Homologue scanning mutagenesis, a strategy chosen to preserve structural integrity of mutant molecules (19), was used to map key residues in CD66 required for Opa protein binding. Residues within the N-domain of CD66e (which binds most Opa variants) were switched to homologous amino acids of the N-domain of CD66b (which does not bind any Opa variant). The key residues identified through loss-of-function mutagenesis were confirmed by gain-of-function experiments, whereby the CD66b protein was converted into a functional receptor for Opa variants.

Materials and Methods

Bacterial Strains. N. gonorrhoeae M11 variants were propagated on gonococcal clear typing agar (20). Wild-type variants M11mk expressing chromosomally encoded Opa proteins were provided by J. Swanson and are designated according to Swanson et al. (21) by capital, e.g., OpaA-OpaK. Recombinant M11 Opa variants (5) were a gift of T.F. Moyer (Max-Planck Institut für Biologie, Tubingen, Germany). Opa protein expression was verified by SDS-PAGE and immunoblotting of bacterial lysates followed by detection with anti-Opa antibody 4B12 (21). Only nonpiliated bacteria were used. For experiments, bacteria were grown for 3 h in 10 ml Hapes medium (10 mM Hepes, 145 mM NaCl, 5 mM KCl, 0.5 mM MgCl2, 1 mM CaCl2, 5 mM glucose, and 1.5% proteose peptone no. 3 [Difco]), pH 7.4, in a gyratory shaker at 37°C. Bacterial suspensions were pelleted and resuspended in 1 ml Hapes buffer (Hapes medium without protease peptone).

Construction of Mutant CD66 N-domains. The construction of 6xHis-tagged N-domains of CD66e and CD66b in the pRSET-A vector was described previously (18). Mutations were introduced by PCR using the mutagenic primer and a common vector-based 3′ primer (pRSET-rev) with the CD66 N-domain construct as template. The pRSET-rev primer was removed by passing the reaction mixture through a 100-kD Centricon device (5 min at 3,000 g; Amicon). A second PCR was performed with the same template plus 17 µl of the 50 µl of 100-kD Centricon retenate as 3′ primer and a common vector-based 5′ primer (pRSET-for). The resultant PCR product was cut with EcoRI and HindIII and ligated into pRSET-A. Constructs were electrooporated into E. coli strain BL21 (DE3; Novagen). Mutations were verified by DNA sequencing through the entire N-domain insert. Primers were purchased from Genosys and restriction enzymes from New England Biolabs.

Binding of CD66 N-domains by G. gonorrhoeae. Cleared lysates of E. coli cells expressing the appropriate CD66 N-domain were prepared as described (18). Gonococci (109 in 200 µl Hapes buffer were incubated with 5–10 µl of cleared lysate for 20 min at 37°C. Bacteria were collected by centrifugation (5 min at 2,000 g), washed twice with 1 ml Hapes buffer, then solubilized in 30 µl SDS-PAGE sample buffer of which 2.5 µl was electrophoresed in 15% SDS-PAGE and transferred onto nitrocellulose. Bound CD66 N-domains were detected by anti-His antibody (1:15,000; Amersham Pharmacia Biotech) followed by peroxidase-conjugated protein A (1:20,000; Sigma Chemical Co.). Blots were developed using the enhanced chemiluminescence (ECL) protocol (Amersham Pharmacia Biotech). Documentation and quantification of bands were performed with an Alpha Imager® 2000 Imaging system (Alpha Innotech).

Construction of CD66b cDNA Mutants. CD66b cDNA in pUC118 was a gift from Motomu Kuroki (Fukuoka University, Fukuoka, Japan). The CD66b insert was subcloned into the eukaryotic expression vector pTracer-CMV2 (Invitrogen). All mutations were made first in the CD66b N-domain construct in pRSET-A as outlined above. To introduce mutated N-domains into the full-length CD66b cDNA, the BspI site present at the start of the N-domain in CD66b cDNA was introduced in the CD66b N-domain construct in the pRSET-A vector by PCR. For introduction of the chimeric CD66e/b N-domain into CD66b, the BspI site present in CD66b (GCT CAGC) was mutated to the BspI site found in CD66e (GCT TAAAGC) by megaprimer PCR, in order to have the N-domain start with the CD66b-derived lysine. The resultant PCR product was cleaved with BspI and NsiI (NsiI cuts at residue 71 in CD66 N-domains) and substituted with the fragment present in the full-length CD66b cDNA in pTracer-CMV2. To substitute the entire CD66b N-domain with the CD66e N-domain, a silent mutation containing a ClaI site was introduced at residue 113 just downstream of the N-domain in CD66b. The CD66b N-domain in pRSET-A was amplified with primers containing a BspI and a ClaI site, respectively, cut and ligated into BspI/ClaI-cut CD66b. The constructs were electrooporated into E. coli DH5α. All mutations in the pTracer-CMV2-CD66b constructs were verified by DNA sequencing.

Transfection Procedure. Chinese hamster ovary (CHO) cells (Pro5) were obtained from the American Type Culture Collection and were grown in RPMI 1640/5% FCS in 25-cm2 flasks to 50% confluency. Plasmid preparations of pTracer-CMV2-CD66b mutants were made by the Wizard miniprep procedure (Promega Corp.). Pro5 cells were incubated in 2 ml DMEM/10% N-1 serum (Collaborative Biomedical Products) containing 4 µg plasmid DNA and 0.2 mg/ml DEAE-dextran (M, 5 × 106; Amersham Pharmacia Biotech) for 4 h at 37°C. Cells were then shocked with 2.5 ml 10% DMSO in PBS for 1 min at room temperature, washed once with HBS, and subsequently cultured overnight in RPMI 1640/5% FCS (23). The next day, the transfectants were trypsinized and seeded onto 12-mm diameter circular glass coverslips in 24-well plates (105 cells per coverslip). Cells were cultured for 2–3 d before infection assays were carried out.
Infection Assay. Gonococci (1.5 × 10^7) were added to 24-well plates containing the transfected cell cultures on coverslips in 1 ml DMEM (without serum) for 45 min at 37°C and 5% CO₂. Non-adherent bacteria were removed by three washes with HBSS. Infected cultures were fixed with 2% formaldehyde in PBS for at least 30 min.

Staining Procedures. Fixed infected cells were incubated with 0.5% Triton X-100 in PBS for 20 min and then blocked with 5% FCS in PBS for 1 h. Antibodies were diluted in PBS/0.05% Tween/0.5% FCS. To detect receptor expression, cells were stained with rabbit anti-CD66a antiserum (1:200; Dako). To subsequently stain gonococci, coverslips were incubated with a mouse mAb against gonococcal LPS, generated in our laboratory by J. Swanson, followed by FITC-conjugated goat anti-mouse (GAM) IgG (1:400; Sigma Chemical Co.). When only receptor expression was evaluated, the permeabilization step with Triton X-100 was omitted. mAb Kat4C was provided by H. Turley (John Radcliffe Hospital, Oxford, UK).

Identification of CD66e N-domain residues critical for binding of Opa-expressing gonococci. The differential recognition of CD66 receptor N-domains by gonococcal Opa variants is shown in Table I. CD66e is recognized by the majority of Opa variants, whereas CD66b does not bind any Opa variant. OpaB, OpaC, OpaG, and Opa pal variants demonstrate the broadest recognition of CD66 receptors. Assays with previously constructed chimeric receptor N-domains consisting of the NH₂-terminal half (residues 1–59) of CD66a fused to the COOH-terminal half of the CD66b N-domain and vice versa, located the critical domain for binding of OpaB, OpaC, OpaG, and Opa pal variants to the first 59 residues of CD66b (18; compare binding of the chimeric N-domains CD66e/b and CD66b/e in Table I). Using homologue scanning mutagenesis, we exchanged regions and single residues between the NH₂-terminal 59 residues of CD66e and CD66b (for sequence differences, see Fig. 1 A) and measured the ability of the mutant proteins to bind to Opa, OpaB, OpaC, and Opa pal-expressing gonococci in order to identify CD66 residues required for Opa protein binding. Since OpaG is nearly identical to OpaB, we did not include this Opa variant in our assays.

As is shown in Table I and Fig. 2 A, mutations in the first 11 residues of the CD66e N-domain did not influence binding of any tested Opa variant. Mutation of the region comprising residues 27–29 (mut3) resulted in a complete loss of recognition by all variants. Evaluation of the individual residues in this region revealed that the loss of binding was caused by the single F29R mutation (mut6). The single mutation S32N (mut7) also abrogated all Opa binding. The double mutation G41A + Q44R (mut9) clearly diminished Opa binding, but when these mutations were tested individually, a difference between Opa variants was noted. For Opa pal interaction, both the G41A and the Q44R mutations were deleterious. This was also the case for interaction with OpaB, although to a lesser extent. The Q44R mutation had a moderate effect on OpaB binding, whereas the G41A change had no effect at all. Mutations in the region between residues 51 and 55 caused only a moderate decrease in Opa binding. Thus, the two residues F29 and S32 in CD66e are critical for binding of all Opa variants. As is shown in Table I and Fig. 2 A, mutations in the first 11 residues of the CD66e N-domain did not influence binding of any tested Opa variant. Mutation of the region comprising residues 27–29 (mut3) resulted in a complete loss of recognition by all variants. Evaluation of the individual residues in this region revealed that the loss of binding was caused by the single F29R mutation (mut6). The single mutation S32N (mut7) also abrogated all Opa binding. The double mutation G41A + Q44R (mut9) clearly diminished Opa binding, but when these mutations were tested individually, a difference between Opa variants was noted. For Opa pal interaction, both the G41A and the Q44R mutations were deleterious. This was also the case for interaction with OpaB, although to a lesser extent. The Q44R mutation had a moderate effect on OpaB binding, whereas the G41A change had no effect at all. Mutations in the region between residues 51 and 55 caused only a moderate decrease in Opa binding. Thus, the two residues F29 and S32 in CD66e are critical for binding of all Opa variants, whereas residues G41 and Q44 are important to different extents for the various Opa proteins.

Gain-of-function by the non-Opa-binding CD66 Family Member CD66b. If the four residues indicated above were indeed responsible for the receptor function of CD66e, it should be possible to impart Opa-binding properties to CD66b by introducing those four residues into CD66b. To test this concept, we changed the residues in question in the CD66b N-domain to the corresponding ones of CD66e in different combinations. As can be seen in Table III, the presence of F29+ S32 (mut13) did not result in detectable binding of any Opa variant. Residue F29 in mut13 is preceded by two CD66b-specific residues, D27 and P28, which may influence the correct conformation of F29. To address whether the F29 residue in a more “CD66e-like” environment would mediate Opa binding, we added residues H27 and L28 to mut13. This molecule (mut14) also failed to bind significantly to any Opa variant. The A41G + R44Q mutant (mut15) did not bind any Opa pal variant either. We then added the G41 and Q44 residues individually to mut14, resulting in mut16 and mut17, and found that each addition slightly enhanced binding of all Opa variants compared with mut14. Only when both G41 and Q44 were present (mut18) was full binding activity by all Opa variants demonstrated.

**Table I.** Recognition of CD66 receptor N-domains by M S11 Opa variants

<table>
<thead>
<tr>
<th>Receptor type</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD66e</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD66a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD66c</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD66d</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD66b</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD66b/e</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD66b/e</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

M S11 Opa variants were incubated with E. coli lysates containing the indicated His-tagged CD66 N-domain and processed for immunoblotting. Blots were probed with anti-His antibody and peroxidase-conjugated protein A followed by ECL. When a significant signal was present on the blot, recognition was defined as +. CD66a-e are native N-domain sequences; CD66b/e and CD66b/e represent chimeric N-domains between CD66b and CD66e, as described in reference 18. Some of these data were shown previously in reference 18.
CD66 Receptor Residues Required for Opa Protein Binding

We then readdressed the question whether the origin of residues 27 and 28 played any role in Opa binding by construction of mut19–21. The presence of F29 and S32 was sufficient for binding of OpaB and OpaI variants; however, for OpaC binding an additional CD66e-derived residue (L28) was necessary, indicating a difference in binding characteristics among Opa variants (Fig. 2 B, and Table III). These data confirm the key role of four residues in the CD66e N-domain for receptor function of CD66e and again show a difference in binding characteristics within the OpaB, OpaC, and OpaI group of variants.

Table II. Binding of CD66e N-domain Mutants to MS11 Opa Variants

<table>
<thead>
<tr>
<th>Mutations introduced in the CD66e N-domain</th>
<th>Binding by Opa variants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OpaB</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>++</td>
</tr>
<tr>
<td>1 K1Q</td>
<td>++</td>
</tr>
<tr>
<td>2 S6A + T7V + F9S + V11A</td>
<td>++</td>
</tr>
<tr>
<td>3 H27D + L28P + F29R</td>
<td>++</td>
</tr>
<tr>
<td>4 H27D</td>
<td>++</td>
</tr>
<tr>
<td>5 L28P</td>
<td>++</td>
</tr>
<tr>
<td>6 F29R</td>
<td>++</td>
</tr>
<tr>
<td>7 S32N</td>
<td>++</td>
</tr>
<tr>
<td>8 R38T</td>
<td>++</td>
</tr>
<tr>
<td>9 G41A + Q44R</td>
<td>++</td>
</tr>
<tr>
<td>10 G41A</td>
<td>++</td>
</tr>
<tr>
<td>11 Q44R</td>
<td>++</td>
</tr>
<tr>
<td>12 G51S + T52N + A55I</td>
<td>++</td>
</tr>
</tbody>
</table>

Table II. Binding of CD66e N-domain Mutants to MS11 Opa Variants

MS11 Opa variants were incubated with E. coli lysates containing the indicated mutant (mut) CD66e N-domain and processed for immunoblotting. Binding was calculated by determining the signal generated by the amount of His-tagged N-domain for each variant, compared with the signal generated in a lane where an amount of His-tagged N-domain was loaded representing 100% binding. Opa variants did not bind any N-domain construct (data not shown). Results represent the mean of two to four independent experiments. ++, >50% binding; +, 20–50% binding; +/-, 5–20% binding; --, <5% binding.

Expression and Recognition of Full-length CD66b Mutants on the Surface of Epithelial Cells. To confirm that these results obtained with soluble recombinant His-tagged N-domains reflect interactions taking place at the cell surface, we introduced the identified key residues into full-length CD66b cDNA, transfected CHO cells with these constructs, and performed infection assays to determine Opa receptor activity. All transfectants stained positive with mAb Kat4C, which recognizes an epitope in internal CD66 domains (26; data not shown), as well as with a polyclonal anti-CD66 serum (Fig. 3), indicating that the receptors were expressed at the surface of the cells.

Infection of the various transfectants with MS11 Opa variants was evaluated by double immunofluorescence staining. Bacteria were found only on receptor-positive cells, which comprised 50–75% of the total cell population. The results of these experiments are shown in Table IV, with Fig. 3 illustrating the designations used in Table IV. Surprisingly, a single mutation in CD66b (N32S) resulted in significant binding of OpaB (Fig. 3 c) and OpaC variants.
Table III. Binding of CD66b N-domain mutants by MS11 Opa variants

<table>
<thead>
<tr>
<th>Mutations introduced in the N-domain of CD66b</th>
<th>Binding by Opa variants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OpaB</td>
</tr>
<tr>
<td>None</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>R29F+N32S</td>
</tr>
<tr>
<td>14</td>
<td>D27H+P28L+R29F+N32S</td>
</tr>
<tr>
<td>15</td>
<td>A41G+R44Q</td>
</tr>
<tr>
<td>16</td>
<td>D27H+P28L+R29F+N32S+A41G</td>
</tr>
<tr>
<td>17</td>
<td>D27H+P28L+R29F+N32S+R44Q</td>
</tr>
<tr>
<td>18</td>
<td>D27H+P28L+R29F+N32S+A41G+R44Q</td>
</tr>
<tr>
<td>19</td>
<td>R29F+N32S+A41G+R44Q</td>
</tr>
<tr>
<td>20</td>
<td>P28L+R29F+N32S+A41G+R44Q</td>
</tr>
<tr>
<td>21</td>
<td>D27H</td>
</tr>
</tbody>
</table>

MS11 Opa variants were incubated with E. coli lysates containing the indicated mutant (mut) CD66b N-domain and processed for immunoblotting. Binding was calculated by determining the signal generated by the amount of His-tagged N-domain present for each variant, compared with the signal generated in a lane where an amount of His-tagged N-domain was loaded representing 100% binding. Opa: variants did not bind any N-domain construct (data not shown). Results represent the mean of two to four independent experiments. ++, >50% binding; +, 20–50% binding; +/–, 5–20% binding; –, <5% binding.
receptor mutants a difference in binding characteristics was noted for variants expressing OpaC compared with OpaB and OpaI variants.

**Discussion**

In this study, we have mapped residues on CD66 receptors that determine Opa protein binding. We have identified three key amino acid residues in CD66e required for maximal binding using homologue scanning mutagenesis and subsequent analysis of both loss-of-function and gain-of-function of CD66 mutants in binding and infection assays. Furthermore, Opa proteins B, C, and I, which bind to an identical spectrum of native CD66 receptors, were found to differ in their recognition of mutant CD66 N-domains and mutant receptors on cells, indicating that their binding characteristics may actually not be identical.

Neisserial Opa proteins are thought to span the bacterial outer membrane in an eight-stranded β-barrel conformation resulting in four extracellular loops. Three of the exposed loops consist of variable sequence domains while a fourth loop near the COOH terminus of the protein is highly conserved. The differential binding of Opa proteins to CD66 receptors is likely a reflection of the ability of these variable domains to interact with the receptors. As we pointed out previously (10), it is remarkable that Opa proteins, such as OpaB, OpaC, and OpaI, that contain heterogeneous variable domains recognize the same subgroup of CD66 family members. However, our present data showing differences in binding of mutant molecules by these Opa proteins may indicate that they actually bind with slightly different characteristics, which would be expected from proteins with such divergent binding domain sequences.

The opa gene family is thought to have arisen from recent gene duplication events and genetic reassortment of variable sequence domains between members of the gene family (25, 27–29). Interestingly, the CD66 family also appears to have arisen recently by duplication of at least one ancestral gene. Sequence comparisons between the rodent and primate CD66 gene families show higher interspecies than intraspecies variation, suggesting that the duplication occurred after mammalian radiation took place (30, 31). In addition, the mutation rate in the CD66 N-domain exons is twice as high as that of the adjacent intron, suggesting...
homology scanning mutagenesis does not address the role of conserved residues among the two homologous proteins in ligand binding. The finding that single, relatively conservative mutations in CD66b, such as N32S and A41G, were sufficient to induce receptor function in CD66b may suggest that CD66b contains conserved residues participating in Opa protein binding. To test whether the actual binding site is comprised in the first 59 residues of CD66e, we constructed a truncated, His-tagged CD66e molecule (residues 1–59) and tested it for binding to Opa variants. This molecule was well expressed by E. coli but failed to bind significantly to any Opa variant (data not shown), indicating that other sites within the N-domain may be required for binding or that the peptide did not adopt the correct conformation for binding activity. The finding that single mutations in CD66b result in functional receptor activity could suggest that in vivo isoforms of CD66b exist that will be recognized by Opa variants. Evidence to support this concept comes from analysis of CD66b cDNAs cloned from normal white blood cells and leukemic cells. These cDNAs differ in two base pairs in the coding region, resulting in two amino acid differences, one in the N-domain (R80K) and one in the COOH-terminal M-domain (V288L) (37, 38). Cloning of another CD66 family member, CD66d, by two different groups resulted in proteins differing in two residues (39, 40). These phenomena fit with the notion that CD66 molecules are subject to sequence variation associated with rapid evolution.

CD66 family members are capable of mediating homophilic and heterophilic intercellular adhesion, like many other IgSF proteins (41). Binding between CAMs is usually of very low affinity, but due to the highly multimeric nature of cell–cell adhesion, sufficient avidity can be achieved to allow detection of the interaction between cells. The weakness of CAM interactions is illustrated by the difficulties in detecting binding of purified, monomeric forms of CAMs. This difficulty arises because binding assays require separation and washing steps, during which time weakly interacting molecules dissociate (42). This phenomenon may explain our observation that binding of Opa variants to soluble CD66b N-domain mutants required more CD66e-derived residues than binding to cell surface CD66 receptors. If each mutation introduced into CD66b enhances binding affinity between Opa and CD66b, as suggested by our infection assay data, then the threshold level of affinity necessary for detection will be reached sooner for the infection assay than for binding in solution. Alternatively, the level of multimerization may be important for Opa-CD66 binding, as has been shown for the binding of IgSF member intercellular adhesion molecule 1 (ICAM-1) to its receptor LFA-1. Recombinant ICAM-1 exists as monomers in so-

### Table IV. Recognition by Opa variants of mutant full-length CD66b receptors expressed by CHO cells

<table>
<thead>
<tr>
<th>Transfectant</th>
<th>Recognition by Opa variants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OpaA</td>
</tr>
<tr>
<td>CD66b</td>
<td>–</td>
</tr>
<tr>
<td>R 29F</td>
<td>+/+</td>
</tr>
<tr>
<td>N 32S</td>
<td>+</td>
</tr>
<tr>
<td>A41G</td>
<td>+/+</td>
</tr>
<tr>
<td>R 44Q</td>
<td>–</td>
</tr>
<tr>
<td>N 32S + R 29F</td>
<td>+</td>
</tr>
<tr>
<td>N 32S + A 41G</td>
<td>+</td>
</tr>
<tr>
<td>N 32S + R 44Q</td>
<td>+</td>
</tr>
<tr>
<td>R 29F+N 32S + A 41G</td>
<td>+/+ fp</td>
</tr>
<tr>
<td>CD66e/b</td>
<td>+/+ fp</td>
</tr>
<tr>
<td>CD66e</td>
<td>+/+ fp</td>
</tr>
</tbody>
</table>

Interaction with Opa variants was determined by evaluating the percentage of receptor-positive cells that had significant amounts of associated gonococci (≥5 bacteria per cell). Presence of receptor and bacteria was determined by immunofluorescence microscopy. The first column indicates the mutations made in CD66b; CD66e/b and CD66e indicate that the entire CD66b N-domain was replaced with either the chimeric N-domain mentioned in Table I or the entire native CD66e N-domain. The results were tabulated as follows: +/+ fp, all receptor-positive cells were infected plus numerous footprints were present; +, 60–90% of receptor-positive cells were infected; +/−, 30–60% of receptor-positive cells were infected; +/−, 10–30% of receptor-positive cells were infected; −, no infected cells present. Infection experiments with recombinant M511 Opa variants (reference 5) resulted in identical recognition patterns as those shown here for wild-type M511 Opa variants. Results represent the mean of two to four independent experiments.

that the CD66 family is still undergoing rapid evolution (30). It is tempting to speculate that the extensive evolution of the Opa gene family in the strictly human pathogen N. gonorrhoeae has taken place in response to the rapidly evolving family of primate-specific CD66 molecules. The observed differential Opa-CD66 binding patterns may be a reflection of this process.

According to the predicted structure of the CD66e N-domain (32), the residues we have identified as important for Opa protein binding, F29, S32, and G41, are located in exposed loops and strands of the GFCC face of the CD66e N-domain (Fig. 1 B). This face of the molecule is not covered by carbohydrate, in contrast to the ABED face, as predicted by a low resolution model for CD66e (34), and would therefore be accessible for protein–protein interactions. The key role of S32 and G41 in binding of Opa proteins is supported by the fact that these residues are conserved among Opa-binding CD66 molecules (Fig. 1 A). Residue F29 is conserved in three out of four of the Opa-binding CD66 molecules (CD66a, d, and e), whereas CD66c contains an I at residue 29. Possibly hydrophobic residues such as F or I at that position support Opa protein binding, while a charged residue, such as R present in CD66b, does not. The role of residues L28 and Q44 is probably minor, since they are not required for Opa recognition of the native molecule, although their presence enhances binding of the soluble N-domain. Several ligand and viral binding sites on IgSF members have been found on the GFCC face of the ligand-binding domain (35, 36), indicating that this domain face is positioned favorably to serve as a ligand-binding platform for IgSF members.

Homologue scanning mutagenesis does not address the role of conserved residues among the two homologous proteins in ligand binding. The finding that single, relatively conservative mutations in CD66b, such as N32S and A41G, were sufficient to induce receptor function in CD66b may suggest that CD66b contains conserved residues participating in Opa protein binding. To test whether the actual binding site is comprised in the first 59 residues of CD66e, we constructed a truncated, His-tagged CD66e molecule (residues 1–59) and tested it for binding to Opa variants. This molecule was well expressed by E. coli but failed to bind significantly to any Opa variant (data not shown), indicating that other sites within the N-domain may be required for binding or that the peptide did not adopt the correct conformation for binding activity. The finding that single mutations in CD66b result in functional receptor activity could suggest that in vivo isoforms of CD66b exist that will be recognized by Opa variants. Evidence to support this concept comes from analysis of CD66b cDNAs cloned from normal white blood cells and leukemic cells. These cDNAs differ in two base pairs in the coding region, resulting in two amino acid differences, one in the N-domain (R80K) and one in the COOH-terminal M-domain (V288L) (37, 38). Cloning of another CD66 family member, CD66d, by two different groups resulted in proteins differing in two residues (39, 40). These phenomena fit with the notion that CD66 molecules are subject to sequence variation associated with rapid evolution.

CD66 family members are capable of mediating homophilic and heterophilic intercellular adhesion, like many other IgSF proteins (41). Binding between CAMs is usually of very low affinity, but due to the highly multimeric nature of cell–cell adhesion, sufficient avidity can be achieved to allow detection of the interaction between cells. The weakness of CAM interactions is illustrated by the difficulties in detecting binding of purified, monomeric forms of CAMs. This difficulty arises because binding assays require separation and washing steps, during which time weakly interacting molecules dissociate (42). This phenomenon may explain our observation that binding of Opa variants to soluble CD66b N-domain mutants required more CD66e-derived residues than binding to cell surface CD66 receptors. If each mutation introduced into CD66b enhances binding affinity between Opa and CD66b, as suggested by our infection assay data, then the threshold level of affinity necessary for detection will be reached sooner for the infection assay than for binding in solution. Alternatively, the level of multimerization may be important for Opa-CD66 binding, as has been shown for the binding of IgSF member intercellular adhesion molecule 1 (ICAM-1) to its receptor LFA-1. Recombinant ICAM-1 exists as monomers in so-

Downloaded from on April 9, 2017
lution, and direct binding to LFA-1 has been impossible to
detect. Only when ICAM-1 was modified to induce
dimerization could LFA-1 binding be detected (43, 44). 
CD66 family members can exist as dimers in the plasma
membrane of eukaryotic cells (45), and recombinant CD66e
N-domains have been shown to form oligomers in solution
(46). Receptor dimers will more likely be found on the
surface of cells, where the receptor concentration is higher
than in solution. Thus, if Opa binding requires a receptor
dimer, one would expect Opa variants to bind more readily
to receptor-expressing cells than to soluble receptors. An-
other possible explanation for the discrepancy between the
two binding detection methods is that in the solution assay
the receptor binding domain exists as an unglycosylated,
single domain, while in the infection assay the binding do-
mend is presented in the context of a complete, glycosylated
molecule. Although Opa binding does not require carbo-
hydrate (18), the presence of sugar moieties may influence
the strength of adhesion, as has been observed for the inter-
action between CD2 and CD58. Human CD2 has a single
carbohydrate addition site in its Ig variable–like N-domain
that is absolutely required for binding to its normal ligand,
CD58. Evidence from solution structure of this carbohy-
drate chain in relation to the GFC binding face indicates
that the glycans is not itself situated in the binding face but is
required to balance an unfavorable negative charge in order
to maintain an active binding configuration (47). In fact,
CD66 N-domains contain a potential glycosylation site at
residue 70 (Fig. 1), which corresponds exactly to the struc-
tural position of the glycosylation site affecting ligand bind-
ing ability of human CD2 (41). Mutation of this site in
CD66e influences CD66e homophilic interactions, which
are based on protein–protein interactions, indicating that in
CD66e also the degree of glycosylation can influence bind-
ing events mediated through CD66e protein sequences (41).
Our data stress the importance of evaluating binding events
in different assays, since differences between assays can re-
veal further details of the molecular interaction between
ligands. Regardless of the molecular basis for the observed
discrepancy, both assays show clearly that OpaC binding
requires more CD66e-derived residues than OpaB or
OpaC, which may indicate that OpaC binding of the recep-
tor is of lower affinity than binding of OpaB or OpaC.

In conclusion, mapping of key residues in CD66 required
for recognition by the various gonococcal Opa adhesins indi-
cates that single amino acid residues in CD66 receptors
determine Opa protein binding. These results may provide
a first step towards resolving the structural requirements
for the Opa-CD66 receptor interaction and thereby help the
development of infection inhibitory strategies, and may
provide insights into the function of CD66 molecules in
normal tissue and in carcinogenesis.

We thank Drs. Jos van Putten, Byron Caughey, and Jim Fox for helpful comments on the manuscript; Jim
Fox for help with the PredictProtein program; and Gary H etrick for assistance in preparation of the figures.

Address correspondence to Martine P. Bos, Rocky Mountain Laboratories, NIH, NIAID, 903 South 4th
St., Hamilton, MT 59840. Phone: 406-363-9301; Fax: 406-363-9204; E-mail: Martine_Bos@nih.gov

Submitted: 25 February 1999 Revised: 20 May 1999 Accepted: 8 June 1999

References

of mucosal epithelial cells by Neisseria gonorrhoeae. Rev.
3. Murphy, G.L., T.D. Connell, D.S. Barritt, M. Koomey, and
J.G. Cannon. 1989. Phase variation of gonococcal protein II:
regulation of gene expression by slipped-strand mispairing of
a repetitive DNA sequence. Cell. 56:539–547.
4. Malorny, B., G. Morelli, B. Kusecek, J. Kolberg, and M.
Achtman. 1998. Sequence diversity, predicted two-dimen-
sional protein structure, and epitope mapping of Neisserieal
Meyer. 1993. Variable opacity (Opa) outer membrane pro-
teins account for the cell tropisms displayed by Neisseria
gonorrhoeae for human leucocytes and epithelial cells. EMBO J.
can-like cell surface proteoglycan receptors is required for
Neisseria gonorrhoeae entry into human mucosal cells. EMBO
7. Chen, T., R.J. Belland, J. Wilson, and J. Swanson. 1995. Ad-
herence of pilus–Opa+ gonococci to epithelial cells in vitro
8. Chen, T., and E.C. Gotschlich. 1996. CGM 1a antigen of
neutrophils, a receptor of gonococcal opacity proteins. Proc.
1996. Carcoemboembrionic antigens (CD66) on epithelial cells
and neutrophils are receptors for Opa proteins of pathogenic
recognition of members of the carcinoembryonic antigen
family by Opa variants of Neisseria gonorrhoeae. Infect.
receptors influence tissue interactions and cellular response to
Carcinoembrionic antigen family: molecular biology and


