Molecular analysis of neisserial Opa protein interactions with the CEA family of receptors: identification of determinants contributing to the differential specificities of binding

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Summary

The carcinoembryonic antigen (CEA) gene family members, CEACAM1, CEACAM3, CEACAM5 and CEACAM6, are bound by the Opa outer membrane proteins of pathogenic Neisseria spp., whereas CEACAM8 is not. In this study, we demonstrate that the closely related CEACAM4 and CEACAM7, which are also members of the CEA family, are not Opa receptors. We exploited the high conservation between CEACAM6 and CEACAM8 to generate an extensive set of chimeric receptors in order to delineate the sequences necessary for Opa binding. Using a transfection-based infection system, we showed that binding of Opa52 involves residues 27–42, which are predicted to form β-strand C and short loops adjacent to it, and residues lying between amino acids 60 and 108 in the amino-terminal domain. The replacement of residues 27–29 in CEACAM6 with the CEACAM1 or CEACAM5 sequences generated recombinant CEACAM6 receptors that are bound by CEACAM1/CEACAM5-specific Opa variants. Together, our data demonstrate that Opa proteins bind to residues exposed on the GFCC face of the N-terminal domain of CEACAM receptors, and identify an amino acid triplet sequence that is responsible for the differential binding of Opa proteins to CEACAM1, CEACAM5 and CEACAM6.

Introduction

Primary attachment of the human pathogen Neisseria gonorrhoeae to mucosal epithelia is mediated by the pilus (McGee et al., 1983; Rudel et al., 1992). The colony opacity-associated (James and Swanson, 1978) Opa outer membrane adhesins then mediate a more intimate attachment, which may ultimately result in neisserial penetration into subepithelial layers (McGee et al., 1983; Wang et al., 1998). The hallmark of gonorrhoea is an elaboration of pus, composed mostly of polymorphonuclear neutrophils (PMNs) containing intra- and extracellular gonococci (Handsfield, 1990). This interaction also appears to be mediated by Opa proteins, as certain variants mediate the enhanced binding, opsonin-independent phagocytosis and induction of respiratory burst by these cells (Virji and Heckels, 1986; Fischer and Rest, 1988).

Individual gonococcal strains have been shown to encode up to 11 highly related, but antigenically distinct, Opa variants (Bhat et al., 1991; Kupsch et al., 1993). Each allele is constitutively transcribed, but their expression is phase variable as a result of RecA-independent changes in the number of pentanucleotide coding repeat units in the leader sequence (Stern et al., 1986; Murphy et al., 1989). This variation continually maintains a heterogeneous population of bacteria, which express no, one or multiple Opa proteins. Certain Opa variants (e.g. Opa50) have been shown to bind cell surface heparan sulphate proteoglycan (HSPG) receptors (Chen et al., 1995; van Putten and Paul, 1995). This binding can itself lead to bacterial engulfment by many epithelial cell lines in vitro (Weel et al., 1991; Kupsch et al., 1993). However, the invasion is greatly facilitated by an additional binding event of Opa50 to extracellular matrix proteins, such as vitronectin and fibronectin, in other cell lines (Duensing and van Putten, 1997; Gomez-Duarte et al., 1997; Dehio, M. et al., 1998; van Putten et al., 1998). Most neisserial Opa variants have been shown to bind to members of the carcinoembryonic antigen (CEA) gene family, which
are differentially expressed on various epithelial, endothelial and haematopoietic cells in vivo (Berling et al., 1990; Thompson et al., 1991; Prall et al., 1996). CEACAM1 (CD66a; biliary glycoprotein, BGP), CEACAM3 (CD66d; CEACAM gene family member 1, CGM1), CEACAM4 (CD66e; carcinoembryonic antigen, CEA), CEACAM5 (CD66c; non-specific cross-reacting antigen, NCA) and CEACAM6 (CD66b; CGM2) can be bound by distinct neisserial Opa variants, while CEACAM8 (CD66b; CGM6) is not recognized by any Opa variant tested to date (Chen and Gotschlich, 1996; Virji et al., 1996a; Bos et al., 1997; Gray-Owen et al., 1997a).

In addition to the CD66 subset, the CEA family also includes the membrane proteins CEACAM7 (CGM2) and CEACAM4 (CGM7), and the soluble serum pregnancy-specific glycoproteins (PSG; Kuroki et al., 1991; Thompson et al., 1991; Beauchemin et al., 1999). Whether these closely related proteins can also function as receptors for the neisserial Opa proteins is still unknown. The CEA gene family belongs to the immunoglobulin (Ig) superfamily (Obrink, 1997). Each CEA family member

Fig. 1. Structure and N-terminal amino acid sequences of cell surface-bound CEA family members.
A. Schematic drawing of CEACAM1–CEACAM8 (Beauchemin et al., 1999) illustrating domain structure and membrane anchorage (according to http://www.cea.uni-freiburg.de). N, amino-terminal IgV-like domain; A/B, IgC2-like domains, AB subsets; †, Asn-linked glycosylation site; - or ¯ indicates binding or no binding by Opa-expressing bacteria.
B. Proposed secondary structural elements of the N-terminal domain of CEACAM5 (Bates et al., 1992) and sequence alignment of the N-terminal domain amino acid sequences of each CEA family member used in our experiments. The single letter amino acid code is used. Bars indicate β-strands; lines represent loops between β-strands. Conserved residues are indicated by a period (;) each tenth residues is marked with a dot (•) above the sequences. Asn-linked glycosylation sites are underlined. Code refers to the predicted orientation of the amino acid residue in the protein according to Bates et al. (1992): +, exposed; −, buried; , semi-buried 60–90%; s, a Pro or Gly residue, residues essential for fold stability; c, predicted to be covered by carbohydrates; i, interface region between domains; n, Asn-linked glycosylation site. Accession numbers: CEACAM1, D12502; CEACAM3, D90277; CEACAM4, D90276; CEACAM5, M17303; CEACAM6, M29541; CEACAM7, X98311; CEACAM8, X52378.
consists of an amino (N)-terminal immunoglobulin variable (IgV)-like domain followed by a different number of IgC2 constant-like domains (Oikawa et al., 1987; Zimmermann et al., 1987; Thompson et al., 1991). CEACAM1, CEACAM3 and CEACAM4 are inserted into the cellular membrane via a carboxy-terminal transmembrane and cytoplasmic domain, while CEACAM5, CEACAM6, CEACAM7 and CEACAM8 instead possess a glycosylphosphatidylinositol (GPI) anchor (Fig. 1A). Each CEA family member can be up to 50% carbohydrate by weight (Oikawa et al., 1987; Paxton et al., 1987; Hefta et al., 1990). However, the cell level of glycosylation may vary depending upon cell type and differentiation state, and multiple glycoforms of the same protein have even been isolated from a single colonic tumour metastasis (Hefta et al., 1990). In clear contrast to HSPG binding by Opa<sub><i>s</i></sub> (Chen et al., 1995; van Putten and Paul, 1995), Bos et al. (1998) have recently demonstrated that carbohydrate structures are not involved in Opa binding to the CEACAM receptors. The fact that CEACAM3 lacks any IgC2-like domains (Nagel et al., 1993; Fig. 1A) indicates that Opa-binding determinants must lie within the highly conserved N-terminal domain. Soluble CEACAM1 and CEACAM5 chimeras that contain only the N-terminal domain have also confirmed that this region is responsible for binding of the Opa proteins to these receptors (Bos et al., 1998; Virji et al., 1996b).

The inability of Opa proteins to bind CEACAM8, despite its close sequence and structural relationship to the other CEACAM receptors, was the basis for our approach to delineate further the binding determinants that are present in the CEACAM protein sequence. By exchanging reciprocal parts between CEACAM6 and CEACAM8 sequences, we generated an extensive set of chimeric receptors, which were tested for Opa protein binding on transiently and stably transfected cell lines. This strategy allowed us to show that Opa proteins bind to residues exposed on the protein face that consists of β-strands C (βC), β′C, βF and βG from the N-terminal domain of CEACAM receptors.

Results

Neisserial Opa proteins do not bind to non-CD66 receptors of the CEA family

Previously, several groups have studied the binding of neisserial Opa proteins to the members of the CD66 subset of the CEA receptor family (Bos et al., 1997; Chen et al., 1997; Gray-Owen et al., 1997b). Although the CEA family members CEACAM4 and CEACAM7 are not recognized by CD66-specific antibodies, they do possess a similar domain structure and significant sequence homologies in common with molecules of the CD66 subset of the CEA family (Fig. 1A and B). We were therefore interested to ascertain whether CEACAM4 and CEACAM7 were also bound by the gonococcal Opa proteins. For this purpose, stably transfected CHO cell lines expressing either CEACAM4 or CEACAM7 were used. The cell line CHO-CEACAM1 was used as a positive control in infection experiments, while untransfected CHO-K1 cells, which do not express any CEA family members, were used as a negative control. Each cell line was infected with the 11 non-piliated recombinant gonococci strains N303–N313, each of which stably expresses a recombinant form of one of the 11 different Opa alleles of <i>N. gonorhoeae</i> MS11. In addition, the non-opaque parental strain N302 was used as a negative control. As these strains can also potentially express Opa proteins from the chromosomal loci, the Opa expression pattern of each strain was analysed by immunoblot analysis to confirm the expression of a single Opa variant (data not shown).

The expression of CEACAM4 and CEACAM7 did not effect the association of any Opa-expressing gonococcal strain with these cells, while CEACAM1 expression resulted in a 100-fold increase in bacterial binding compared with the untransfected controls. Figure 2 is a representative result showing the association of gonococcal strain N309 (Opa<sub><i>s</i></sub>) with the parental and recombinant CHOK1 cell lines.

Residues in the predicted β-strand C (amino acids 31–36) are involved in CEACAM binding by the neisserial Opa proteins

Opa protein binding to CEACAM1, CEACAM3, CEACAM5 and CEACAM6, but not to the closely related molecules CEACAM4, CEACAM7 and CEACAM8 (Fig. 1A), suggests that a highly specific interaction exists. The fact that Opa proteins recognize CEACAM3 indicates that all elements necessary for binding are present in the conserved N-terminal domain of CEACAM molecules. This premise has also been confirmed by binding experiments performed using soluble recombinant CEACAM1 and CEACAM5 N-domains (Virji et al., 1996b; Bos et al., 1998). CEACAM8 is not recognized by any Opa protein tested to date, despite the fact that it shares the same domain organization and has about 70% sequence identity with the other CEACAM receptors (i.e. 78 out of 108 amino acids in the N-terminal domain are identical in CEACAM6 and CEACAM8; Fig. 1A and B). In order to determine whether the binding domain could be delineated further by creating chimeras between CEACAM6 and CEACAM8, we first introduced a junction that allowed us to switch the N-terminal 59 amino acids between these two proteins (chimeras χA and χB; Fig. 3). Bacterial adherence assays performed with transfected cell lines expressing these constructs clearly indicated that <i>N. gonorhoeae</i>
N309 (Opa_{52}) binding correlated with the presence of the first 59 amino acids of CEACAM6 (Fig. 3). This result is consistent with the work of Bos et al. (1998), who recently showed a similar result using soluble CEACAM5/CEACAM8 chimeras. We generated a subsequent set of chimeras that further subdivided the region between amino acids 1 and 59, and fusions at amino acids 15/16 (chimeras \( \chi C_1, \chi C_2, \chi D_1, \chi D_2 \)), 30/31 (chimeras \( \chi E_1, \chi E_2, \chi F_1, \chi F_2 \)) and the conserved residue 42 (chimeras \( \chi G_1, \chi G_2, \chi H_1, \chi H_2 \)) were constructed (Fig. 3). Initially, we had planned to generate stably transfected cell lines expressing each chimeric construct for use in the bacterial infection assays. However, we were unable to generate stably transfected cell lines expressing some of the chimeric constructs despite repeated attempts. We therefore assessed the utility of using transiently transfected cells in infection assays instead. In each case in which constructs could be tested in both stable and transient transfection assay systems, the results were consistent (i.e. compare Figs 3 and 4), and transient transfection assays were therefore used to test all subsequent chimeric constructs. Strong bacterial binding correlated with the presence of residues 31–41 from CEACAM6 (i.e. compare binding to chimeras \( \chi C_1, \chi C_2, \chi E_1, \chi H_1, \chi H_2 \) with non-binding to chimeras \( \chi D_1, \chi D_2, \chi F_1, \chi F_2, \chi G_1, \chi G_2 \)), implying that this region is directly involved in binding by the Opa proteins.

Owing to the conservation between CEACAM6 and CEACAM8, chimera pairs \( \chi F_1/\chi H_1 \) and \( \chi E_1/\chi G_1 \) only differ by three residues (amino acids 32, 38 and 41; Fig. 3). Chimera pairs \( \chi I_1/\chi K_1 \) and \( \chi I_2/\chi K_2 \), which fuse CEACAM6 and CEACAM8 between residues 33 and 34, were therefore generated in order to define further the residues involved in binding. Remarkably, the single-residue (i.e. Asn-32→Ser) difference between chimeras \( \chi F_1 \) and \( \chi K_1 \) and between chimeras \( \chi F_2 \) and \( \chi K_2 \), respectively, is sufficient to restore full binding function upon the chimeric receptor (Fig. 3). The presence of residues 38 and 41 from CEACAM6 also facilitates some weaker binding, as demonstrated by comparing \( \chi G_1 \) and \( \chi I_1 \). Ser-32 thus appears to be of critical importance for bacterial binding to these constructs, while the others are of secondary importance. Site-directed mutagenesis to generate a Ser-32→Asn exchange in CEACAM6 (chimera \( \chi L \)) is, however, not sufficient to abrogate bacterial binding, and an Asn-32→Ser exchange in CEACAM8 does not result in CEACAM8 being recognized by Opa_{52} (chimera \( \chi M \); Fig. 3). Ser-32 is therefore not the only residue involved in binding Opa proteins, but rather lies within a context of residues that together form the binding site. According to the model for CEACAM5 (CEA) proposed by Bates et al. (1992), Ser-32 lies within predicted \( \beta \)C. The conclusion that this region is involved in binding is consistent with the fact that each CEACAM receptor and chimeric construct that is strongly bound by Opa_{52} has a conserved \( \beta \)C with a Ser at position 32 (Figs 1B and 3).

**Sequences outside of \( \beta \)-strand C contribute to Opa protein interactions with the CEACAM receptors**

The fact that \( \chi E_2 \), which has \( \beta \)C with Ser-32, is not recognized by Opa_{52} indicates that the binding site does not only consist of this element. Additional determinants that contribute either directly or indirectly to the binding process must therefore exist. Bacterial binding to \( \chi E_1 \), but not to \( \chi E_2 \), indicates that sequences C-terminal to residue 59 must influence Opa binding (Fig. 3). These determinants are of secondary importance when certain residues between amino acids 1 and 59 are derived from CEACAM6 (e.g. chimeras \( \chi A, \chi C_2, \chi H_1, \chi K_1 \)). However, when these residues are derived instead from CEACAM8, the downstream sequence between residues 60 and 108 becomes important (e.g. compare \( \chi E_1/\chi E_2 \) and \( \chi I_1/\chi I_2 \); Fig. 3). This may result from the fact that amino acids 85–108 are proposed to form \( \beta \)F and \( \beta \)G, which lie directly adjacent to \( \beta \)C, together forming with \( \beta \)C’ the GFCC’ \( \beta \)-sheet of the N-terminal domain (Bates et al., 1992; Fig. 6). Therefore, \( \beta \)F and \( \beta \)G could contribute to the structural integrity of the binding site. Alternatively, parts of the binding site may actually exist on \( \beta \)F and \( \beta \)G, as their surface-exposed residues should lie in direct proximity to those of \( \beta \)C (e.g. Ser-32) and \( \beta \)C’ and of the C–C’ loop (Bates et al., 1992; Fig. 6).
Reduced bacterial binding was evident when the first 15 residues of CEACAM8 were used to replace the CEACAM6 sequence (x1C1), but the reciprocal construct (x1D1) did not result in CEACAM8 gaining any binding function. As these residues are predicted to be on a protein surface covered by carbohydrates (Bates et al., 1992), this change may have affected bacterial binding indirectly by altering the protein's folded conformation. Consistent with this, the effect does not occur when either residues 16–30 (x1E1) or residues 60–108 (x1C2) are also derived from CEACAM8, as neisserial binding to chimeras x1E1 and x1C2 was indistinguishable from its binding to the native CEACAM6 (Fig. 3). Bacterial binding to x1C2, but not to x1E2, indicates that between residues 16 and 30 lies a structure that is important for Opa52 binding. The simple exchange of residue 32 between CEACAM6 and CEACAM8 had no effect on Opa52 binding (chimeras x1L and x1M). Instead, we therefore exchanged residue 32 together with the variable residues 27–29 between x1A and x1B to generate chimeras x1N2 and x1O2 (Fig. 3). Bacterial binding to cells expressing x1O2 but not to cells expressing x1N2 clearly demonstrates the importance of this sequence to Opa interactions. When residues 27–32 were exchanged instead between the parental CEACAM6 and CEACAM8 receptors, some binding to both chimeras was seen (i.e. compare binding to x1N1/x1O1 with that to x1N2/x1O2). An exchange of these four residues thus converts CEACAM8 to a receptor that mediates binding by Opa52-expressing gonococci, but a corresponding exchange in CEACAM6 is not sufficient to abrogate binding. In order to determine

Fig. 3. Opa-mediated binding to CEACAM6, CEACAM8 and chimeric receptor proteins. Alignment of the first 59 amino acids of CEACAM5, CEACAM6 and CEACAM8 N-terminal domain amino acid sequences in single-letter code, along with a schematic representation of the predicted secondary structural elements for CEACAM5 (Bates et al., 1992). Letters within the rectangles represent β-strands, Trip refers to amino acid residues 27–29. Identical residues are indicated by a period (.), and a dot (·) marks every tenth residue above the sequence. Residues 60–108 of the N-terminal domain are indicated schematically, but are not drawn to scale. Code refers to the predicted orientation of the amino acid residue in the native protein, with abbreviations as outlined in the legend to Fig. 1B. The three changes in the protein sequence that we found in our CEACAM6 and CEACAM8 clones with regard to previously published data (Arakawa et al., 1990; Berling et al., 1990; Neumaier et al., 1998) are introduced (CEACAM6, position 48; CEACAM8, positions 8 and 80; see Experimental procedures). Schematic diagrams representing generated CEACAM receptor constructs. Dark grey fill indicates sequences derived from CEACAM6; bright grey fill indicates regions derived from CEACAM8. IgC2-like domains and membrane anchors (see Fig. 1A) for each construct were derived from CEACAM6 or CEACAM8 according to the region between amino acids 60 and 108. Constructs in the left and right columns share the same residues between amino acids 1 and 59 but are different in their amino acid sequences C-terminal to amino acid residue 59. The binding phenotype of recombinant N. gonorrhoeae N309 (Opa52) to cells transiently transfected with each construct is indicated on the right side of the chimeras: ++, strong binding to cells irrespective of receptor expression level; +, binding only to strongly expressing cells; −, no binding. Binding assays were performed in duplicate on at least three separate occasions.
whether exchanging the variable triplet of residues (amino acids 27–29) alone between c2 and c2 was sufficient to influence binding, p and q were produced (fig. 3). d2 was chosen as the basis for this exchange, as it is one of the receptors that contain the most ceacam6-derived sequence, while still not being bound by opa52. conversely, c2 is the receptor that possesses the most ceacam8-derived sequence, while still binding opa52-expressing gonococci to a level that is indistinguishable from that of ceacam6. as a control, residues 27–29 together with residue 32 were exchanged in c2 and d2, resulting in r and s. opa52-mediated binding to chimera p is reduced compared with binding with c2, but was completely abrogated in r, and no binding was seen to cells expressing chimera q, whereas s permits strong binding of opa52-expressing gonococci. together, these results indicate that residues 27–29 contribute to, but are not sufficient to mediate, opa52 binding.

residues 27–29 are responsible for the binding specificities of ceacam1- and ceacam5-specific opa proteins

individual opa protein variants differ in their ability to bind to the ceacam receptors. for example, opa52, opa57, opa58 and opa60 bind to ceacam1, ceacam3, ceacam5 and ceacam6, while binding of opa51, opa53, opa54, opa55, opa56 and opa59 was shown to be restricted to ceacam1 and/or ceacam5 (bos et al., 1997; 1998; chen et al., 1997; gray- Owen et al., 1997b). as βc and the c–c’ loop are completely conserved between ceacam1, ceacam3, ceacam5 and ceacam6, and as both ceacam1 and ceacam6 diverge by only one residue between amino acids 60 and 108 (fig. 1b), sequence differences outside of these regions must be responsible for the different opa specificity. residues 27–29 are the most divergent part of the binding site between ceacam1 (qlf), ceacam5 (hlf) and ceacam6 (nri; fig. 1b). our finding that these residues play a role in opa binding prompted us to replace the ceacam6 triplet sequence with the corresponding sequences from ceacam1 and ceacam5 to generate chimeras t and u respectively (fig. 5). although the binding of opa52-expressing gonococci to stably transfected cells expressing either ceacam6, t or u was indistinguishable, the ceacam1- and ceacam5-specific opa51, opa54, opa55, opa56 and opa59 proteins bound only the chimeric proteins (i.e. t and u; fig. 5). this indicates that the insertion of residues 27–29 from ceacam1 or ceacam5 into ceacam6 is sufficient to allow the latter receptor to be recognized by ceacam1/ceacam5-specific opa proteins. although the amount of opa53 expressed by strain n304 was indistinguishable from the amount of opa expressed by the other strains, no binding of opa53 was seen to t and u. the observed specificity of opa53 for ceacam5 (gray- Owen et al., 1997b) must therefore be determined by other binding determinants.
Fig. 6. Model of the binding site of Opa52 on CEACAM6. The crystal structure of domain I of CD2 (Jones et al., 1992) was used as a model for the N-terminal domain of CEACAM6. Graphics were produced using the SETOR program (Evans, 1993) by replacing and displaying residues in the CD2 crystal structure that correspond to residues 27–29 and 32 of CEACAM6 with those present in CEACAM6. Regions predicted to comprise the binding site of Opa52 are coloured and labelled in order to demonstrate the spatial relationship of sequences that influence Opa binding in the folded protein structure. Opa52 binding involves residues 27–29 (red), βC and the C–C‘ loop (blue), with a crucial residue at position 32. The residues at positions 27–29 play an essential role in the differential binding of Opa51, Opa54, Opa55, Opa56 and Opa59 to CEACAM1, CEACAM5 and CEACAM6. βF, the F–G loop and βG (green; amino acids 85–108) lie in direct proximiy to the other parts of the binding site and are therefore most probably the region between amino acids 60 and 108, which is involved in Opa protein binding. βA, βB, βD and βE, which lie in the background are in grey and shaded. No binding was associated with this part of the molecule or to βG‘ in our experiments. See Discussion for a more detailed description of binding interactions.

Discussion

Ten out of the 11 different Opa variants of N. gonorrhoeae MS11 are capable of binding to the CEA family members, CEACAM1, CEACAM3, CEACAM5 and CEACAM6 (reviewed by Dehio, C et al., 1998). Owing to the tissue distribution of these receptors, the Opa–CEACAM interactions could potentially mediate neisserial binding to a wide variety of target tissues, including some epithelial, endothelial and phagocytic cells (Virji et al., 1996a; Bos et al., 1997; Chen et al., 1997; Gray-Owen et al., 1997a,b; Wang et al., 1998). The closely related proteins CEACAM4 and CEACAM7 are expressed on colorectal epithelia and leucocytes, respectively (Berling et al., 1990; Thompson et al., 1997), both of which are putative targets for gonococcal infection (Handsfield, 1990). Despite the fact that these proteins share significant homology to the CD66 subset of the CEA family, we demonstrate in this study that CEACAM4 and CEACAM7 are not bound by the Opa variants of N. gonorrhoeae MS11.

Clear evidence exists that the binding site of neisserial Opa proteins is present in the highly conserved N-terminal domain of the CEACAM molecules (Virji et al., 1996b; Bos et al., 1998). Bates et al. (1992) proposed a structural model of the N-terminal domain of CEACAM5 (CEA) based on the known X-ray and nuclear magnetic resonance (NMR) structures of the Ig superfamily members, human CD4, rat CD2, and the Bence Jones protein REI. According to this model, the N-terminal domain of CEACAM5 is predicted to be composed of eight β-strands (see Figs 1B and 6), which build up a stacked pair of two anti-parallel β-sheets with βA, βB, βD and βE forming one β-sheet and βC, βC‘, βF and βG forming the other. Owing to the high identities between CEACAM5 and CEACAM6 (89%, including all residues predicted to be essential for the stability of each fold) and CEACAM5 and CEACAM8 (70% identity; Fig. 1B), this structure of CEACAM5 should provide a good working model for the structure of other CEACAM receptors. By examining sequences reciprocally between CEACAM6 and CEACAM8, we were able to identify regions in the N-terminal domain that are necessary for binding by Opa52. The infection-based cellular system used to assess gonococcal binding to each chimera provided a relevant assay for detecting and quantifying bacterial adherence in the context of the cell surface. It also reduces the chance that misfolding or different post-translational processing by a heterologous system would influence Opa–CEACAM interactions. As Opa52 has the capacity to bind four of the CEACAM receptors, it is likely that contacts responsible for Opa52 binding to CEACAM6 are conserved among all Opa–CEACAM interactions.

Based upon the results presented here, the sequences that are predicted to form βC and the C–C‘ loop are crucial for Opa binding (Fig. 6). Consistent with this, all natural CEACAM molecules that are bound by Opa proteins are conserved over this region (i.e. residues 31–42; Fig. 1B), whereas all that are not bound are different. Similarly, avid binding to the CEACAM6/CEACAM8 chimeric constructs generated in this study generally correlated with the presence of Ser-32 from CEACAM6. It is likely that adjacent surface-exposed residues that are conserved between CEACAM6 and CEACAM8 also contribute to binding. However, these would not be detected here, as our current goal was to identify residues that are responsible for the difference in Opa binding to these two receptors. This postulated binding site fits well with the recent demonstration that Opa binding to CEACAM...
receptors is a protein–protein interaction (Bos et al., 1998), as the GFCC’ face is predicted to be devoid of carbohydrate structures, while the ABDE face is predicted to be covered by carbohydrates (Bates et al., 1992; Boehm et al., 1996).

The region between residues 60 and 108 can clearly influence Opa binding (e.g. compare binding to chimeras $\chi_{N1}/\chi_{N2}$ and chimeras $\chi_{E1}/\chi_{E2}$; Fig. 3). The absence of binding to chimera B does, however, confirm that this region is not sufficient for binding of Opa$_{32}$, and it is not required for Opa$_{52}$ binding when residues 27–32 are derived from CEACAM6 (chimera $\chi_{O1}$). The proposed structure of CEACAM5 predicts that residues 85–108 form $\beta$F and $\beta$G, which lie directly adjacent to $\beta$C in the GFCC’ $\beta$-sheet of the N-terminal domain (Bates et al., 1992; Fig. 6). It is therefore possible that residues exposed from this region are involved in Opa protein binding. Alternatively, changes in $\beta$F and $\beta$G may influence the orientation and/or structure of the other parts of the Opa binding site that include residues 27–32, which lie at the distal end of $\beta$C (Fig. 6).

Some Opa protein variants are able to bind to CEACAM1, CEACAM3, CEACAM5 and CEACAM6, while the binding of other variants is restricted to CEACAM1 and CEACAM5 (Bos et al., 1997; 1998; Chen et al., 1997; Gray-Owen et al., 1997b). These different binding specificities may have important implications for the pathogenic process of Neisseria, as the distribution pattern of each CEACAM receptor may influence the cellular tropism of neisserial strains expressing different Opa variants in vivo. In addition, very different cellular processes have been linked to individual CEAC family members (Thompson et al., 1991; Kuijpers et al., 1992; Eidelman et al., 1993; Klein et al., 1996; Kleinerman et al., 1996), suggesting that the cellular response to neisserial binding depends upon the specific combination of CEACAM receptors bound. The exchange of residues 27–29 in CEACAM6 with the corresponding sequences from either CEACAM1 or CEACAM5 is sufficient to allow binding by the CEACAM1- and CEACAM5-specific Opa protein variants (i.e. Opa$_{51}$, Opa$_{54}$, Opa$_{55}$, Opa$_{56}$ and Opa$_{59}$; see Fig. 5). In general, the Opa proteins bind more strongly to chimeras possessing the CEACAM1 (QLF) than to those with the CEACAM5 (HLF) sequences at this site. This may be caused by the fact that the N-terminal domain of CEACAM6 is more similar to the N-terminal domain of CEACAM1 than it is to the N-terminal domain of CEACAM5, suggesting that alternative interactions or structural differences are also influencing the binding specificity. This may help to explain why some of the Opa proteins can distinguish between CEACAM1 and CEACAM5. However, this may also depend on the experimental approach used for infection assays (Bos et al., 1997; 1998; Gray-Owen et al., 1997b).

Activation of the cellular response to neisserial binding therefore one more fascinating example of the exquisite adaptation of Neisseria sp. to life within humans, their only known host. Whether this interaction can be exploited for the prophylactic and/or therapeutic treatment of neisserial diseases is therefore an important question for future research.

Experimental procedures

Bacterial strains

The recombinant N. gonorrhoeae MS11 strains used in this study have a chromosomal deletion in the opaC locus that encodes the HSPG-specific Opa$_{30}$ and constitutively expresses single defined Opa variants from strain MS11: Opa$_{30}$ (strain N303); Opa$_{31}$ (strain N305); Opa$_{32}$ (strain N309); Opa$_{33}$ (strain N304); Opa$_{54}$ (strain N311); Opa$_{55}$

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Published was a kind gift from Dr J. Thompson (Institut für Immunobiologie, University of Freiburg, Germany). All cell lines were cultured in a humidified atmosphere at 37°C. Synthesis of chimeric CEACAM receptor expression constructs was performed by preparative agarose gel electrophoresis, purified using a QiAquick DNA purification kit (Qiagen), and then 5' and 3' overlapping fragments were mixed in equal amounts for SOEing of the PCR products. The SOEing products were generated by performing five thermocycles under the conditions outlined above, but lacking any oligonucleotide primers. The products were then amplified by adding the T7 and SP6 primers (Table 1) and then continuing the amplification reaction for a further 25 cycles. Vent DNA polymerase (New England Biolabs) with proof-reading activity and Perkin-Elmer Thermocycler Gene Amp 2400 were used for the generation of these chimeras. The hybrid molecules generated were cloned into the expression vector pRC/C MV. The amino-terminal domains of all chimeric constructs were then sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) to confirm their identity and to detect any mutations generated during cloning. Three changes in the protein sequence of CEACAM6 and CEACAM8 with regard to previously published data (Neumaier et al., 1988; Arakawa et al., 1990; Berling et al., 1990) were found in our clones by sequencing: CEACAM6: Tyr-48!Asn; CEACAM8: Pro-8!Gln. As these clones were used to generate the stably transfected cell lines used for all previous Neisseria binding studies (Bos et al., 1997; Chen et al., 1997; Gray-Owen et al., 1997a,b), they were also used for all cloning and transfection experiments performed in this study. All chimeras that carry the corresponding parts of CEACAM6 and CEACAM8 will therefore share these amino acid changes. Sequencing revealed additional point mutations in chimeras χC1, Gly-102!Arg; χE1, Pro-108!Leu; χF2 and χK2, Glu-37!Ala; and in chimeras χ1, and χK1, Gly-36!Ala.

Stable and transient transfection procedures

Polyclonal stable transfecants were generated by transfecting COS-7 cells with the pRC/C MV expression constructs expressing either CEACAM6, CEACAM8 or chimeras χD1, χD2, χE1, χF1, χG1, χG2, χH1, χH2, χI2, χK2, χT or χU were filled using T4 DNA polymerase. Subsequent religation generated the modified plasmid pRC/C MV-Apal. CEACAM8 cDNA was modified by polymerase chain reaction (PCR) leading to CEACAM88, which lacks the Apal site in the leader sequence but has a new Apal site generated at the position corresponding to the Apal site in the N-domain of CEACAM6. The plasmids pRC/C MV-Apal CEACAM6 and pRC/C MV-Apal CEACAM8 were then digested with Apal and Xbal. The resulting vector DNAs with the N-terminal portion of either CEACAM6 or CEACAM8 cDNA and the liberated gene fragments were each isolated and ligated in a reciprocal manner to generate the two constructs, chimeras χA and χB (Fig. 3). All other chimeric proteins generated in this study were synthesized using the PCR-based splicing by overlap extension (SOEing) approach (Horton et al., 1990) using oligonucleotide primer pairs and templates outlined in Tables 1 and 2. The thermocycle profile used for the generation of DNA fragments for the subsequent SOEing reactions included an initial 1 min incubation at 95°C, followed by 25 cycles of 10 s at 95°C, 10 s at 50°C and 1 min at 72°C; the final cycle was followed by an incubation at 72°C for 2 min to extend completely all amplified products to the 3' termini. Appropriate PCR products were isolated by preparative agarose gel electrophoresis, purified using a QiAquick DNA purification kit (Qiagen), and then 5' and 3' overlapping fragments were mixed in equal amounts for SOEing of the PCR products. The SOEing products were generated by performing five thermocycles under the conditions outlined above, but lacking any oligonucleotide primers. The products were then amplified by adding the T7 and SP6 primers (Table 1) and then continuing the amplification reaction for a further 25 cycles. Vent DNA polymerase (New England Biolabs) with proof-reading activity and Perkin-Elmer Thermocycler Gene Amp 2400 were used for the generation of these chimeras. The hybrid molecules generated were cloned into the expression vector pRC/C MV. The amino-terminal domains of all chimeric constructs were then sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) to confirm their identity and to detect any mutations generated during cloning. Three changes in the protein sequence of CEACAM6 and CEACAM8 with regard to previously published data (Neumaier et al., 1988; Arakawa et al., 1990; Berling et al., 1990) were found in our clones by sequencing: CEACAM6: Tyr-48!His; CEACAM8: Pro-8!Leu and Lys-80!Arg. As these clones were used to generate the stably transfected cell lines used for all previous Neisseria binding studies (Bos et al., 1997; Chen et al., 1997; Gray-Owen et al., 1997a,b), they were also used for all cloning and transfection experiments performed in this study. All chimeras that carry the corresponding parts of CEACAM6 and CEACAM8 will therefore share these amino acid changes. Sequencing revealed additional point mutations in chimeras χC1, Gly-102!Arg; χE1, Pro-108!Leu; χF2 and χK2, Glu-37!Ala; and in chimeras χ1, and χK1, Gly-36!Ala.

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Synthesis of chimeric CEACAM receptor expression constructs

The construction of pRC/C MV-CEACAM6 (Jantschew et al., 1996) has been described previously. The unique Apal site in the multiple cloning site of plasmid pRC/C MV (Invitrogen) was removed by cleavage with Apal, and the resulting ends...
Table 2. SOEing strategy used to generate the chimeric constructs.

| Chimera | Template 1 | Primer pair 1<sup>a</sup> | Template 2 | Primer pair 2<sup>a</sup> |
|---------|------------|-----------------|------------|-----------------|
| xG<sub>C1</sub> | CEACAM8 | SGO002 | CEACAM6 | SGO001 |
| xG<sub>C2</sub> | CEACAM6 | SGO002 | xA | SGO001 |
| xG<sub>D1</sub> | CEACAM6 | SGO002 | yB | SGO001 |
| xG<sub>E1</sub> | CEACAM8 | SGO006 | CEACAM6 | SGO005 |
| xG<sub>E2</sub> | CEACAM6 | SGO002 | AP011 | xB |
| xG<sub>F1</sub> | CEACAM6 | SGO004 | CEACAM8 | SGO003 |
| xG<sub>F2</sub> | CEACAM8 | SGO007 | xB | SGO006 |
| xG<sub>G1</sub> | CEACAM6 | SGO010 | CEACAM6 | SGO009 |
| xG<sub>H1</sub> | CEACAM6 | SGO008 | xA | SGO007 |
| xG<sub>H2</sub> | CEACAM6 | SGO008 | xB | SGO007 |
| xG<sub>I1</sub> | CEACAM8 | AP005 | CEACAM6 | AP004 |
| xG<sub>I2</sub> | CEACAM6 | AP005 | xA | AP004 |
| xG<sub>K1</sub> | CEACAM6 | AP003 | CEACAM8 | AP002 |
| xG<sub>K2</sub> | CEACAM6 | AP003 | xB | AP002 |
| xG<sub>L</sub> | CEACAM6 | AP007 | CEACAM6 | AP006 |
| xG<sub>M</sub> | CEACAM8 | AP011 | CEACAM6 | AP010 |
| xG<sub>N1</sub> | CEACAM6 | AP031 | CEACAM6 | AP030 |
| xG<sub>O1</sub> | CEACAM8 | AP033 | CEACAM6 | AP032 |
| xG<sub>O2</sub> | CEACAM6 | AP033 | xA | AP030 |
| xG<sub>P</sub> | CEACAM6 | AP033 | xB | AP032 |
| xG<sub>Q</sub> | CEACAM8 | AP027 | CEACAM6 | AP026 |
| xG<sub>R</sub> | CEACAM8 | AP029 | CEACAM6 | AP028 |
| xG<sub>S</sub> | CEACAM6 | AP031 | CEACAM6 | AP030 |
| xG<sub>T</sub> | CEACAM8 | AP023 | CEACAM6 | AP022 |
| xG<sub>U</sub> | CEACAM6 | AP025 | CEACAM6 | AP024 |

<sup>a</sup> Corresponding primers for primer pair 1 and 2 were (+) T7 and (−) SP6.
using Lipofectamine reagent (Life Technologies) and Opti-MEM medium (Life Technologies), essentially as outlined by the manufacturers. Transfected cells were selected for using 500 \( \mu \)g/ml \(-1\) geneticin and then isolated using either polyclonal rabbit anti-CEA antisera (Dako) coupled with sheep anti-rabbit-conjugated Dynabeads (Dynal) or monoclonal antibodies 80H3 and D14HD11 coupled to rat anti-mouse-conjugated Dynabeads. Transient transfections were performed by seeding CHO-K1 or CHO-pgs677 cells onto glass coverslips in a 24-well plate, transfecting with Lipofectamine reagent and Opti-MEM medium as outlined above, and then infecting 2 days later.

**Bacterial infection assays**

The infection of stably transfected cell lines was performed by the saponin lysis and dilution plating technique described previously (Gray-Owen et al., 1997a,b). Infection of transiently transfected cells was performed as for the stable cell lines, except that cells were initially seeded onto 12 mm glass coverslips and fixed after the final washing step of the infection experiment by incubation in 3.7% paraformaldehyde in 200 mM HEPES buffer (pH 7.4) for 30 min. Fixed samples were then immunocytochemically stained, and binding interactions were analysed by confocal laser scanning microscopy as described previously (Gray-Owen et al., 1997a).

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