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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 Opa_{52} 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.

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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. Opa_{50}) 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 Opa_{50} 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

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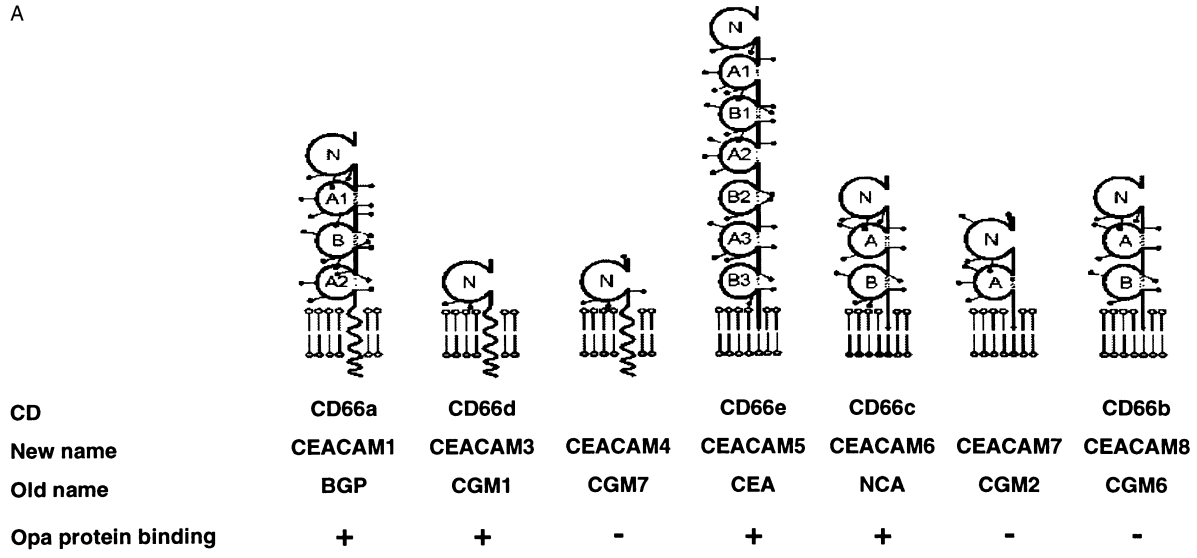

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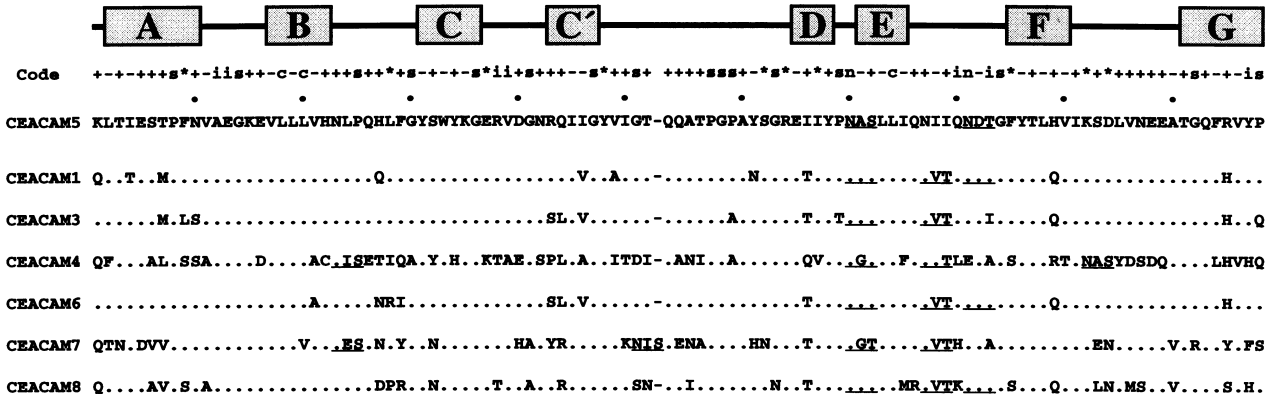

Fig. 1. Structure and N-terminal amino acid sequences of cell surface-bound CEA family members.
A. Schematic drawing of CEACAM1–CEACAM8 (Beauchemin *et al*., 1999) illustrating domain structure and membrane anchorage (according to http://www.cea.uni-freiburg.de). N, amino-terminal IgV-like domain; A/B, 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.

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; CEA gene family member 1, CGM1), CEACAM5 (CD66e; carcinoembryonic antigen, CEA) and CEACAM6 (CD66c; non-specific cross-reacting antigen, NCA) 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

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 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_{50} (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), βC′, 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 *N. gonorrhoeae* 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_{52}) 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 *N. gonorrhoeae*

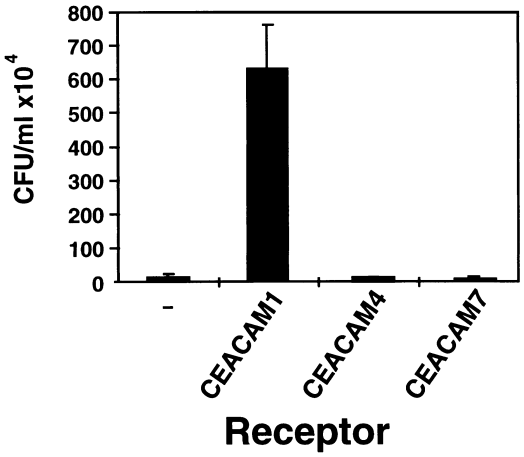

Fig. 2. Neisserial Opa protein interactions with the CEA family members CEACAM4 and CEACAM7. Stably transfected CHOK1 cell lines expressing the indicated CEA family members were infected with Opa_{52}-expressing *N. gonorrhoeae* (N309) for 3 h. Viable bacteria that remained associated with the cells after washing were quantified by lysis of the eukaryotic cell membranes with 1% saponin in PBS followed by dilution plating (Gray-Owen *et al.*, 1997b). Assays were performed in triplicate on at least two separate occasions, and data displayed illustrate the mean ± SD of one representative experiment. Although they are not shown here, neither CEACAM4 nor CEACAM7 affected binding of any of the 11 *N. gonorrhoeae* MS11 Opa protein variants.

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 χC_1, χC_2, χD_1, χD_2), 30/31 (chimeras χE_1, χE_2, χF_1, χF_2) and the conserved residue 42 (chimeras χG_1, χG_2, χH_1, χH_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 χC_1, χC_2, χE_1, χH_1, χH_2 with non-binding to chimeras χD_1, χD_2, χF_1, χF_2, χG_1, χG_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 χF_1 and χK_1 and between chimeras χF_2 and χK_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 χG_1 and χI_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 χL) 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 χM; 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 βC. 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 βC with a Ser at position 32 (Figs 1B and 3).

Sequences outside of β-strand C contribute to Opa protein interactions with the CEACAM receptors

The fact that χE_2, which has βC 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 χE_1, but not to χE_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 χA, χC_2, χH_1, χK_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 βF and βG, which lie directly adjacent to βC, together forming with βC′ the GFCC′ β-sheet of the N-terminal domain (Bates *et al.*, 1992; Fig. 6). Therefore, βF and βG could contribute to the structural integrity of the binding site. Alternatively, parts of the binding site may actually exist on βF and βG, as their surface-exposed residues should lie in direct proximity to those of βC (e.g. Ser-32) and βC′ and of the C–C′ loop (Bates *et al.*, 1992; Fig. 6).

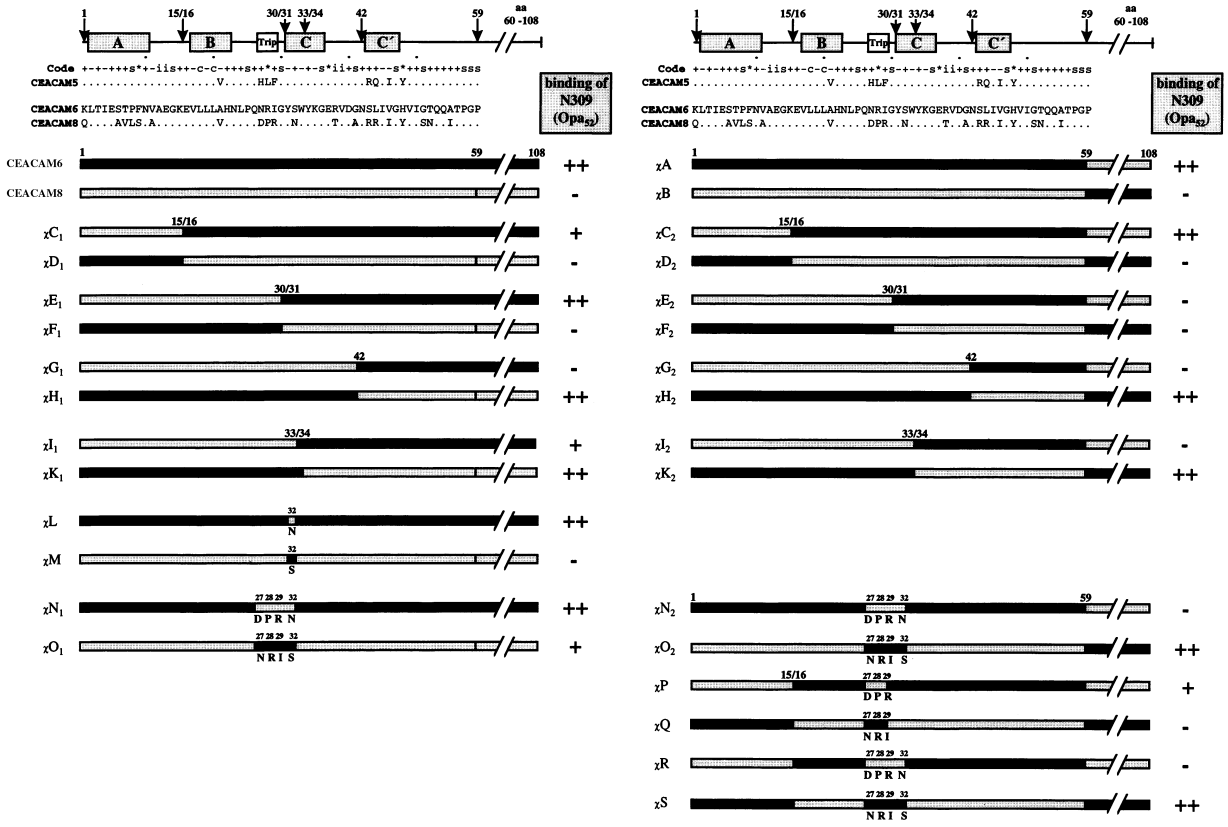

Fig. 3. Opa-mediated binding to CEACAM6, CEACAM8 and chimeric receptor proteins. Alignment of the first 59 amino acids of CEACAM5, CEACAM6 and CEACAM8 N-terminal domain amino acid sequences in single-letter code, along with a schematic representation of the predicted secondary structural elements for 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 (Opa_{52}) 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.

Reduced bacterial binding was evident when the first 15 residues of CEACAM8 were used to replace the CEACAM6 sequence (χC_1), but the reciprocal construct (χD_1) 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 (χE_1) or residues 60–108 (χC_2) are also derived from CEACAM8, as neisserial binding to chimeras χE_1 and χC_2 was indistinguishable from its binding to the native CEACAM6 (Fig. 3). Bacterial binding to χC_2, but not to χE_2, indicates that between residues 16 and 30 lies a structure that is important for Opa_{52} binding. The simple exchange of residue 32 between CEACAM6 and CEACAM8 had no effect on Opa_{52} binding (chimeras χL and χM). Instead, we therefore exchanged residue 32 together with the variable residues 27–29 between χA and χB to generate chimeras χN_2 and χO_2 (Fig. 3). Bacterial binding to cells expressing χO_2 but not to cells expressing χN_2 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 $\chi N_1/\chi O_1$ with that to $\chi N_2/\chi O_2$). An exchange of these four residues thus converts CEACAM8 to a receptor that mediates binding by Opa_{52}-expressing gonococci, but a corresponding exchange in CEACAM6 is not sufficient to abrogate binding. In order to determine

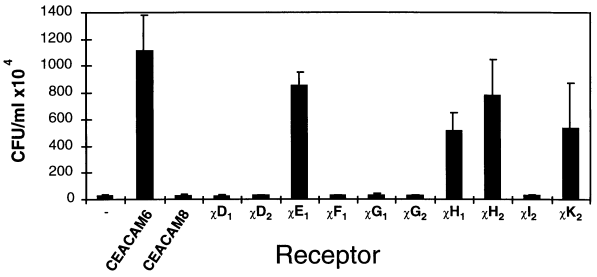

Fig. 4. Attachment of *N. gonorrhoeae* N309 (Opa_{52}) to stably transfected COS cell lines expressing various CEACAM6/CEACAM8 chimeras. Cell lines were infected for 4 h with a multiplicity of infection (MOI) of 50–100 in DMEM alpha medium without FCS. After thorough washing, total cell-associated bacteria were quantified by lysis of the eukaryotic cell membrane with 1% saponin in PBS followed by dilution plating (Gray-Owen *et al.*, 1997b). The infection experiments were performed in triplicate on at least three separate occasions, and data displayed illustrate the means ± SD of one representative experiment.

whether exchanging the variable triplet of residues (amino acids 27–29) alone between χC_2 and χD_2 was sufficient to influence binding, χP and χQ were produced (Fig. 3). χD_2 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 Opa_{52}. Conversely, χC_2 is the receptor that possesses the most CEACAM8-derived sequence, while still binding Opa_{52}-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 χC_2 and χD_2, resulting in χR and χS. Opa_{52}-mediated binding to chimera χP is reduced compared with binding with χC_2, but was completely abrogated in χR, and no binding was seen to cells expressing chimera χQ, whereas χS permits strong binding of Opa_{52}-expressing gonococci. Together, these results indicate that residues 27–29 contribute to, but are not sufficient to mediate, Opa_{52} 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, Opa_{52}, Opa_{57}, Opa_{58} and Opa_{60} bind to CEACAM1, CEACAM3, CEACAM5 and CEACAM6, while binding of Opa_{51}, Opa_{53}, Opa_{54}, Opa_{55}, Opa_{56} and Opa_{59} 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 χT and χU respectively (Fig. 5). Although the binding of Opa_{52}-expressing gonococci to stably transfected cells expressing either CEACAM6, χT or χU was indistinguishable, the CEACAM1- and CEACAM5-specific Opa_{51}, Opa_{54}, Opa_{55}, Opa_{56} and Opa_{59} proteins bound only the chimeric proteins (i.e. χT and χU; 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 Opa_{53} expressed by strain N304 was indistinguishable from the amount of Opa expressed by the other strains, no binding of Opa_{53} was seen to χT and χU. The observed specificity of Opa_{53} for CEACAM5 (Gray-Owen *et al.*, 1997b) must therefore be determined by other binding determinants.

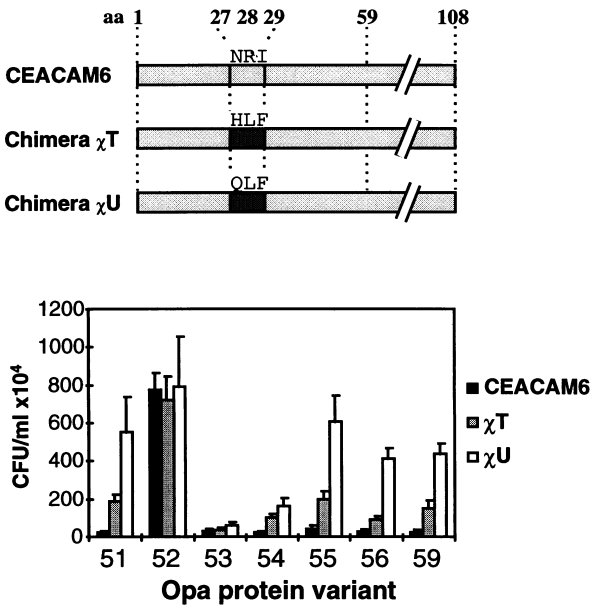

Fig. 5. Differential binding of Opa protein variants to CEACAM6 receptors containing CEACAM1 or CEACAM5 tripeptide sequence insertions at residues 27–29 of the N-terminal domain (chimeras χT and χU). Stably transfected COS cell lines were infected for 4 h with an MOI of 50–100 in DMEM alpha without FCS. After thorough washing, total cell-associated bacteria were quantified by lysis of the eukaryotic cell membranes with 1% saponin in PBS followed by dilution plating (Gray-Owen *et al.*, 1997b). Assays were performed in triplicate on at least three separate occasions, and data displayed illustrate the means ± SD of one representative experiment.

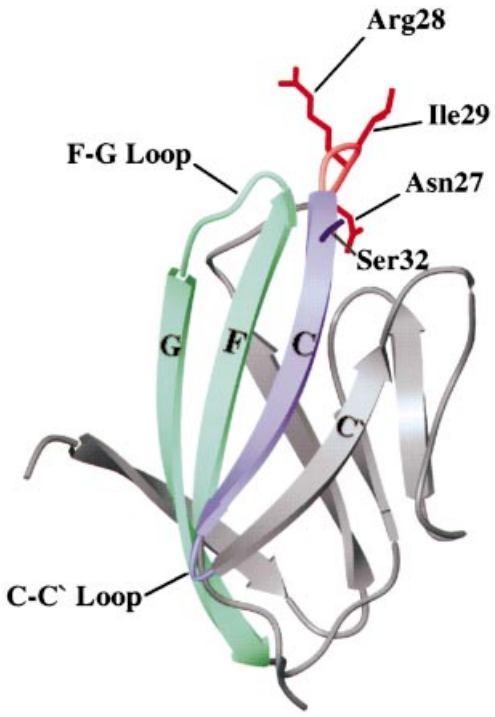

Fig. 6. Model of the binding site of Opa_{52} on CEACAM6.The crystal structure of domain I of CD2 (Jones *et al*., 1992) was used as a model for the N-terminal domain of CEACAM6. Graphics were produced using the SETOR program (Evans, 1993) by replacing and displaying residues in the CD2 crystal structure that correspond to residues 27–29 and 32 of CEACAM6 with those present in CEACAM6. Regions predicted to comprise the binding site of Opa_{52} are coloured and labelled in order to demonstrate the spatial relationship of sequences that influence Opa binding in the folded protein structure. Opa_{52} 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 Opa_{51}, Opa_{54}, Opa_{55}, Opa_{56} and Opa_{59} to CEACAM1, CEACAM5 and CEACAM6. βF, the F–G loop and βG (green; amino acids 85–108) lie in direct proximity 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 βC′ 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 exchanging sequences reciprocally between CEACAM6 and CEACAM8, we were able to identify regions in the N-terminal domain that are necessary for binding by Opa_{52}. 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 Opa_{52} has the capacity to bind four of the CEACAM receptors, it is likely that contacts responsible for Opa_{52} 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 $\chi N_1/\chi N_2$ and chimeras $\chi E_1/\chi E_2$; Fig. 3). The absence of binding to chimera B does, however, confirm that this region is not sufficient for binding of Opa_{52}, and it is not required for Opa_{52} binding when residues 27–32 are derived from CEACAM6 (chimera χO_1). The proposed structure of CEACAM5 predicts that residues 85–108 form βF and βG, which lie directly adjacent to βC in the GFCC′ β-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 βF and βG 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 βC (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 CEA 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). In clear contrast, the binding specificity of Opa_{53} is not influenced by residues at positions 27–29, indicating that other portions of the CEACAM molecules determine its binding specificity. It has been shown previously that the ability of each Opa protein to bind to CEACAM6 generally correlates with its ability to bind to CEACAM3 (Bos *et al*., 1997; 1998; Chen *et al*., 1997; Gray-Owen *et al*., 1997b). The importance of residues 27–29 for the specificity of Opa binding to CEACAM6 is therefore surprising based on the fact that these residues are identical between CEACAM3 and CEACAM5 (Fig. 1B). CEACAM3 differs at positions 59, 69 and 108, which are predicted to be essential for fold stability of CEACAM1, CEACAM5 and CEACAM6, and also lacks an additional Asn-linked glycosylation site at position 81 (Fig. 1B). Structural differences may therefore be the reason why CEACAM3 is not recognized by some Opa variants. It is interesting in this respect that the number and type of IgC2-like domains can influence mouse hepatitis virus binding to the mouse homologue of CEACAM1, despite the fact that the virus only directly contacted the receptor's N-terminal domain (Wessner *et al*., 1998). Whether the absence of an IgC2-like domain in CEACAM3 influences its structure and affects Opa protein binding in an analogous fashion is still uncertain.

Various CEACAM receptors have previously been suggested to function in homotypical and heterotypical interactions (Thompson *et al*., 1991). These interactions appear to involve contacts between residues on the GFCC′ face of CEACAM receptors (Oikawa *et al*., 1991). Other membrane-associated members of the immunoglobulin superfamily (i.e. CD2 and CD4) also typically bind their native ligands at this surface (Ryu *et al*., 1990; Wang *et al*., 1990; Jones *et al*., 1992; Bodian *et al*., 1994). Opa protein binding to the residues that are involved in the natural function of CEACAM receptors should prevent any adaptive mutations within the host that could abrogate neisserial binding, as such a change would probably also abrogate CEACAM function. It is 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_{50} (strain N303); Opa_{51} (strain N305); Opa_{52} (strain N309); Opa_{53} (strain N304); Opa_{54} (strain N311); Opa_{55}

(strain 307); Opa_{56} (strain N308); Opa_{57} (strain N313); Opa_{58} (strain N312); Opa_{59} (strain N306); or Opa_{60} (strain N310). Daily subculture of all neisserial strains was carried out using a binocular microscope to select for desired Opa phenotypes. The construction of all strains used has been described previously (Kupsch *et al*., 1993). Confirmation of Opa protein expression was verified by SDS–PAGE and immunoblot analysis of total bacterial extracts obtained from cultures used for infection. The monoclonal antibody 4B12C11 (Achtman *et al*., 1988), generously provided by Dr Mark Achtman, Berlin, Germany, was used for the detection of Opa proteins.

Cell lines

COS-7 green monkey kidney (ATCC CRL1651), Chinese hamster ovary (CHOK1; ATCC CRL9618) and CHO-*pgs*677, which is defective in proteoglycan biosynthesis (Esko *et al*., 1988) and was a generous gift from Dr J. D. Esko (University of Alabama at Birmingham, USA), have all been described previously. Stably transfected HeLa cell lines expressing the CEACAM1 (CD66a; BGP), CEACAM3 (CD66d; CGM1), CEACAM5 (CD66e; CEA), CEACAM6 (CD66c; NCA) and CEACAM8 (CD66b; CGM6) receptors (Berling *et al*., 1990; Nagel *et al*., 1993; Gray-Owen *et al*., 1997a) as well as the stably transfected CHO-CEACAM4 (CGM7) and CHO-CEACAM1 (Kuroki *et al*., 1991; Oikawa *et al*., 1991) have been described previously. The CHO-CEACAM7 (CGM2) cell line (Schoelzel, S *et al*., data to be published) was a kind gift from Dr J. Thompson (Institut für Immunbiologie, University of Freiburg, Germany). All cell lines were cultured in a humidified atmosphere at 37°C with 5% CO_2. RPMI-1640 medium supplemented with L-glutamine, 10% fetal calf serum (FCS) and 500 $\mu g\,ml^{-1}$ geneticin was used to propagate stably transfected HeLa cells; MEMα without ribonucleotides supplemented with 10% FCS was used for CHOK1 cells; Ham's F12 with 10% FCS for CHO-*pgs*677 cells; and DMEMα with 10% FCS for COS cells. All media for cell culture were obtained from Life Technologies. The expression of recombinant and chimeric CEACAM molecules in all stably transfected cells used in this study was confirmed by fluorescence-activated cell sorter (FACS) analysis (FACScan; Becton Dickinson). The CD66b-specific, mouse-derived monoclonal antibody (mAb) 80H3 (Immunotech), the CD66a-, CD66c-, CD66d-, CD66e- and CEACAM4-specific mAb D14HD11 (Knapp *et al*., 1989), polyclonal rabbit anti-CEA antisera (Dako) and the CEACAM7-specific mAb BAC2 (Schoelzel, S *et al*., unpublished) were used for primary labelling of the transfected cells for this purpose. BAC2 was kindly provided by Dr J. Thompson, University of Freiburg, Germany. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit and goat anti-mouse antibodies (Dianova) were used as secondary antibodies.

Synthesis of chimeric CEACAM receptor expression constructs

The construction of pRc/CMV-CEACAM6 (Jantscheff *et al*., 1996) has been described previously. The unique *Apa*I site in the multiple cloning site of plasmid pRc/CMV (Invitrogen) was removed by cleavage with *Apa*I, and the resulting ends were filled using T4 DNA polymerase. Subsequent religation generated the modified plasmid pRc/CMV-*Apa*I. CEACAM8 cDNA was modified by polymerase chain reaction (PCR) leading to CEACAM8$^{\geq}$, which lacks the *Apa*I site in the leader sequence but has a new *Apa*I site generated at the position corresponding to the *Apa*I site in the N-domain of CEACAM6. The plasmids pRc/CMV-*Apa*I CEACAM6 and pRc/CMV-*Apa*I CEACAM8$^{\geq}$ were then digested with *Apa*I and *Xba*I. 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/CMV. 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 χC_1: Gly-102→Arg; χE_1: Pro-108→Leu; χF_2 and χK_2: Glu-37→Ala; and in chimeras χI_1 and χI_2: Gly-36→Ala.

Stable and transient transfection procedures

Polyclonal stable transfectants were generated by transfecting COS-7 cells with the pRc/CMV expression constructs expressing either CEACAM6, CEACAM8 or chimeras χD_1, χD_2, χE_1, χF_1, χG_1, χG_2, χH_1, χH_2, χI_2, χK_2, χT or χU

Table 1. Oligonucleotide primers used for the generation of the chimeric constructs.

Primer	Sequence
T7	TAATACGACTCACTATAGGG
SP6	ATTTAGGTGACACTATAG
SGO001	GCAGAGGGGAAGGAGGTTCTTCTAC
SGO002	GTAGAAGAACCTCCTTCCCCTCTGC
SGO003	GAATCGTATTGGTTACAACTGGTACAAAGG
SGO004	CCTTTGTACCAGTTGTAACCAATACGATTC
SGO005	GGACCCTCGTGGCTACAGCTGGTACAAAGC
SGO006	GCTTTGTACCAGCTGTAGCCACGAGGGTCC
SGO007	AAAGAGTGGATGGCAACCGTCGAATTATAG
SGO008	CTATAATTCGACGGTTGCCATCCACTCTTT
SGO009	AAACAGTGGATGCCAACAGTCTAATTGTAG
SGO010	CTACAATTAGACTGTTGGCATCCACTGTTT
AP002	ATTGGTTACAGCTGGTACAAAGGGGAAAC
AP003	GTTTCCCCTTTGTACCAGCTGTAACCAAT
AP004	CGTGGCTACAACTGGTACAAAGGCGAAAG
AP005	CTTTCGCCTTTGTACCAGTTGTAGCCACG
AP006	CGTATTGGTTACAACTGGTACAAAGGC
AP007	GCCTTTGTACCAGTTGTAACCAATACG
AP010	CCTCGTGGCTACAGCTGGTACAAAGGG
AP011	CCCTTTGTACCAGCTGTAGCCACGAGG
AP022	CAACCTGCCCCAGCATCTTTTTGGTTACAGCTGG
AP023	CCAGCTGTAACCAAAAAGATGCTGGGGCAGGTTG
AP024	CAACCTGCCCCAGCAACTTTTTGGTTACAGCTGG
AP025	CCAGCTGTAACCAAAAAGTTGCTGGGGCAGGTTG
AP026	CAACCTGCCCCAGGACCCTCGTGGTTACAGCTGG
AP027	CCAGCTGTAACCACGAGGGTCCTGGGGCAGGTTG
AP028	CAATCTGCCCCAGAATCGTATTGGCTACAACTGG
AP029	CCAGTTGTAGCCAATACGATTCTGGGGCAGATTG
AP030	CTGCCCCAGGACCCTCGTGGCTACAACTGGTACAAAG
AP031	CTTTGTACCAGTTGTAGCCACGAGGGTCCTGGGGCAG
AP032	CTGCCCCAGAATCGTATTGGTTACAGCTGGTACAAAG
AP033	CTTTGTACCAGCTGTAACCAATACGATTCTGGGGCAG

Table 2. SOEing strategy used to generate the chimeric constructs.

Chimera	Template 1	Primer pair 1[a] (+)	Template 2	Primer pair 2[a] (−)
χC_1	CEACAM8	SGO002	CEACAM6	SGO001
χC_2	χB	SGO002	χA	SGO001
χD_1	CEACAM6	SGO002	CEACAM8	SGO001
χD_2	χA	SGO002	χB	SGO001
χE_1	CEACAM8	SGO006	CEACAM6	SGO005
χE_2	χI_2	AP011	χI_2	AP010
χF_1	CEACAM6	SGO004	CEACAM8	SGO003
χF_2	χK_2	AP007	χK_2	AP006
χG_1	CEACAM8	SGO010	CEACAM6	SGO009
χG_2	χB	SGO010	χA	SGO009
χH_1	CEACAM6	SGO008	CEACAM8	SGO007
χH_2	χB	SGO008	χB	SGO007
χI_1	CEACAM8	AP005	CEACAM6	AP004
χI_2	χB	AP005	χA	AP004
χK_1	CEACAM6	AP003	CEACAM8	AP002
χK_2	χB	AP003	χB	AP002
χL	CEACAM6	AP007	CEACAM6	AP006
χM	CEACAM8	AP011	CEACAM8	AP010
χN_1	CEACAM6	AP031	CEACAM6	AP030
χO_1	CEACAM8	AP033	CEACAM8	AP032
χN_2	χA	AP031	χA	AP030
χO_2	χB	AP033	χB	AP032
χP	χC_2	AP027	χC_2	AP026
χQ	χD_2	AP029	χD_2	AP028
χR	χC_2	AP031	χC_2	AP030
χS	χD_2	AP033	χD_2	AP032
χT	CEACAM6	AP023	CEACAM6	AP022
χU	CEACAM6	AP025	CEACAM6	AP024

a. 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 CHOK1 or CHO-*pgs*677 cells onto glass coverslips in a 24-well plate, transfecting with Lipofectamine reagent and OptiMEM 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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