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'Small' talk: Opa proteins as mediators of *Neisseria*–host-cell communication

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Opa proteins are variable outer membrane proteins of *Neisseria gonorrhoeae* and *Neisseria meningitidis* that mediate tight interaction of these pathogens with human cells. They have emerged as a paradigm of a bacterial toolbox allowing recognition of different host receptors and orchestrating the cell type tropism displayed by pathogenic *Neisseriae*. Recent work has highlighted the molecular basis of Opa-protein–host-receptor interaction and has shed new light on the functional consequences of this interaction with regard to bacterial attachment, invasion, and responses elicited in particular host cells.

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Abbreviations

ASM	acid sphingomyelinase
CEA	carcinoembryonic antigen
CEACAM	CEA-related cell adhesion molecule
ECM	extracellular matrix
GPI	glycosylphosphatidylinositol
HSPG	heparansulphate proteoglycan
HV	hypervariable domain
Ig	immunoglobulin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
Opa	opacity-associated
Opa_{CEA}	Opa proteins recognising CEA or related molecules
Opa_{HS}	heparansulphate-recognising Opa protein
PC	phosphatidylcholine
PKC	protein kinase C
PLC	phospholipase C

Introduction

Neisseria gonorrhoeae and *N. meningitidis* are Gram-negative bacterial pathogens highly adapted to survive within a single host population, humans. Both microorganisms colonise mucosal surfaces in different body parts, reflecting the route of transmission: either the genito-urinary tract following sexual transmission of gonococci; or the nasopharynx after airborne spread of meningococci. Colonisation with these pathogens often (gonococcus) or in the majority of cases (meningococcus) remains without symptoms; however, invasive disease can develop involving breaching of epithelial and endothelial barriers, purulent inflammation, and haematogenic spread, resulting in severe and often life-threatening medical conditions (reviewed in [1,2]).

A characteristic feature of both species is the enormous variability in the antigenic properties and expression of surface components. The modulation of outer membrane lipooligosaccharide and pili is thought primarily to reflect an immune evasion mechanism, allowing the bacteria to escape innate and acquired host defences and to outmanoeuvre immune memory. However, research over the past decade has demonstrated that variation of at least one family of outer membrane proteins serves the purpose of facilitating the interaction of the bacteria with different host cell types (reviewed in [3]). This family of neisserial proteins has been termed 'opacity-associated' (Opa) proteins, since they are responsible for an opaque phenotype of agar-grown colonies.

In this review, we summarise the current knowledge with regard to Opa protein biology and the nature of Opa receptors present on human cells, emphasising the molecular basis of the interaction and the potential role of the Opa-initiated pathogen–host communication during the infection process.

Opa protein structure and variation

Opa proteins are integral outer membrane proteins that are synthesised as precursors containing signals for inner membrane transport. A single gonococcal strain can harbour up to 12 *opa* genes, whereas meningococci usually encode three to four Opa proteins. Opa protein expression undergoes phase variation (i.e. expression of each Opa protein can be independently switched to an 'on' or 'off' state). The translation-based form of Opa protein phase variation is due to pentameric sequence repeats within the coding region of the amino-terminal leader peptide. Since the number of repeats is critical for the correct reading frame, modulation of the repeat number by slipped-strand mispairing is thought to result in the frequent phase shifts in Opa protein expression [4]. As a result, any given natural neisserial population should comprise a mixture of individual bacteria expressing

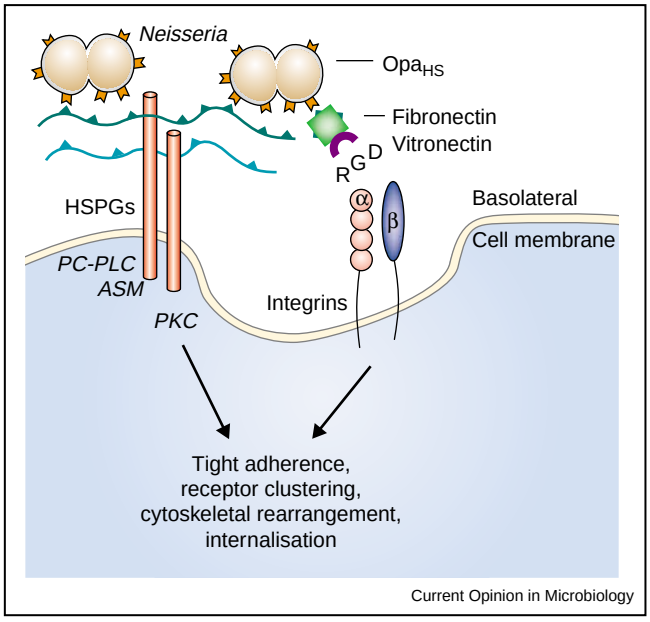
either none, one or multiple Opa proteins. It is important to note that Opa proteins, in contrast to neisserial pili, are not required for the initial colonisation of the host, as human volunteer challenge experiments with non-opaque strains result in successful colonisation. However, colonies re-isolated from these volunteers almost invariably express Opa proteins, suggesting a strong selection pressure towards the expression of Opa proteins *in vivo* [5,6].

Secondary structure predictions suggest that mature Opa proteins possess eight membrane-spanning domains arranged as anti-parallel β strands, giving rise to a membrane-embedded β barrel with four extracellular loops [7,8]. As sequence variation between Opa proteins is observed predominantly within the central two loops, they have been termed 'hypervariable domain 1' (HV-1) and 2 (HV-2). New Opa protein variants constantly emerge not only because of point mutations within HV-1 and HV-2, but also by modular exchange of domains between different Opa proteins [9]. Interestingly, the interaction of Opa proteins with different receptors on human cells has also been pinned down to the HV-1 and HV-2 regions [10–12], posing the puzzling question of how specific receptor recognition is achieved in the context of sequence alterations. Despite variation in amino acid sequence, almost all currently characterised meningococcal and gonococcal Opa proteins can be grouped into two major classes, according to their binding specificity for human surface receptors: the Opa_{HS}-type proteins (the term 'Opa_{HS}' denotes heparansulphate-recognising Opa proteins such as OpaA/Opa_{30} of gonococcal strain MS11 or $Opa_{27.5}$ of strain VP1); and the large group of Opa_{CEA}-type proteins (the term 'Opa_{CEA}' comprises all Opa proteins recognising carcinoembryonic antigen (CEA) or related molecules such as Opa_{52} of gonococcal strain MS11 or Opa_{132} of meningococcal strain C751). (For Opa nomenclature, refer to [8].)

Opa_{HS}-type proteins

The Opa_{HS} group of proteins was identified by their ability to mediate gonococcal attachment to and invasion *in vitro* of several epithelial cell lines such as HeLa and ME180 cervix carcinoma cells, Chang conjunctiva cells, Hec1B endometrial adenocarcinoma cells, and Hep2 larynx carcinoma cells, as well as into Chinese hamster ovary (CHO) cells (Figure 1, [13–15]). Heparansulphate proteoglycans (HSPGs) were identified as receptors on human cells, since Opa_{HS}-mediated attachment and invasion is abolished in the presence of heparin, a soluble glycosaminoglycan that competes with HSPGs for Opa_{HS} binding [16,17]. HSPGs are membrane proteins that bind to a variety of growth factors and extracellular matrix proteins and function as accessory molecules in cell attachment, cell growth, signal transduction and cytoskeletal organisation. They are characterised by glycosaminoglycan sidechains comprising negatively charged heparansulphates and chondroitin sulphates. Recent work

Figure 1



Opa_{HS}-mediated interactions with human epithelial cells. Pathogenic *Neisseriae* employ Opa_{HS} to engage the glycosaminoglycan sidechains of HSPGs. This contact is sufficient to stimulate the activity of PC-PLC and ASM, two events that are required for internalisation of the microorganisms. In addition, transmembrane syndecan-type HSPGs seem to connect to conventional PKCs regulating cytoskeletal rearrangements that lead to bacterial engulfment. In several epithelial cell lines, efficient internalisation of the bacteria requires indirect interactions of Opa_{HS} with integrins mediated by the ECM proteins vitronectin or fibronectin. This interaction is sensitive to RGD (arginine–glycine–aspartic acid) peptides that mimic the binding site of ECM proteins for integrins and is thought to occur at the basolateral side of polarised epithelial cells.

has suggested that it is the arrangement and number of positively charged amino acids in HV-1 of Opa_{HS} that confers recognition of HSPGs [11]. Interestingly, HSPGs come in different flavours, either as glycosylphosphatidylinositol (GPI)-linked proteins (glypicans) or as transmembrane receptors (syndecans). Although syndecan family members are already present on most epithelial cell lines, overexpression of syndecan-2 and -4 in HeLa cells can further elevate adhesion and strongly enhance invasion of Opa_{HS}-expressing bacteria [18]. HSPG-mediated internalisation of inert particles or gonococci can be blocked by pharmacological inhibitors of actin dynamics, protein tyrosine kinases and conventional protein kinase C (PKC) isoforms, suggesting that HSPGs are linked to intracellular signalling networks and the actin cytoskeleton [18–22]. Expression of syndecan-4 constructs containing carboxy-terminal deletions or point mutations in the cytoplasmic domain not only abolishes the enhanced Opa_{HS}-initiated uptake, but also blocks internalisation via endogenous HSPGs [18]. This dominant-negative effect points to a critical role for syndecan

oligomerisation or receptor association with cytoplasmic proteins in bacterial internalisation. Indeed, the carboxy-terminal EFYA motif of syndecans is critical for recruitment of PDZ-domain-containing proteins such as syntenin [23], which could provide a link to the remodelling of the actin cytoskeleton.

Interestingly, interference with the lipid-modifying enzymes phosphatidylcholine-dependent phospholipase C (PC-PLC) and acid sphingomyelinase (ASM) by pharmacological and genetic means blocks Opa_{HS}-initiated invasion via HSPGs in Chang conjunctiva cells and fibroblasts [24]. As the Opa-induced activity of PC-PLC and ASM leads to release of the second messengers diacylglycerol and ceramide, respectively, these signalling intermediates could contribute to the modulation of the cytoskeleton during gonococcal invasion [21,22]. However, localised PC-PLC and ASM activity could also modify the lipid composition of the plasma membrane at the site of invasion, facilitating receptor clustering and bacterial internalisation. Indeed, syndecan-4 partitions to a Triton-insoluble membrane microdomain upon receptor clustering [25], suggesting that the rapid increase in PC-PLC and ASM activities seen after gonococcal infection contributes at this early step [24].

In addition to interacting with HSPG, Opa_{HS} is also able to bind extracellular matrix (ECM) proteins such as vitronectin and fibronectin [15,26,27]. By using these ligands as bridging molecules, Opa_{HS} can indirectly engage integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ on human cells, inducing integrin-mediated uptake into epithelial cells. Importantly, this indirect connection seems to require prior association of Opa_{HS} and HSPGs, because integrin-mediated adherence and invasion can be reduced by pretreatment of the cells with heparitinase that removes heparin sulfate moities from HSPGs [15]. Since vitronectin and fibronectin are components of normal serum, serum leads to enhanced interaction of gonococci and meningococci with several cell lines *in vitro* that show only low levels of HSPG-mediated invasion in the absence of serum [27,28]. Clearly, the contribution of these matrix proteins to the infection process *in vivo* might be unrelated to their presence in serum. However, it is conceivable that during systemic spread of gonococci and meningococci, binding of vitronectin or fibronectin to Opa_{HS} provides the basis for additional bacteria–host-cell contact points and allows invasion into cells with low HSPG expression levels. In polarised epithelial cells, HSPGs and integrins are expressed on the basolateral surface. Therefore, it seems likely that Opa_{HS} mediates attachment and invasion after bacteria have managed to transcytose through the intact mucosal epithelium or after disruption of epithelia in the course of the inflammatory process.

It is noteworthy that meningococci express an unrelated outer membrane protein, the Opc protein, that seems to constitute a functional homologue of Opa_{HS}, as Opc has been shown to bind HSPGs and to mediate, via human fibronectin or vitronectin, the attachment to human umbilical vein or brain microvascular endothelial cells, respectively [29–31].

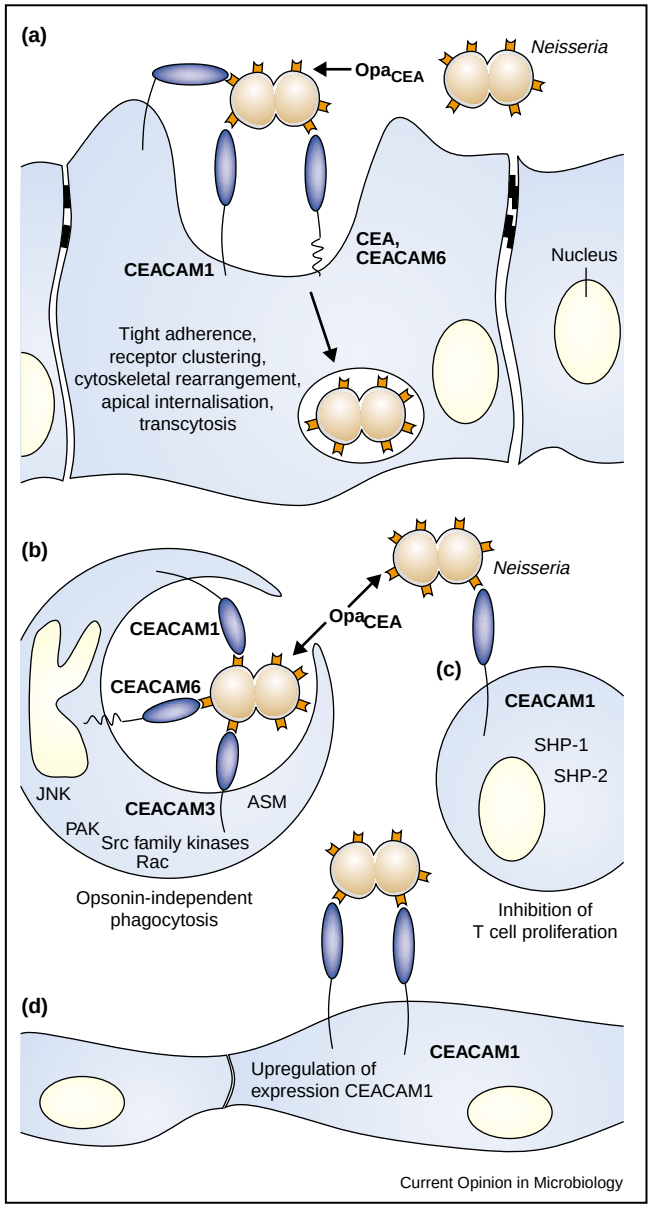
Opa_{CEA}-type proteins

A second group of Opa proteins mediates attachment to members of the CEA-related cell adhesion molecule (CEACAM) family that harbour the CD66 epitope [32–34]. CEACAMs contribute to the adhesive properties of cells by homophilic binding to CEACAMs on adjacent cells or by presenting sialylated $Lewis^X$ antigen for recognition by E-selectin [35]. The human CEACAM family comprises seven members (CEACAM1, CEACAM3–8) that are characterised by a single amino-terminal immunoglobulin (Ig) variable-like domain and a varying number (zero to six) of IgC2 constant-like domains [36,37]. In addition, *CEACAM1*, *CEACAM3* and *CEACAM7* genes can be expressed in several splice variants, affecting the number of IgC2 domains or the length and presence of cytoplasmic domains. Although the amino-terminal Ig_v-like domain is conserved in all CEACAM family members, and although this domain contains the Opa_{CEA} protein binding site [38,39], CEACAM4, CEACAM 7 and CEACAM8 are not recognised by any Opa protein characterised so far [40]. On the basis of Opa_{CEA}-binding and non-binding CEACAM amino-terminal domains, several receptor chimeras and mutants have been constructed to delineate the Opa_{CEA} binding site on the non-glycosylated C'CFG face of the Ig-domain fold (reviewed in [41]).

A second area of interest has been the characterisation of molecular determinants with respect to the Opa_{CEA} proteins that mediate CEACAM recognition. For *N. gonorrhoeae* strain MS11, the binding profile of the complete Opa protein repertoire (with a total of 11 variants) has been determined. Several variants, such as Opa_{52}, recognise CEACAM1, CEACAM3, CEA (the product of the *CEACAM5* gene) and CEACAM6, whereas other Opa proteins only bind to CEACAM1 and CEA [42,43]. Such a differential binding pattern has also been observed for Opa_{CEA} proteins from other gonococcal and meningococcal strains [12,44]. Interestingly, the binding specificity of Opa_{CEA} for subgroups of CEACAM receptors is not reflected by sequence homologies between the respective Opa proteins, but seems to depend on the proper combination of the two HV domains [10].

Upon CEACAM binding, Opa_{CEA}-positive bacteria can be internalised by professional phagocytes (granulocytes and macrophages), but also by other cell types such as epithelial and endothelial cells (Figure 2). Thus, infection of human granulocytes expressing GPI-linked CEACAM6 and transmembrane forms of CEACAM1 and CEACAM3 with Opa_{CEA} gonococci or stimulation with

Figure 2



CEACAM-dependent interactions of Opa$_{CEA}$-expressing *Neisseriae* with different human cell types. **(a)** CEACAMs expressed on epithelial cells are clustered by tightly adhering Opa$_{CEA}$-positive bacteria. Rearrangements of the actin cytoskeleton are involved in the subsequent internalisation. In polarised epithelia, this process takes place at the apical surface and can lead via transcytosis to the release of the bacteria at the basolateral side. Opa$_{CEA}$ further allows multiple interactions with different human cell types. **(b)** Binding to CEACAMs on polymorphonuclear granulocytes results in opsonin-independent phagocytosis accompanied by activation of ASM, Src-family kinases, the small GTPase Rac and the serine/threonine kinases p21-activated kinase (PAK) and c-Jun amino-terminal kinase (JNK). **(c)** In activated T cells, binding of the Opa$_{CEA}$-positive microorganisms to CEACAM1 inhibits T-cell proliferation and activation, presumably by recruiting the tyrosine phosphatases SHP-1 and SHP-2. **(d)** Presence of bacteria-derived lipooligosaccharide or bacteria-induced pro-inflammatory cytokines such as tumour necrosis factor α stimulate the expression of CEACAM1 on endothelial cells, allowing enhanced adhesion of Opa$_{CEA}$-expressing *Neisseriae*.

anti-CD66-epitope antibodies result in elevated levels of Src family tyrosine kinase activity [45]. The increase in tyrosine kinase activity is accompanied by reduced levels of SHP1 tyrosine phosphatase activity, leading to a pronounced accumulation of tyrosine-phosphorylated cellular proteins in response to Opa$_{CEA}$-expressing, but not non-opaque, piliated gonococci [46]. As inhibition of tyrosine kinases blocks CEACAM-mediated uptake in granulocytes, Src kinase activity seems to be essential for the internalisation process, presumably by modulating the actin cytoskeleton. In addition, the importance of actin dynamics is highlighted by the involvement of the small GTPase Rac, a master regulator of the actin cytoskeleton [45,47•]. As is true for Opa$_{HS}$, Opa$_{CEA}$-initiated *Neisseria*–host-cell contact leads to a rapid stimulation of ASM, suggesting bacteria-triggered receptor clustering [48].

To address the role of individual CEACAMs in *Neisseria*-induced internalisation and signalling, various members of the CEACAM family have been overexpressed in HeLa, COS or CHO cells. These experiments have demonstrated that both transmembrane-type (CEACAM1 and CEACAM3) as well as GPI-linked receptors (CEA and CEACAM6) can mediate internalisation. However, individual receptors support different levels of internalisation via a particular Opa$_{CEA}$, with CEACAM1, CEACAM6 and CEA displaying lower levels of uptake compared with CEACAM3 [43,44]. In this regard, it is interesting to note that CEACAM1 phosphorylation and internalisation have been linked to insulin clearance by hepatocytes, suggesting that endocytosis of certain CEACAMs could have a physiological function and is not solely triggered by pathogenic bacteria [49].

Although the intracellular signals mediating internalisation by GPI-anchored CEA and CEACAM6 have remained elusive, CEACAM1 and CEACAM3 connect to intracellular signalling networks via their cytoplasmic tails. Several phosphorylation-independent associations of CEACAM1 and CEACAM3 with cytoplasmic proteins such as actin, tropomyosin, and calprotectin have been reported [50,51]. Nevertheless, receptor-initiated signalling seems to rely to a large extent on post-translational modifications involving the phosphorylation of tyrosine and serine residues [52–54]. Deletion or site-specific mutation of a tyrosine-based sequence motif in the cytoplasmic tail of CEACAM3 leads to a profound reduction in bacterial internalisation [47•,55]. Similar motifs have been detected in several receptors of the Ig superfamily and have been termed 'immunoreceptor tyrosine-based activation motifs' (ITAMs). Interestingly, ITAM tyrosine residues are usually phosphorylated by Src-family kinases, further indicating a critical role for Src-family kinases in CEACAM3-mediated internalisation. It is important to note that CEACAM3 expression is limited to granulocytes; therefore, it is tempting to speculate that

this human-specific receptor enables efficient phagocytosis, and probably enables elimination of CEACAM-binding bacteria by these immune effector cells.

Interestingly, the cytoplasmic domain of a CEACAM1 splice variant harbours an immunoreceptor tyrosine-based inhibition motif (ITIM) that functions to down-regulate intracellular signalling events such as calcium ion influx [56•]. The role of the ITIM motif in bacterial internalisation, however, has not been addressed so far. CEACAM1 has the broadest tissue expression of all CEACAMs and is not only abundantly expressed on epithelia (ranging from stomach, colon, kidney, gall bladder, liver, urinary bladder, prostate to cervix and endometrium), sweat and sebaceous gland cells, endothelia and granulocytes, but is also found on B and T cells. Recently, it has been demonstrated that gonococcal stimulation of the ITIM-bearing CEACAM1 expressed on T cells arrests the activation and proliferation of these cells in response to cytokines *in vitro*, suggesting that Opa_{CEA}-type gonococci are able to modulate T-cell responses via CEACAM1 *in vivo* [57••].

Although the lack of expression of CEACAMs on a small panel of isolated primary cells from target tissues of gonococci has cast doubt on a role for these receptors for the attachment and invasion at the primary infection site, it is important to stress that expression of CEACAMs is upregulated on epithelial and endothelial cells in response to inflammatory stimuli [58•,59•,60]. In addition, CEACAM expression is directed to the apical surface of polarised epithelia allowing, in contrast to HSPGs, access of the bacteria to the receptor from the lumenal side. In agreement with the apical exposure of CEACAMs, Wang *et al*. [61] observed efficient transcellular traversal through a tight-junction-forming epithelial cell layer by gonococci applied to the apical side. Interestingly, transcytosis through these epithelial cells that express CEACAM1, CEA, and CEACAM6 was dependent on expression of Opa_{CEA}-type proteins by the gonococci [61]. Taken together, these findings suggest that Opa_{CEA}-mediated binding to CEACAMs on the apical surface of polarised cells could be a means by which the bacteria tightly anchor themselves to the host cells. It might also provide an entry point to allow access to the inside of the cell or a gateway to deeper host tissues. As CEACAM recognition is not limited to pathogenic *Neisseria*, the biological implications of Opa_{CEA}-initiated cellular events could also apply to other nonpathogenic and pathogenic bacteria sharing the same ecological niche [62–65].

Conclusions

Although we have witnessed significant progress in the molecular understanding of neisserial virulence factors over the past few decades, research on the contribution of the identified factors to the infection process *in vivo* has been hampered by the lack of animal models for these human-specific pathogens. This is particularly true for the Opa proteins that have been shown to engage multiple host receptors expressed on a variety of cell types. Clearly, a critical role for Opa proteins in the infection process can be inferred from the selective survival advantage of opaque organisms *in vivo* [5,6]. However, it will be an important goal of future research to decipher which of the multiple interactions mediated by Opa proteins *in vitro* takes place at a certain location *in vivo*, and how Opa protein function is orchestrated with other known virulence factors of meningococci and gonococci. A step toward this goal might be the development of transgenic animals that express specific human receptors in defined tissues. Such engineered *in vivo* models might provide new insights into the complex interplay between these highly specialised pathogens and their human host.

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- • of special interest
- •• of outstanding interest

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