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CEACAM3: An innate immune receptor directed against human-restricted bacterial pathogens

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Abstract

Carcinoembryonic antigen-related cell adhesion molecule 3 (CEACAM3) is an immunoglobulin-related glycoprotein exclusively expressed on granulocytes. In contrast to other members of the CEACAM family, CEACAM3 does not support cell–cell adhesion, but rather mediates the opsonin-independent recognition and elimination of a restricted set of human-specific Gram-negative bacterial pathogens including *Neisseria gonorrhoeae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. Within the last 4 years, molecular determinants of CEACAM3 function and CEACAM3-initiated signaling pathways have been elucidated. Sequence comparison between CEACAM3 and other CEACAM family members points to a chimeric origin of this receptor with the bacteria-binding extracellular domain and the function-promoting intracellular domain derived from different genes. This review summarizes the current knowledge about the structure–function relationship of CEACAM3 and tries to combine these molecular aspects with a plausible scenario concerning the evolutionary origin of this phagocyte receptor in the light of host–pathogen adaptation.

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Keywords: ITAM; Signal transduction; Phagocytosis; Host–pathogen coevolution

Introduction

CEACAM3 belongs to the family of carcinoembryonic antigen (CEA)-related cell adhesion molecules (CEACAMs) that are characterized by a highly similar amino-terminal immunoglobulin variable (Ig_v)-like do-

main (Beauchemin et al., 1999; Kuespert et al., 2006). In humans, the CEACAM family comprises 12 closely related proteins, with several family members expressed by epithelial cells (e.g. CEACAM1, CEA, CEACAM6) (see also <http://cea.klinikum.uni-muenchen.de/>). These glycoproteins can mediate homotypic and heterotypic binding to CEACAMs located on neighboring cells, an interaction that requires the amino-terminal Ig_v-like domain and contributes to cell–cell adhesion as well as modulation of signal transduction (Kuespert et al., 2006; Obrink, 1997). In addition to their physiological roles, CEACAMs on epithelial cells are utilized by a number of human-specific bacterial pathogens, including *Neisseria gonorrhoeae*, *Haemophilus influenzae*, and

Abbreviations: CEA, carcinoembryonic antigen; CEACAM, carcinoembryonic antigen-related cell adhesion molecule; ITAM, immunoreceptor tyrosine-based activation motif.

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Moraxella catarrhalis, to contact their human host cells. Tight binding to CEACAMs expressed on the apical side of epithelia not only mediates intimate attachment of these microorganisms to the mucosa, but also triggers endocytosis of the bacteria, alters gene expression patterns in the host cells, and enhances extracellular-matrix binding of the infected cells (Bradley et al., 2005; Muenzner et al., 2005).

In contrast to these epithelial CEACAMs, the expression of other family members, such as CEACAM3, CEACAM4 and CEACAM8, is restricted to primate granulocytes, a cell type involved in the clearance of bacterial infection. In particular, CEACAM3 has not been found to engage in cell–cell adhesion with other CEACAM family members, despite the high sequence similarity of the amino-terminal Ig_V-like domain of CEACAM3 with CEACAM1 (87% amino acid sequence identity), CEA (92% amino acid sequence identity), or CEACAM6 (89% amino acid sequence identity) (Kuroki et al., 1991). The finding that *N. gonorrhoeae* can be phagocytosed in an opsonin-independent, but CEACAM-dependent manner by human granulocytes (Chen and Gotschlich, 1996; Gray-Owen et al., 1997a; Hauck et al., 1998; Virji et al., 1996), together with the demonstration that CEACAM3 is the main receptor responsible for this process (Schmitter et al., 2004, 2007b), has led to the suggestion that CEACAM3 is not involved in cell–cell adhesion, but rather represents a germline-encoded innate immune receptor targeted against human-specific pathogens. In this review, we will discuss the current understanding of CEACAM3 function for phagocytosis of bacteria and speculate about the evolutionary origin of this particular CEACAM family member.

Initial identification of CEACAM3

CEACAM3 was identified during an analysis of granulocyte-expressed non-specific cross-reacting antigen (NCA). NCA was initially defined by its cross-reactivity with antibodies against CEA, a well-established tumor marker, and, at the end of the 1980s, NCA was molecularly characterized (Neumaier et al., 1988). Using a cDNA probe corresponding to the amino-terminal domain of NCA-90 (now known as CEACAM6), several clones were isolated from a pooled human leukocyte-derived cDNA library. One clone (clone W264) encoded a glycoprotein of ~35 kDa (Kuroki et al., 1991). This glycoprotein, which corresponds to CEACAM3, was termed CEA gene family member 1 (CGM1), and three splice variants have been described (Nagel et al., 1993). The longest isoform, CGM1a, encodes a transmembrane protein with a 76-amino-acids long carboxy-terminal cytoplasmic domain.

The isoform CGM1c is similar to CGM1a, but due to alternative splicing harbors a 36-residue long cytoplasmic domain with distinct amino acid sequence (Nagel et al., 1993). In contrast, the cDNA clone of CGM1b lacks a transmembrane domain and would presumably lead to a secreted protein (Kuroki et al., 1991).

Already during the initial characterization, it was noticed that the cDNA for CGM1 is only found in human granulocytes and in white blood cells isolated from chronic myeloid leukemia (CML) patients. Furthermore, the CGM1a isoform could be detected in both normal granulocytes and leukemia cells, whereas the short isoform (CGM1c) was only present in leukocytes from a single CML patient and the cDNA of CGM1b could not be detected (Nagel et al., 1993). On the protein level, CGM1a has been assigned to the CD66 antigen family as it reacts with a number of CD66-specific monoclonal antibodies and was designated CD66d. Finally, in an overdue revision of the CEA-family nomenclature in 1999, the CGM1 protein and CD66d antigen have been termed CEACAM3 (Beauchemin et al., 1999). Interestingly, the murine and dog genome sequences do not encode a homolog of CEACAM3 (Kammerer et al., 2007; Zebhauser et al., 2005). Furthermore, cDNA cloning of sequences encoding CEACAM amino-terminal domains from African green monkey (*Cercopithecus aethiops*) and baboon (*Papio hamadryas*) failed to reveal an ortholog of human CEACAM3 (Zhou et al., 2001). These results suggest that human CEACAM3 is a rather recent invention in evolution, as it presumably occurred after the divergence of *Cercopithecidae* and *Hominoidae* approximately 24 million years ago (Kumar and Hedges, 1998).

CEACAM3 domain organization

CEACAM3 is a type 1 transmembrane protein encompassing an amino-terminal 34-residue signal peptide, a single extracellular Ig_V-like domain, a hydrophobic transmembrane domain as well as a cytoplasmic sequence (Fig. 1A). Whereas the Ig_V-like domain of CEACAM3 is closely related to CEACAM1, the cytoplasmic sequence differs from CEACAM1. The most distinctive feature within the cytoplasmic part of CEACAM3 is the presence of an immunoreceptor tyrosine-based activation motif (ITAM)-like sequence that is characterized by two precisely spaced tyrosine residues in a particular sequence context (Fig. 1B). ITAMs are known from a number of leukocyte receptors including the B cell receptor Ig α and Ig β chains, the Fc ϵ receptor I γ chain (Fc ϵ R1 γ), and the T cell receptor ζ chain (Cambier, 1995). Tyrosine phosphorylation within the ITAM is a critical event in signal

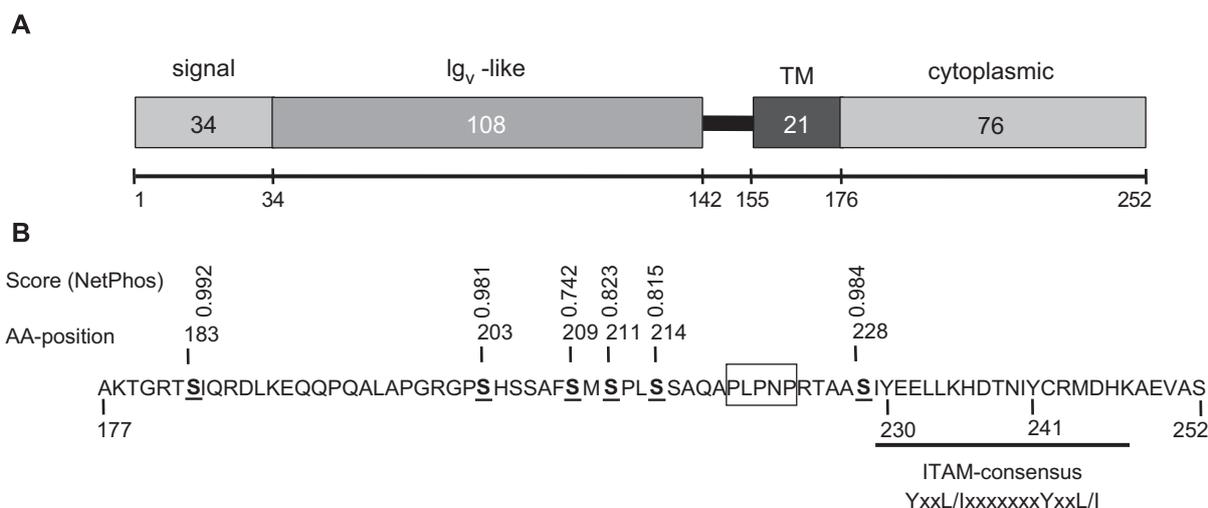


Fig. 1. Primary structure and sequence determinants of CEACAM3. (A) Primary structure of CEACAM3. CEACAM3 is a 252-amino acid (aa) type 1 transmembrane protein. The amino-terminal 34 aa signal peptide is cleaved off, leaving an extracellular part of 121 aa containing the Ig_v-like domain (108 aa) and a 13 aa linker connecting to the 21 aa transmembrane (TM) domain. CEACAM3 also possesses a 76 aa carboxy-terminal cytoplasmic tail. (B) Sequence motifs in the cytoplasmic domain of CEACAM3. The most distinctive feature of the cytoplasmic domain of CEACAM3 is an immunoreceptor tyrosine-based activation motif (ITAM)-like sequence (underlined) that closely resembles the indicated ITAM-consensus sequence. The tyrosine residues within this motif (Y-230 and Y-241) have been shown to be phosphorylated by Src family protein tyrosine kinases. In addition, the cytoplasmic domain includes several predicted serine phosphorylation sites (indicated in bold and underlined) (predicted by NetPhos; available online at <http://www.cbs.dtu.dk/services/NetPhos/>; [Blom et al., 1999]). These sites could be the target of PKA, PKC, or PKG activity. A proline-rich sequence may serve as a docking site for SH3 domain-containing proteins (boxed).

transduction by these receptors and appears to be important for CEACAM3-initiated cellular responses as well (see below). Besides the ITAM-like sequence, the cytoplasmic part of CEACAM3 contains a short proline-rich motif as well as numerous predicted serine phosphorylation sites (Fig. 1B).

CEACAM3-mediated pathogen recognition

Chen and Gotschlich (1996) identified CEACAM3 (at that time still termed CGM1a) as a granulocyte receptor responsible for the opsonin-independent recognition and uptake of Opa protein-expressing *Neisseria gonorrhoeae*. Stable transfection of HeLa cells with CEACAM3 cDNA allowed these cells to recognize and internalize *N. gonorrhoeae* as well as *E. coli* expressing neisserial Opa proteins. These results already highlighted the fact that CEACAM3 can operate in an autonomous manner in different cell types as apparently no other granulocyte-specific factors are needed to allow CEACAM3-mediated internalization. Infection of cell lines exposing distinct CEACAM family members on their surface with bacteria expressing a defined Opa protein demonstrated that CEACAM3 in particular leads to efficient internalization of microorganisms (Billker et al., 2002; Schmitter et al., 2004).

Early studies had shown that CEACAM-dependent interactions between granulocytes and gonococci pro-

mote the stimulation of cytoplasmic tyrosine kinases, reduce the activity of tyrosine phosphatases, increase tyrosine phosphorylation of cellular proteins, and stimulate the small GTPase Rac (Hauck et al., 1998, 1999). Therefore, it seemed likely that CEACAM3-mediated efficient uptake of bacteria might be regulated by these biochemical responses of the infected cells.

Regulation of CEACAM3-initiated phagocytosis

To dissect the molecular determinants of CEACAM3-initiated phagocytosis, mutations of the characteristic ITAM-like motif within the cytoplasmic domain of the receptor have been generated. Disabling the phosphorylation of the YxxLx₍₇₎YxxM motif in the CEACAM3 cytoplasmic domain by exchanging tyrosine for phenylalanine impairs CEACAM3-mediated responses (Billker et al., 2002; Chen et al., 2001; McCaw et al., 2003; Schmitter et al., 2004). Already a single conservative exchange of either Tyr-230 or Tyr-241 for phenylalanine reduces CEACAM3-dependent internalization of bacteria and the combination of these two mutations shows an additive effect (Billker et al., 2002; Chen et al., 2001; McCaw et al., 2003; Schmitter et al., 2004). Deletion of the complete cytoplasmic domain of CEACAM3 further reduces the capacity of the receptor to mediate uptake of bacteria, suggesting that intracellular determinants in

addition to the ITAM-like sequence contribute to this process (McCaw et al., 2003; Schmitter et al., 2004). The tyrosines within the ITAM-like sequence serve as targets for protein tyrosine kinases (PTKs) of the Src family that are activated upon bacterial engagement of CEACAM3 (McCaw et al., 2003). In fibroblasts deficient for the Src family PTKs c-Src, Fyn and c-Yes, CEACAM3 is not able to promote efficient uptake of Opa-expressing gonococci, whereas re-expression of c-Src in these cells re-constitutes bacterial internalization (Schmitter et al., 2007b). Upon receptor phosphorylation, the SH2 domain of c-Src is able to associate with CEACAM3 (Schmitter et al., 2007b). Though c-Src is not strongly expressed in human granulocytes, the closely related PTK Hck is also able to bind to CEACAM3 in a phosphorylation-dependent manner via its SH2 domain, and Hck is found to be recruited and strongly activated in phagocytes upon encounter with Opa protein-expressing gonococci (Hauck et al., 1998; Schmitter et al., 2007b).

Interestingly, the cytoplasmic tyrosine kinase Syk, which is known to critically contribute to ITAM-initiated uptake in the case of the Fc γ receptor, is not

required to stimulate CEACAM3-initiated phagocytosis (Sarantis and Gray-Owen, 2007). This is in line with previous reports showing that Syk is not activated upon CEACAM stimulation in human phagocytic cells (Hauck et al., 1998). A molecular explanation for this surprising finding is provided by the fact that the cytoplasmic domain of CEACAM3 can directly interact with the guanine-nucleotide exchange factor (GEF) Vav (Schmitter et al., 2007a). In particular, phosphorylated Tyr-230 of CEACAM3 selectively associates with the SH2 domain of Vav, suggesting a short circuit between bacterial engagement of CEACAM3 and the local stimulation of guanine nucleotide exchange activity (Fig. 2). Indeed, the recruitment of Vav appears as the critical molecular event required for CEACAM3-initiated GTP loading of Rac (Schmitter et al., 2007a). Interference with either Rac or Vav in primary human granulocytes blocks the efficient uptake of Opa-expressing gonococci (Schmitter et al., 2004, 2007a). GTP-loaded Rac operates as a master regulator of actin polymerization and NADPH oxidase function in phagocytes, suggesting that the signaling events initiated by CEACAM3 are responsible for the uptake as well as

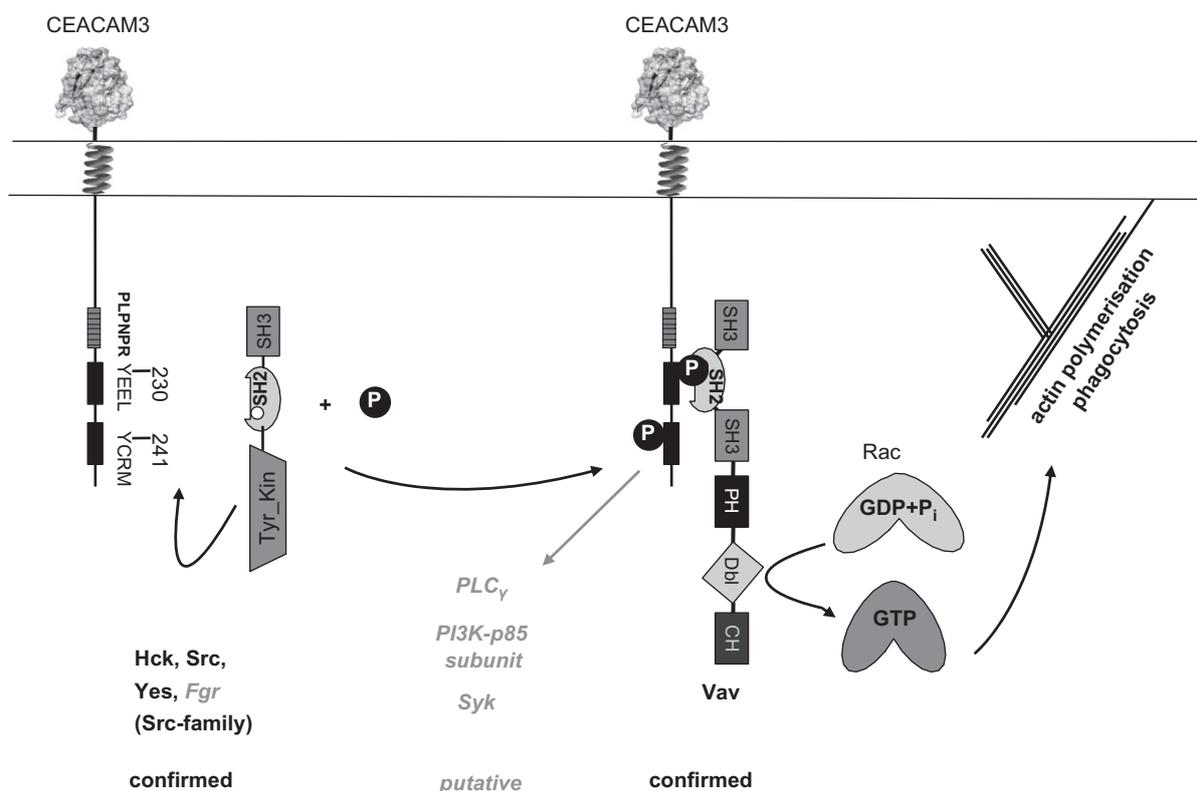


Fig. 2. Signaling connections of CEACAM3. Upon receptor engagement, the tyrosine residues in the ITAM-like sequence of CEACAM3 are phosphorylated by Src-family kinases (such as Src, Hck, or Fgr). The phosphorylated residues can then serve as docking sites for SH2 domain-containing molecules, and several interactions have been biochemically confirmed. The Rac-directed guanine-nucleotide exchange factor Vav has been shown to bind selectively to phosphorylated Y230 via its SH2 domain, linking CEACAM3 engagement to stimulation of the small G-protein Rac. GTP-bound Rac in turn will initiate the local formation of actin-based lamellipodia resulting in uptake of CEACAM3-bound bacteria. Several other signaling molecules are putative binding partners of the phosphorylated ITAM, as they have been found in proximity of bacteria-engaged CEACAM3 (shaded).

elimination of gonococci. Interestingly, interfering with the function of the closely related small GTPase Cdc42 in transfected human cells or primary human granulocytes does not impair CEACAM3-initiated phagocytosis pointing to a highly specific linkage between this receptor and Rac GTP loading.

In line with the critical role of Rac, actin cytoskeleton dynamics are essential for CEACAM3-initiated bacterial uptake, whereas other CEACAMs appear to be internalized via lipid raft-dependent processes (Muenzner et al., 2008; Schmitter et al., 2007b). Lipid raft association and, therefore, sensitivity to cholesterol depletion, discriminate between bacterial internalization via CEACAM1 or glycosylphosphatidylinositol (GPI)-anchored CEACAMs (CEA and CEACAM6) and CEACAM3-mediated uptake (Muenzner et al., 2008). In this regard it is interesting to point out that the opsonin-independent phagocytosis of Opa protein expressing bacteria by human granulocytes is not sensitive to cholesterol depletion (Schmitter et al., 2007b). These findings further support the idea that, despite the presence of multiple CEACAMs on granulocytes, CEACAM3 is the critical family member responsible for the efficient uptake of CEACAM-binding bacteria.

Several other molecules have been localized by fluorescence light microscopy together with bacteria-bound CEACAM3 such as the isolated SH2 domains of phosphatidylinositol-3 kinase or phospholipase C γ (Booth et al., 2003). However, it is currently not clear if these molecules interact directly with CEACAM3 and how they contribute to bacterial internalization by human granulocytes. Nevertheless, from the combined biochemical and functional studies described above it becomes apparent that the specific combination of an extracellular CEACAM Ig ν -like domain with a phagocytosis-promoting cytoplasmic domain in a single molecule allows human granulocytes to recognize and

eliminate CEACAM3-binding bacteria in the absence of opsonizing factors. Therefore, CEACAM3 could be seen as an evolutionarily optimized receptor to combat pathogens that exploit epithelial CEACAMs such as CEACAM1, CEA, or CEACAM6.

Ancestry and evolution of CEACAM3

Given the functional implication of CEACAM3-mediated recognition and elimination of pathogens it is interesting to ask for the origin of this protein within the rapidly evolving CEACAM family. Interestingly, human granulocytes express another CEACAM family member, CEACAM4, which perfectly matches the domain structure of CEACAM3. Both proteins feature a single extracellular Ig ν -like domain followed by a transmembrane region and a cytoplasmic domain encompassing ITAM-like sequences (Kuespert et al., 2006) (see also <http://cea.klinikum.uni-muenchen.de/>). Moreover, on a genomic level, the number of exons is the same for both the *CEACAM3* and the *CEACAM4* gene, but the length of the introns and therefore the entire loci are different: The *CEACAM3* gene covers about 7 kb more than the *CEACAM4* gene (based on total length of exons and introns) (Fig. 3). As with other *CEACAM* genes, the first two exons and the first intron are highly similar in length. Most of the length variation is due to intron 2, the large intron separating the exons encoding the Ig ν -like domain from the exons encoding the transmembrane and the cytoplasmic domain. Intron 2 contains sequences homologous between *CEACAM3* and *CEACAM4*, but the central segments are largely made up of LINE, SINE, and LTR repetitive elements (Fig. 3). Interestingly, the degree of sequence divergence between the *CEACAM3* and the *CEACAM4* gene

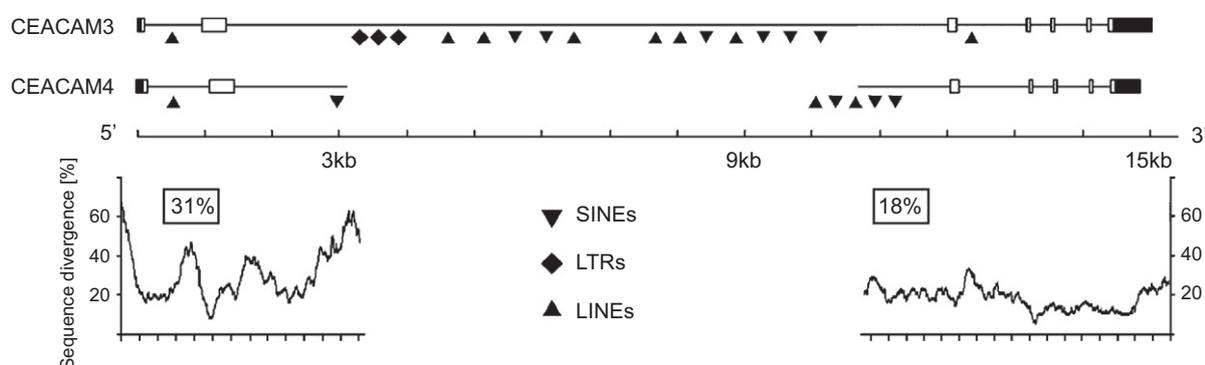


Fig. 3. Schematic alignment of *CEACAM3* and *CEACAM4* genomic regions. The position of exons is shown (boxes) with untranslated regions in black. The percentage sequence divergence (Jukes–Cantor method) is shown below the alignable 5' and 3' sections graphed from a sliding window of 200 bp (every 10 bp). The average pairwise distances for the 5' and 3' regions are also given in percent (boxed). Repetitive elements: the positions of short interspersed nuclear elements (SINEs), long terminal repeats (LTRs), and long interspersed nuclear elements (LINEs) are indicated. Insertions and deletions, including those representing repetitive elements, are not included in the pairwise difference calculations.

differs between the regions either side of the repeat-filled intron 2 (Fig. 3). This is also true on the protein level: whereas the first part of the carboxy-terminal intracellular domain is highly similar between CEACAM3 and CEACAM4 (identity 73%), the amino-terminal Ig_V-like domain differs considerably between these two family members (identity 49%) and is most similar between CEACAM3 and CEA (identity 92%). Therefore, CEACAM3 appears to be a natural chimera, with the Ig_V-like domain derived from a bacteria-recognizing CEACAM such as CEACAM1, CEA, or CEACAM6, but the cytoplasmic tail of the receptor that is able to induce phagocytosis via an ITAM-like sequence is most likely inherited from an ancestor of CEACAM4.

Together, these data about a potential chimeric origin of CEACAM3 nicely support the biochemical and functional analyses that have divided this receptor into a bacteria-recognizing extracellular module and a phagocytosis-promoting intracellular part.

CEACAM3 as an example of host–pathogen co-evolution

CEACAM-binding human pathogens, including *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Neisseria meningitidis*, can cause life-threatening conditions such as sepsis and bacterial meningitis (Erwin and Smith, 2007; Stephens, 1999; Verduin et al., 2002). However, the severe forms of disease are a dead-end for the pathogens, as these bacteria use the mucosa and not the blood or the cerebrospinal fluid of the infected person, as a platform for further transmission. Indeed, these bacteria are most often found as harmless inhabitants of the nasopharyngeal mucosa and only a subset of the known strains is associated with disease. Given this epidemiological background, it is not easy to reconcile how such microbes should pose a strong selective pressure that results in the evolution of a germline-encoded innate immune receptor. Interestingly, the pathogen *Neisseria gonorrhoeae*, which appears to have evolved rather recently from *N. meningitidis* (Feavers and Maiden, 1998; Vazquez et al., 1993), might have provided a strong impetus for our immune system to develop a novel innate defense mechanism.

On the one hand, gonococci possess an extraordinary antigenic variability of surface components and secrete an immunoglobulin A1 protease allowing these bacteria to readily escape acquired humoral immune defenses. The antigenic variability is also the major reason why there is still no vaccine against *N. gonorrhoeae* available. Furthermore, these bacteria also manage to inactivate or delude the complement system of human serum by recruiting the inhibitory complement component C4b-binding protein or by sialylation of their outer membrane lipooligosaccharide

with host-derived cytidine 5'-monophosphate-*N*-acetylneuraminic acid (Mandrell and Apicella, 1993; Ngampasutadol et al., 2005; Putten, 1993).

On the other hand, urogenital tract infections with *N. gonorrhoeae* can lead to infertility or early abortion. Though gonococci are rarely lethal, they can severely affect the reproductive success of individuals and, therefore, might have represented a strong selective pressure during human evolution. Of course this is highly speculative, but the advent of gonococci in the human population might have been the reason for the adaptive evolution of CEACAM3 as an innate immune receptor being able to recognize and eliminate potentially harmful CEACAM-binding gonococcal variants. As we are only able to evaluate the current status of the human–gonococcal co-evolution, we cannot determine who has an edge in this competing arms race. But the expansion of *opa* genes in the gonococcus (10–12 copies) compared to the meningococcus (2–4 copies) as well as the presence of Opa proteins in gonococci binding to CEA and CEACAM1, but avoid recognition by CEACAM3 (Bos et al., 1997; Gray-Owen et al., 1997b; Kuespert et al., 2007), already hint at the fact that the gonococcus itself is also adapting to this defense mechanism of its sole natural host.

Conclusions

In this review, we tried to summarize the current knowledge about the function and biochemical features of the phagocyte receptor CEACAM3 and speculated about the evolutionary origin of this molecule. CEACAM3 emerges as a specific adaptation of the innate immune system to cope with a small set of host-specific pathogens. A common feature of the recognized pathogens is their antigenic variability, allowing them to escape acquired immune responses. However, their ability to engage human CEACAMs by structurally diverse adhesins makes these microbes vulnerable to recognition by CEACAM3. The use of granulocyte CEACAM3 as a germline-encoded death trap for CEACAM-binding pathogens is tightly coupled to the cytoplasmic domain of this receptor. Importantly, signaling connections emanating from the ITAM-like sequence, in particular the direct binding to the Rac GEF Vav, render CEACAM3 a highly efficient phagocytotic receptor. The involvement of CEACAMs and in particular CEACAM3, in an arms race with specific bacterial pathogens, makes this an interesting system to study bacterial–host co-evolution.

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