Profiling of bacterial adhesin — host receptor recognition by soluble immunoglobulin superfamily domains

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Abstract

Several Gram-negative human pathogens recognize members of the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family. Pathogenic *Neisseriae* employ distinct isoforms of the colony opacity-associated proteins (Opa_{CEA} proteins) to bind to the amino-terminal domains of CEACAMs. Here we present a novel approach to rapidly determine the CEACAM-binding properties of single bacteria. Expression of the isolated amino-terminal domains of various CEACAMs in eukaryotic cells yields soluble probes that selectively recognize Opa_{CEA}-expressing bacteria in a pull-down assay format. Furthermore, by expressing soluble CEACAMs as fusions to green-fluorescent protein (CEACAM-N-GFP), CEACAM-binding bacteria can be decorated with a fluorescent label and analysed by flow cytometry allowing the specific detection of receptor binding events on the level of single bacteria. Besides its potential for rapid and quantitative analysis of pathogen–receptor interactions, this novel approach allows the detection of receptor recognition in heterogeneous bacterial populations and might represent a valuable tool for profiling the host binding capabilities of various microorganisms.

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1. Introduction

Bacterial invasion, the pathogen-induced entry into eukaryotic cells, affords bacteria protection from immune effector mechanisms and facilitates access to deeper tissues. Invasion into host cells is often triggered by interaction of specialized bacterial surface proteins, so-called adhesins, with eukaryotic cell surface receptors (Finlay and Cossart, 1997). In many instances, invasive bacteria, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Shigella flexneri*, *Neisseria meningitidis* and *Neisseria gonorrhoeae* exploit cellular adhesion molecules, such as cadherins, integrins, or members of the immunoglobulin superfamily of cell adhesion molecules (IgCAMs), to gain entry into the cell (Hauck et al., 2006). In the case of the human-specific pathogens *N. gonorrhoeae* (Ngo) and *N. meningitidis* (Nme), invasion can be mediated by colony opacity associated (Opa) proteins that are integral membrane proteins of the outer membrane of these Gram-negative bacteria (Hauck and Meyer,

2003). Whereas the meningococcal genome encodes up to 4 distinct Opa proteins, gonococci harbour up to 11 copies of Opa genes (Bhat et al., 1992). Expression of Opa proteins is subject to phase variation due to a RecA independent insertion or deletion of pentanucleotide repeats within the leader peptide coding sequence which leads to translational reading frame shifts in these constitutively transcribed *opa* genes (Stern et al., 1986). Phase variation of individual Opa proteins occurs at a frequency of $\sim 10^{-3}$ and results in a heterogeneous population of bacteria expressing none, one or multiple Opa proteins. Interestingly, recent studies from Toleman et al. (2001) demonstrated the expression of Opa-like proteins in diverse commensal strains of *Neisseria*, including *N. lactamica* and *N. subflava*. Besides a few Opa protein variants that recognize cell surface expressed heparansulfate proteoglycans (Opa_{HSPG}) (Chen et al., 1995; van Putten and Paul, 1995), most Opa proteins of diverse strains of Nme and Ngo recognize one or more members of the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family (Chen and Gotschlich, 1996; Gray-Owen et al., 1997a; Virji et al., 1996b). These Opa proteins bind to the non-glycosylated face of the immunoglobulin-variable (IgV)-like amino-

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terminal domain of CEACAMs and have been collectively referred to as Opa_{CEA} (Hauck and Meyer, 2003). CEACAMs are composed of a single IgV-like amino-terminal domain followed by up to 6 Ig-constant (IgC2)-like domains (Kuespert et al., 2006). These highly glycosylated proteins are anchored to the membrane either by glycosylphosphatidylinositol (GPI) moieties (CEA, CEACAM6, CEACAM7, CEACAM8) or by trans-membrane and cytoplasmic domains (CEACAM1, CEACAM3, CEACAM4). Opa_{CEA}–CEACAM interaction has been found for four members of the CEACAM-family, namely CEACAM1, CEACAM3, CEA and CEACAM6 (Bos et al., 1997; Gray-Owen et al., 1997b; Muenzner et al., 2000). Depending on the CEACAM isoform involved, engagement of these receptors can result in different outcomes for the bacteria. For example, engagement of CEACAM1 or CEACAM6, two family members found on the apical membrane of human epithelial cells, can lead to enhanced matrix adhesion of the infected host cell, a process that could counteract the exfoliation response of epithelia and promote colonization of the human mucosa (Muenzner et al., 2005). Furthermore, neisserial binding to CEACAM1 expressed on CD4-positive T-cells can arrest T-cell proliferation *in vitro* and might influence the acquired immune response against gonococci *in vivo* (Boulton and Gray-Owen, 2002). In contrast, binding of Opa_{CEA} proteins to CEACAM3, a receptor present only on human granulocytes, results in recognition, efficient phagocytosis, and elimination of the bound pathogens (Schmitter et al., 2004). Interestingly, human Gram-negative pathogens apart from *Neisseria*, such as *Haemophilus influenzae* (Virji et al., 2000) and *Moraxella catarrhalis* (Hill and Virji, 2003) have also been shown to recognize CEACAMs by diverse adhesins.

Accordingly, the analysis of the receptor binding profile of CEACAM-recognizing bacteria is critical to predict the potential cellular interactions mediated by these bacterial strains. Though several approaches such as adhesion and invasion assays with CEACAM-transfected cell lines (Bos et al., 1997; Gray-Owen et al., 1997b) as well as receptor overlay assays (Virji et al., 1996a) have been employed, they all involve time consuming handling and/or produce only semi-quantitative data.

Therefore, we describe in this study a receptor-binding assay which allows rapid analysis of Opa_{CEA}–CEACAM interaction. Our method is based on recognition of CEACAM-binding bacteria by soluble, GFP-tagged N-terminal domains of CEACAMs. Bacterial–CEACAM association with soluble CEACAM domains can be analysed either by Western blot or by flow cytometry allowing rapid detection of bacteria–CEACAM interaction. In addition to profiling the CEACAM-binding potential of various microorganisms, this approach is useful for detecting CEACAM-binding microbes in heterogeneous bacterial populations.

2. Materials and methods

2.1. Cell culture and bacteria

The human embryonic kidney cell line 293T (293 cells) was grown in DMEM/10% calf serum (CS) at 37 °C, 5% CO₂. Cells were subcultured every 2–3 days. Neisserial strains were grown at 37 °C, 5% CO₂ on GC agar (Gibco BRL, Paisely, UK) supplemented with 1% vitamin-mix (500 mM dextrose, 70 mM L-glutamine, 150 mM L-cystein–HCl, 0.2 mM cocarboxylase,

Table 1
Primers and templates used in this study to construct soluble CEACAM variants

Primer	Sequence (5'–3')	Template	Product
CEA1-sense	GAAGTTATCAGTCGACCAGCTCACTACTGAATCCATGCC	cDNA human CEACAM1	CEA1-NA1BA2
CEA1-NA1BA2-anti	ATGGTCTAGAAAGCTTGGGTCGCTTTGGTTCTTACTGATTGG		
CEA1-sense	see above	cDNA human CEACAM1	CEA1-NA1B
CEA1-NA1B-anti	ATGGTCTAGAAAGCTTGGTGTGGTCTCTGTTGCAGC		
CEA1-sense	see above	cDNA human CEACAM1	CEA1-N
CEA1NT-anti	ATGGTCTAGAAAGCTTTTGAAGTACTCTGGCCCGTATACATGGAAGTGTCCAGT		
CEA3NT-sense	GAAGTTATCAGTCGACAAGCTCACTATTGAATCCATGCC	cDNA human CEACAM3	CEA3-N
CEA3NT-anti	ATGGTCTAGAAAGCTTTTCACTATACATGGAAGTGTCCAGTTGC		
CEA4NT-sense	GAAGTTATCAGTCGACACCATGGGCCCCCTCAGCC	cDNA human CEACAM4	CEA4-N
CEA4NT-anti	ATGGTCTAGAAAGCTTTGCTTGAAGTACTCTGGGTGTACGTGGAGCTGGCCAG		
CEA5NT-sense	GAAGTTATCAGTCGACACCATGGAGTCTCCTCGGCC	cDNA human CEACAM5	CEA5-N
CEA5NT-anti	ATGGTCTAGAAAGCTTTGCTTGAAGTACTCTGGGTGTACCCGGAAGTGGCCAGTTGC		
CEA6NT-sense	GAAGTTATCAGTCGACACCATGGGACCCCCCTCAGCC	cDNA human CEACAM6	CEA6-N
CEA6NT-anti	ATGGTCTAGAAAGCTTTGCTTGAAGTACTCTGGGTGTACATGGAAGTGTCCGGTTGC		
CEA8NT-sense	GAAGTTATCAGTCGACACCATGGGGCCCCATCTCAGCC	cDNA human CEACAM8	CEA8-N
CEA8NT-anti	ATGGTCTAGAAAGCTTTTGAAGTACTCTGGCCCATGTACGCTGAACTGGCCAGT		

50 μM $\text{Fe}(\text{NO}_3)_3$, 9.0 μM thiamine–HCl, 0.4 mM NAD, 7.5 μM vitamin B₁₂, 0.7 mM L-arginine, 95 μM *p*-aminobenzoic acid, 4.5 mM L-cystine, 5.5 mM adenine, 4.5 mM uracil, 0.2 mM guanine) and the appropriate antibiotics (7 $\mu\text{g}/\text{ml}$ erythromycin and/or 10 $\mu\text{g}/\text{ml}$ chloramphenicol). The phenotype of bacterial colonies (opaque or non-opaque) was visually examined using a stereomicroscope and the bacteria were subcultured every 24 h. Opa_{HSPG} (Opa₅₀) - and Opa_{CEA} (Opa₅₂-, Opa₅₅-, and Opa₆₀)-expressing, non-piliated *N. gonorrhoeae* MS11 strains, an Opa-negative, non-piliated *N. gonorrhoeae* MS11 strain as well as commensal neisserial strains including *N. cinerea*, *N. lactamica*, *N. mucosa*, and *N. sicca* were obtained from T.F. Meyer (MPI Infektionsbiologie, Berlin, Germany). Opa-expressing, non-capsulated *N. meningitidis* strain MC58 (*siaD* mutant) was provided by M. Frosch (Institut für Hygiene und Mikrobiologie, Universität Würzburg, Germany). Opa-negative, non-capsulated *N. meningitidis* MC58 strain was isolated from Opa-positive MC58 strain by visual screening for colonies with non-opaque phenotype.

2.2. Recombinant DNA constructs

Plasmids encoding cDNA of human CEACAM1, CEACAM3, CEACAM4, CEA (the product of the *CEACAM5* gene), CEACAM6 and CEACAM8 were provided by W. Zimmermann (Universitätsklinikum Grosshadern, München, Germany) and used as a template for PCR amplification of amino-terminal domains of diverse CEACAMs and soluble variants of CEACAM1. For PCR amplification, appropriate primers (see Table 1) and a mixture of Taq- and Vent-polymerase (New England Biolabs, Beverly, MA) were used. The resulting PCR fragments were cloned into pDNR-Dual using the In-Fusion Dry-down PCR Cloning Kit (Clontech, Mountain View, CA), verified by sequencing, and transferred by Cre-mediated recombination into pLPS-3'EGFP (Clontech) resulting in GFP fused to the carboxy-terminus of the expressed proteins.

2.3. Transfection and cell culture supernatants

Transfection of 293 cells was accomplished by standard calcium phosphate co-precipitation using 8 μg plasmid/10 cm culture dish as previously described (Schmitter et al., 2004). 24 h after transfection, culture medium was replaced by OptiMem (Gibco BRL). Culture supernatants were collected 3 days after transfection and purified from cell debris by centrifugation (2500 $\times$ g, 4 °C, 10 min). Supernatants were adjusted for equal levels of soluble CEACAMs and used for pull-down assays. Control supernatants were prepared from cells transfected with the empty expression vector.

2.4. Bacterial pull-down

Bacteria were grown overnight for 18 h on GC agar plates. Bacteria were scraped from plates, suspended in PBS, and colony forming units (cfu) were estimated by OD₅₅₀ readings according to a standard curve. Bacteria were washed twice with PBS containing 0.9 mM CaCl₂ and 0.5 mM MgCl₂ (PBS⁺) and

4 $\times$ 10⁶ cfu were suspended in 1 ml cell culture supernatant containing the indicated receptor protein. Bacteria were incubated with the soluble receptor domains for 30 min at 20 °C with head-over-head rotation. After incubation, bacteria were washed twice with PBS⁺ and either boiled in SDS sample buffer prior to SDS-PAGE and Western blotting or taken up in PBS/2% FCS (flow buffer) and analysed by flow cytometry.

2.5. SDS-PAGE and Western Blot

Proteins were separated by SDS-PAGE using 12.5% polyacrylamid gels. Western blots were performed as described previously (Schmitter et al., 2004) using mAbs against GFP (clone JL-8; BD Biosciences, Heidelberg, Germany) or against Opa proteins (clone 4B12; provided by M. Achtmann; MPI Infektionsbiologie, Berlin, Germany).

2.6. Flow cytometry

For flow cytometry, 4 $\times$ 10⁶ bacteria were resuspended in 1 ml flow buffer. Samples were analysed on a FACSCalibur (BD Biosciences) by gating on the bacteria based on forward and sideward scatter and measuring bacteria-associated GFP-fluorescence in fluorescence channel 1. In each case, 20,000 events per sample were acquired.

3. Results

3.1. Soluble CEACAM1-GFP constructs recognize neisserial Opa_{CEA} proteins

Functional studies with transfected cell lines and receptor binding assays have indicated that the amino-terminal domains of CEACAMs are sufficient for the association with neisserial Opa proteins (Virji et al., 1999, 1996b). To confirm these data, we cloned distinct portions of the extracellular domain of human CEACAM1 in frame with the coding sequence for green fluorescence protein (GFP) omitting the transmembrane and cytoplasmic domain of CEACAM1 (Fig. 1A). The resulting proteins were secreted in the cell culture supernatant of transfected 293T cells and contained the amino-terminal domain of CEACAM1, followed by either none (CEA1-N-GFP), two (CEA1-NA1B-GFP) or three (CEA1-NA1BA2-GFP) IgC2-like extracellular domains of CEACAM1 fused to GFP (Fig. 1B). The cell culture supernatants containing recombinant soluble CEA1-NA1BA2-GFP, CEA1-NA1B-GFP or CEA1-N-GFP were employed in pull-down experiments. For this purpose, Opa_{CEA}-expressing *N. gonorrhoeae* or non-opaque gonococci were incubated with the supernatants for 30 min, then washed and analysed for CEACAM binding by Western blotting with anti-GFP antibodies. As demonstrated in Fig. 1C, Opa-positive gonococci bound each soluble CEACAM1 variant, whereas Opa-negative gonococci did not associate with any of these proteins. These data demonstrate that soluble CEACAM1 variants bind in an Opa protein-dependent manner to gonococci and the amino-terminal domain of CEACAM1 is sufficient to mediate binding to intact bacteria.

Fig. 1. Association of neisserial Opa_{CEA} proteins with soluble CEACAM1-GFP constructs. (A) Schematic drawing of CEACAM1 wildtype (WT) and soluble CEACAM1-GFP constructs employed in this study. Each soluble CEACAM1 construct contains the amino-terminal IgV-like domain of CEACAM1 followed by either one (CEA1-N-GFP), two (CEA1-NA1B), or three (CEA1-NA1BA2) IgC2-like extracellular domains of CEACAM1 fused to GFP. Additional soluble CEACAM constructs used in subsequent experiments (CEA3-N-GFP, CEA4-N-GFP, CEA5-N-GFP, CEA6-N-GFP and CEA8-N-GFP) are constructed analogous to CEA1-N-GFP. (B) Expression of soluble CEACAM1-GFP constructs. Culture supernatants of transiently transfected 293 cells were harvested and analysed for soluble CEACAM1-GFP constructs by Western blotting using anti-GFP antibody. (C) Bacterial pull-down assays with soluble CEACAM1-GFP constructs. Opa₅₂-expressing (Ngo Opa₅₂) or non-opaque *N. gonorrhoeae* (Ngo Opa⁻) were incubated with culture supernatants containing either CEA1-N-GFP, CEA1-NA1B-GFP or CEA1-NA1BA2-GFP, then washed and analysed for CEACAM binding and Opa-expression by Western blotting using anti-GFP or anti-Opa antibody, respectively.

3.2. The amino-terminal domain of CEACAM8 does not associate with Opa_{CEA}-expressing gonococci

In contrast to CEACAM1, the closely related CEACAM8 does not bind to any Opa protein characterized so far. Therefore, to test the specificity of the observed Opa–CEACAM interaction, a soluble form of the amino-terminal domain of CEACAM8 (CEA8-N-GFP) was produced in 293 cells (Fig. 2A). Pull-down experiments employing CEA1-N-GFP and CEA8-N-GFP demonstrated that Opa₅₂-expressing gonococci were able to bind CEA1-N-GFP, whereas no binding to CEA8-N-GFP was observed (Fig. 2B). Again, Opa-negative gonococci did not bind to CEA1-N-GFP nor CEA8-N-GFP (Fig. 2B). Together, these data support the view that the soluble amino-terminal IgV-like domain of CEACAM1 fused to GFP displays the binding specificity known for the intact receptor.

3.3. Diverse commensal *Neisseria* spp. do not associate with the amino-terminal domain of CEACAM1

As the amino-terminal domain of CEACAM1 is sufficient to mediate association with Opa_{CEA}-proteins of gonococci and

Fig. 2. Selectivity of Opa_{CEA} recognition by soluble CEACAM amino-terminal domains. (A) Culture supernatants from 293 cells transfected with CEA1-N-GFP, CEA8-N-GFP, or the empty control vector (pcDNA) were harvested and analysed for the presence of soluble amino-terminal IgV-like domains by Western blotting using anti-GFP antibody. (B+C) Bacterial pull-down assays with CEA1-N-GFP and CEA8-N-GFP. (B) Opa₅₂-expressing or non-opaque *N. gonorrhoeae* (Ngo) were incubated with culture supernatants containing either CEA1-N-GFP or CEA8-N-GFP, then washed and analysed for CEACAM binding and Opa-expression by Western blotting using anti-GFP antibody and anti-Opa antibody, respectively. (C) Diverse commensal *Neisseria* species were incubated with culture supernatants containing CEA1-N-GFP and analysed as in (B) for CEACAM binding and Opa-expression. Opa₅₂-expressing gonococci and non-opaque gonococci served as a positive and negative control, respectively.

Fig. 3. Binding profile of GFP-tagged amino-terminal CEACAM domains to distinct neisserial Opa_{CEA} proteins. (A) The indicated soluble GFP-tagged amino-terminal CEACAM domains were expressed in 293 cells and culture supernatants were analysed for soluble CEACAM-GFP constructs by Western blotting using anti-GFP antibody. (B) *N. gonorrhoeae* (Ngo) strains expressing Opa_{HSPG} protein (Opa₅₀), distinct Opa_{CEA} proteins (Opa₅₂, Opa₅₅, or Opa₆₀), as well as Opa-positive and Opa-negative *N. meningitidis* (Nme) were incubated with culture supernatants containing the indicated soluble CEACAM variants, then washed and analysed for CEACAM binding by Western blotting using anti-GFP antibody. Bacterial lysates were analysed for Opa-expression with anti-Opa antibody. Non-opaque gonococci served as a negative control.

binding is specific for Opa_{CEA}-recognizing CEACAMs, we further analysed the binding capacity of CEA1-N-GFP to commensal *Neisseria*, which in part express Opa-like proteins that are cross-reactive with Opa protein-specific monoclonal antibodies (Toleman et al., 2001). Western blot analysis of the commensal neisserial strains used in this study confirmed the existence of possible Opa-like proteins. In particular, *N. lactamica* expressed a protein with an approximate size of 27 kDa, which is similar to the size of gonococcal and meningococcal Opa proteins, that cross-reacted with an Opa-specific monoclonal antibody (Fig. 2C). Pull-down experiments with commensal *Neisseriae* incubated with cell culture supernatant containing CEA1-N-GFP demonstrated that, regardless of the presence or absence of proteins reacting with monoclonal anti-Opa protein antibodies, none of the investigated strains associated with the amino-terminal domain of CEACAM1 (Fig. 2C). These data demonstrate that soluble receptor domains can be employed to screen for receptor-recognizing pathogens.

3.4. Soluble amino-terminal domains of human CEACAMs allow profiling of neisserial Opa adhesins

Previous investigations have demonstrated that individual Opa_{CEA} proteins mediate adhesion to distinct sets of cell surface exposed CEACAMs. For example, Opa₅₂ has been shown to recognize CEACAM1, CEACAM3, CEA, and CEACAM6, whereas Opa₆₀ and Opa₅₅ have a more limited binding spectrum by associating only with CEACAM1 and CEA or with CEA alone, respectively (Gray-Owen et al., 1997b). To determine, if soluble receptors also discriminate in their binding to Opa_{CEA} proteins, first, the N-terminal IgV-like domains of all available human CEACAMs, including CEACAM1, CEACAM3, CEACAM4, CEA, CEACAM6, and CEACAM8 were produced in 293 cells in the form of GFP-fusion proteins (Fig. 3A). These constructs were used for interaction studies with defined gonococcal strains expressing distinct Opa_{CEA} proteins (Opa₅₂, Opa₅₅, Opa₆₀) or expressing Opa_{HSPG} (Opa₅₀). Isogenic, non-opaque gonococci served as a negative control.

Fig. 4. Detection and quantification of CEACAM-binding bacteria by flow cytometry. Non-opaque gonococci (Ngo Opa⁻), Opa₅₂-expressing gonococci (Ngo Opa₅₂), or a heterogeneous gonococcal population (prepared by mixing Opa-negative and Opa-positive bacteria in a 1:2 ratio) were incubated with culture supernatants containing CEA1-N-GFP or with supernatants derived from control transfected cells. After washing, bacteria were analysed for CEACAM binding by flow cytometry by measuring GFP-derived fluorescence associated with bacteria. In each case, 20,000 events were measured. Shown are the original dot blots of a representative experiment. The numbers indicate the percentage of gonococci associated with CEA1-N-GFP.

In addition, Opa-positive and Opa-negative *N. meningitidis* (Nme) were analysed. Pull-down assays revealed that with the exception of CEA4-N-GFP and CEA8-N-GFP all recombinant proteins bound at least to one neisserial strain (Fig. 3B). Furthermore, the binding pattern of the isolated amino-terminal domains fused to GFP reflected the specificity known from cell-based assays of gonococcal–host cell adhesion. In particular, gonococci expressing Opa₅₂ interacted with four of the investigated amino-terminal domains, whereas Opa₆₀-expressing bacteria only associated with the amino-terminal domains of CEACAM1 and CEA, and Opa₅₅-expressing gonococci selectively bound to CEA (Fig. 3B). Importantly, *N. gonorrhoeae* expressing the heparansulphate proteoglycan-binding Opa protein (Opa₅₀) did not associate with a single CEACAM amino-terminal domain further confirming the specificity of the assay. The pull-down assay also allowed the profiling of CEACAM-recognition by *N. meningitidis*. Interestingly, Opa-expressing meningococci selectively bound to the amino-terminal domain of CEACAM1, but not to any other family member (Fig. 3B). Together, these analyses demonstrated that soluble GFP-fusions of CEACAM amino-terminal IgV-like domains were able to discriminate between different Opa proteins. Furthermore, pull-down experiments with these recombinant receptor domains allow the profiling of CEACAM binding capabilities of human pathogens such as gonococci and meningococci.

3.5. GFP-tagged amino-terminal domains are powerful tools to analyse CEACAM association in heterogeneous microbial populations

Soluble amino-terminal domains of various CEACAMs provide a convenient means to analyse the Opa protein binding specificity of *Neisseriae* by pull-down assays and Western blotting. However, the fluorescent nature of the GFP-tag, which is linked to the used amino-terminal CEACAM domains, should also allow fast detection and quantitative analysis of Opa_{CEA}–CEACAM interactions by flow cytometry. To explore this possibility, Opa-positive and Opa-negative gonococci were incubated with cell culture supernatants containing CEA1-N-GFP or with supernatants derived from control transfected cells. After washing, bacteria were subjected to flow cytometric analysis. In the absence of a GFP-tagged CEACAM1 amino-terminal domain, both non-opaque as well as Opa_{CEA}-expressing gonococci did only show a weak background fluorescence (Fig. 4). However, in the presence of a GFP-tagged amino-terminal domain of CEACAM1 an about 100-fold increase in fluorescence of the pathogens was observed and more than 99% of the bacterial population associated with the fluorescent protein (Fig. 4). In contrast, only about 2% of the bacteria in the non-opaque population bound to CEA1-N-GFP (Fig. 4) suggesting that the bacteria associated GFP-fluorescence detected with the flow cytometer can be used as a direct measure of Opa_{CEA}–CEACAM interaction. Moreover, when non-opaque and Opa_{CEA}-expressing gonococci were mixed in a 1:2 ratio, the obtained fluorescence signal almost perfectly reflected the heterogeneous population (Fig. 4). Indeed, in the mixed popu-

lation about 66% of the bacteria were found to associate with CEA1-N-GFP, whereas the rest of the microbes displayed low background fluorescence (Fig. 4). Both gonococcal variants could be observed as clearly distinct populations based on their GFP-fluorescence signal that differed about 100-fold between the CEA1-N-GFP-binding and non-binding fraction. Together, these data highlight the potential of GFP-tagged soluble CEACAM amino-terminal domains to detect and quantify pathogen–receptor interactions on a single cell level. Therefore, this approach allows the analysis of bacterial populations with heterogeneous Opa protein expression that reflects the natural status observed in clinical isolates.

4. Discussion

Profiling of the receptor-binding capacity of bacterial adhesins is an important pre-requisite to determine the host cell contacts that are potentially exploited by a specific pathogen isolate. Widely used methods for defining adhesin–receptor interactions such as adhesion and invasion assays with mutant bacteria and transfected cell lines as well as receptor-overlay assays have considerable disadvantages with respect to the labour-intensive procedures and/or the semi-quantitative data yielded by the assay. In the current study, we present a rapid and simple method that utilizes soluble receptor domains as a molecular tool for quantitative analysis of bacterial–receptor association. Briefly, incubation with soluble GFP-fusions of the involved receptor domains decorates the bacteria with a fluorescent label, which can be either detected by Western blotting or directly analysed by flow cytometry. Remarkably, implementation of flow cytometry for exploring bacterial–receptor association enables a rapid profiling within less than an hour and allows the sensitive detection of receptor-binding microorganisms in heterogeneous populations.

To demonstrate the feasibility of our method we took advantage of the well characterized recognition of human CEACAMs by Opa proteins of pathogenic *Neisseriae*. Importantly, the soluble GFP-tagged amino-terminal CEACAM domains not only specifically associated with neisserial Opa_{CEA} protein variants, but also reproduced the pattern of CEACAM isoform recognition known from adhesion assays with CEACAM transfected HeLa cells (Gray-Owen et al., 1997b). Hence, soluble GFP-tagged CEACAM amino-terminal domains constitute selective molecular probes to characterize CEACAM-binding Opa proteins. An additional application of our approach is the rapid profiling of CEACAM recognition by various bacterial strains. A screen for CEACAM association by diverse commensal neisserial strains, which in some instances have been reported to express Opa-like proteins (Tolman et al., 2001), demonstrated that, contrary to their pathogenic relatives, none of the investigated commensal neisserial strains recognized the IgV-like amino-terminal domain of CEACAM1. Further analyses have shown that none of the amino-terminal domains derived from other human CEACAMs binds to these commensal strains (data not shown). Apart from screening within the group of *Neisseria*, amino-terminal domains of CEACAMs might be useful tools to quickly analyse the prevalence of CEACAM

binding in other bacterial genera. In this context, bacteria sharing ecological niches and lifestyles similar to pathogenic *Neisseria* could be interesting candidates to screen for CEACAM recognition. Indeed, several Gram-negative pathogens colonizing the human mucosa, such as *M. catarrhalis* and *H. influenzae* have evolved distinct surface antigens that are also able to engage human CEACAMs (Hill et al., 2001; Hill and Virji, 2003; Virji et al., 2000). As a large number of bacterial pathogens targets cellular receptors to establish an intimate contact with host cells, the described method might be a useful approach for analysing additional adhesin–receptor interaction pairs. However, it is important to keep in mind that the method described here utilizes bacterial adhesins able to recognize monomeric forms of the receptor. In such cases, the adhesin binding domain of the involved host receptor can be produced in a soluble form and, either upon coupling with a fluorescent dye or genetically fused to a fluorescent protein, might be used as a powerful tool for analysing adhesin binding. One potential further application of our method might be the analysis of the interaction between InternalinA of *L. monocytogenes* and human E-cadherin. InternalinA is a surface exposed adhesin of the facultative intracellular pathogen *L. monocytogenes* that binds with high affinity to the extracellular amino-terminal domain of the epithelial cell receptor E-cadherin (Lecuit et al., 1999; Schubert et al., 2002). Hence, soluble, fluorescently-labeled amino-terminal domains of E-cadherin should enable a rapid profiling of the binding capabilities of diverse *Listeria* species and strains.

An outstanding feature of our novel approach is not only its ability to rapidly determine pathogen–receptor interactions, but also its capacity to detect and quantify pathogen–receptor interactions on a single cell level. Such a sensitive quantification can be achieved by analysing pathogen–receptor interactions using flow cytometry. Indeed, the fluorescence associated with each individual microorganism is recorded allowing the discrimination between bacteria binding the GFP-tagged soluble receptor and bacteria not binding the receptor. As demonstrated by using a defined heterogeneous population of Opa protein expressing as well as non-opaque bacteria, the two phenotypes could be correctly distinguished based upon their interaction with the CEACAM1 amino-terminal domain. Interestingly, the flow cytometric analysis also detected about 2% of CEACAM-binding bacteria in the non-opaque gonococcal population. As in each experiment the non-opaque and the Opa-expressing gonococci were visually screened for their colony phenotype, it can be assumed that indeed a small percentage of Opa protein expressing organisms are contained within non-opaque colonies. This is due to the on/off phase switching of Opa protein expression that occurs independently for each of the 11 *opa* gene loci encoded within the *N. gonorrhoeae* MS11 genome. Though expression of one Opa protein (Opa₃₀ or OpaA) is genetically disrupted in the used MS11 strains by a chloramphenicol resistance cassette, non-opaque bacteria can still revert to the opaque phenotype at one of the remaining 10 loci and this reversion seems to occur at a frequency of 10^{-3} (Kupsch et al., 1993; Stern et al., 1986). The detection of a very low percentage of CEACAM-binding

bacteria in the visually selected non-opaque neisserial population therefore highlights the enormous sensitivity of this novel approach allowing the recognition of minute amounts of positive phenotypes in heterogeneous mixtures of bacteria. In principle, the method could be expanded to cover the detection of multiple adhesive interactions by tagging different soluble receptor molecules with distinct fluorophores such as cyan fluorescent protein, yellow fluorescent protein, or red fluorescent protein (Verkhusha and Lukyanov, 2004). In a kind of multiplexing, binding of several soluble receptors, tagged with distinct fluorophores and added to a single sample, could be rapidly analysed and quantified by flow cytometers that allow optical separation of the different fluorescence signals.

Taken together, we present a novel method for exploring pathogen–host association. Besides its potential for rapid and quantitative analysis of pathogen–receptor interactions, this method allows the detection of receptor recognition by single bacteria in heterogeneous populations and might represent a valuable tool to profile the host binding capabilities of microorganisms.

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