

Universität Konstanz

**Internalization of
pathogens in mammalian
cells via CEACAM1**

Dissertation

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ABBREVIATIONS

%	percent
°C	degree Celcius
Amp	ampicillin
APS	ammonium persulfate
bp	base pairs
BSA	bovine serum albumin
Cam	chloramphenicol
CEACAM	carcinoembryonic antigen-related cell adhesion molecule
CFSE	carboxy-fluorescein succinimidylester
cm	centimetre
CS	calf serum
DAG	diacylglycerol
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
EDTA	ethylene diamine tetraacetate
Eph	ephrin
EphA2	Eph receptor A2
FACS	Fluorescence activated cell sorting
FCS	fetal calf serum
Fn	Fibronectin
g	gramm
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
h	hour
HA	hemagglutinine
HBS	Hepes buffered saline
HMW	High molecular weight marker
IP ₃	Inositol(1,4,5)trisphosphate
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
kbp	kilobase pairs
kDa	kilodalton
l	litre
LB	Luria-Bertani
LMW	Low molecular weigth marker
M	molar
mA	milliampere
mg	milligramms
ml	millilitre
mM	millimolar
OD	optical density
opa/opc	opacity associated
PBS	phosphate buffered saline
PBS++	phosphate buffered saline, suppl. with Ca ²⁺ and Mg ²⁺
PCR	polymerase chain reaction

Abbreviations

PFA	paraformaldehyde
PI	Phosphatidylinositol
PI(4,5)P	Phosphatidylinositol-4,5-bisphosphate
PI(3,4,5)P	Phosphatidylinositol-3,4,5-trisphosphate
PL	Poly-Lysine
pmol	picomol
PTK	protein tyrosine kinase
PVDF	polyvinylidene fluoride
Rho	Ras homology Growth-related
RT	room temperature
s	second
SDS	Sodium dodecyl sulfate
SH2	Src-homology 2 domain
SH3	Src-homology 3 domain
TAE	Tris-acetate-EDTA
TEMED	Tetramethyl ethylenediamine
rpm	rounds per minute
ON	over night
UV	ultraviolet
V	Volt
WASP	Wiskott Aldrich Syndrome Protein
WAVE	WASP family verprolin-homologues (Protein)
µg	micrograms
µl	microlitre

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1 Summary

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) belongs to the immunoglobulin superfamily and has several physiological functions in the body, including cell-cell adhesion, angiogenesis, insulin metabolism, and regulation of immune cell functions. Within the CEACAM family, CEACAM1 has the widest tissue distribution and is expressed on endothelial, epithelial, and hematopoietic cells. It also serves as receptor for human specific pathogens like *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Escherichia coli* together with CEACAM3, CEA and CEACAM6. These pathogens express distinct adhesins to associate with the CCFG interface of the N-terminal domain of CEACAMs; in addition we could demonstrate the selective recognition of human CEACAMs, which presumably explains the absence of non-human hosts. Engagement of CEACAM1 by pathogens has several advantages, based on its physiological functions. Receptor binding leads also to internalization and transcytosis of the bacteria in mammalian cells, causing increased survival of the pathogens in the host. During invasion of the bacteria via the epithelial CEACAMs (CEACAM1, CEA and CEACAM6) the actin cytoskeleton plays only a minor role, but it is highly dependent on receptor relocalization into lipid rafts. Also, the cytoplasmic domain of CEACAM1 is not involved in uptake process. In contrast, binding of the pathogens to the granulocyte specific CEACAM3 induces fast and highly efficient phagocytosis and killing of the bacteria, leading to the suggestion that CEACAM3 might function as immune receptor to get rid of these highly adapted pathogens. For CEACAM3-mediated uptake of pathogens, the uptake is regulated by phosphorylation of two tyrosines in the ITAM-like motif of the cytoplasmic domain of CEACAM3 by Src kinases. The guanine nucleotide exchange factor Vav and the adaptor protein

Nck are recruited to these phosphorylated tyrosines. Vav catalyzes the nucleotide exchange of the small GTPase Rac1, which activates the Nck-associated WAVE complex, inducing massive actin cytoskeleton rearrangements and efficient uptake of CEACAM3-bound pathogens. The activity of PI3Ks is not required during CEACAM3-mediated uptake of pathogens, which is surprising because of the involvement during Fc γ receptor mediated uptake in granulocytes. One of the major part of this dissertation was to examine the role of PI(3,4,5)P during pathogen uptake via the epithelial CEACAMs in comparison to CEACAM3. We could clearly demonstrate an important role of this phosphoinositide during the endocytotic process. Further investigations revealed the participation of the extracellular immunoglobulin constant type 2-like (Ig_{C2}-like) domains of the epithelial CEACAMs for PI3K dependent uptake of pathogens. This led us to the suggestion, that another membrane protein might be involved in the signaling process as co-receptor, responsible for signal transduction into the mammalian cell. Another important observation was the involvement of the small GTPase RhoG in endocytotic processes via epithelial CEACAMs in contrast to CEACAM3, regulating the uptake upstream of PI3K. Furthermore, we could identify Ephexin4 as responsible guanine nucleotide exchange factor for RhoG after CEACAM engagement by pathogens and we investigated the involvement of the known upstream regulator of Ephexin4, the receptor tyrosine kinase EphA2. Finally, we could clearly demonstrate the important role of EphA2 during CEACAM1-mediated endocytosis of pathogens, confirming our hypothesis of a possible co-receptor in this signaling process. To sum up, the data obtained from this study provide new mechanistic insight in the barley clarified pathogen uptake via epithelial CEACAMs and the involvement of another membrane protein together with CEACAM1 during uptake could be shown for the first time.

2 Zusammenfassung

CEACAM1 (*carcinoembryonic antigen-related cell adhesion molecule 1*) ist ein Mitglied der Immunglobulin-Superfamilie und spielt eine wichtige Rolle bei zahlreichen physiologischen Funktionen im Körper, wie Zell-Zell Adhäsion, Angiogenese, Insulin-Stoffwechsel und Regulation von Immunzell-Funktionen. CEACAM1 wird in vielen unterschiedlichen Geweben exprimiert, zum Beispiel in Endothel-, Epithel- und Hämatopoetische Zellen. Einige human-spezifische Pathogene wie *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Moraxella catarrhalis* und *Escherichia coli* binden durch verschiedene Adhäsine an das CC'FG Motiv in der N-terminalen IgV-ähnlichen Domäne einiger Mitglieder der CEACAM Familie (CEACAM1, CEACAM3, CEA und CEACAM6). Diese Pathogene erkennen ausschließlich humane CEACAMs, was die strenge Wirtsspezifität der Pathogene erklären könnte. Die Bindung an CEACAM1 ist für die Pathogene von Vorteil, basierend auf den physiologischen Funktionen des Rezeptors. Die Bindung führt zur Internalisierung und Transzytose der Bakterien, um die Überlebensrate im Wirt zu erhöhen. Das Aktinzytoskelett spielt während der Endozytose der Bakterien über epitheliale CEACAMs (also CEACAM1, CEA und CEACAM6) eine untergeordnete Rolle, aber der Prozess ist stark abhängig von der veränderte Lokalisation der Rezeptoren in Membran Mikrodomänen (*Lipid Rafts*). Die zytoplasmatische Domäne von CEACAM1 ist nicht in die Bakterienaufnahme involviert. Im Gegensatz dazu induziert die Bindung der Pathogene an den Granulozyten-spezifischen Rezeptor CEACAM3 eine hocheffiziente Aufnahme und Abtötung der Bakterien, was vermuten lässt, dass CEACAM3 als Immunrezeptor zu verstehen ist, um die stark an den Wirt angepassten Bakterien zu bekämpfen. Die Aufnahme der Bakterien über CEACAM3 ist durch Src Kinasen reguliert, die zwei Tyrosinresten im ITAM-

ähnlichen Motiv der zytoplasmatischen Domäne phosphorylieren. Dies führt zu einer Rekrutierung des Guanosin-Austausch Faktors Vav und des Adaptormoleküls Nck. Vav katalysiert den Nukleotid-Austausch der GTPase Rac1, die wiederum den Nck-assoziierten WAVE Komplex aktiviert, um die massiven Aktinzytoskelett-Umlagerungen für effiziente Bakterienaufnahme zu induzieren. Die CEACAM3-vermittelte Aufnahme von Pathogenen benötigt nicht die Aktivität von PI3Ks. Ein großer Teil dieser Dissertation beinhaltet die Untersuchung der Rolle von PI(3,4,5)P während der Endozytose von Pathogenen durch epitheliale CEACAM Rezeptoren und wir konnten deutlich die wichtige Funktion dieses Phosphoinositids in diesem Prozess zeigen. Weitere Untersuchungen demonstrierten die Beteiligung der extrazellulären Ig_{C2}-ähnlichen Domänen der epithelialen CEACAM Rezeptoren bei der PI3K abhängigen Aufnahme der Pathogene, was auf eine Beteiligung eines weiteren Membranproteins als Ko-Rezeptor hinweist, der für die Signalweiterleitung in die Zelle verantwortlich ist. Außerdem konnten wir zeigen, dass die kleine GTPase RhoG an der Endozytose von Pathogenen durch epitheliale CEACAMs beteiligt ist, und zwar im Signalweg oberhalb der PI3K, reguliert durch den Guanosin-Austausch Faktor Ephexin4. Ephexin4 ist assoziiert mit der Rezeptor-Tyrosin-Kinase EphA2, deren wichtige Rolle in der Endozytose von Pathogenen über epitheliale CEACAMs ebenfalls demonstriert werden konnten. Somit wurde der beteiligte Ko-Rezeptor in der Endozytose von Pathogenen über die epithelialen CEACAMs identifiziert. Zusammenfassend kann man sagen, dass die Daten aus dieser Studie neue mechanistische Einblicke in die kaum aufgeklärte Pathogenaufnahme über epitheliale CEACAMs gibt, außerdem konnte zum ersten Mal die Beteiligung eines weiteren Rezeptors zusammen mit CEACAM1 während der Aufnahme gezeigt werden.

3 GENERAL INTRODUCTION

3.1 CEACAMs

3.1.1 CEACAM family

In 1965, the first member of the carcinoembryonic antigen family, CEA (also named CEACAM5) was discovered from Gold and Freedman as tumor associated antigen in colon cancer (Gold and Freedman 1965). Today, the CEACAM family in humans is subdivided into the CEA-related cell adhesion molecules (CEACAMs) with 12 known members and the pregnancy-specific glycoprotein (PSG) family with 11 members, encoded by 18 genes and 11 pseudogenes on chromosome 19q13.2. PSGs were discovered in 1970 and were synthesized in the placenta. They accumulate in maternal during pregnancy (Zimmermann, Weiss et al. 1989; Teglund, Olsen et al. 1994), however the function is not really clarified until now. One possible function is modulation of the maternal immune system to tolerate the fetus (Snyder, Wessner et al. 2001; Waterhouse, Ha et al. 2002). So far, CEACAMs are only known in mammals and contain to the immunoglobulin superfamily of cell adhesion molecules (IgCAMs) (Obrink 1997; Beauchemin, Draber et al. 1999). Some members of the family have orthologues in distantly related mammals like CEACAM1 and CEACAM16-20, whereas the other CEACAM family members are restricted to specific mammalian lineages (Zebhauser, Kammerer et al. 2005; Kammerer, Popp et al. 2007; Kammerer and Zimmermann 2010). Sequence comparisons lead to the suggestion, that CEACAMs have independently duplicate and diversified in each mammalian lineage, resulting in an expanded family of closely related surface molecules (Kammerer, Popp et al. 2004;

Kammerer and Zimmermann 2010). All CEACAMs contain at least one immunoglobulin variable-region-like (Ig_V-like) domain (which is conserved within the CEACAM family), mostly followed by up to six immunoglobulin constant region type 2-like (Ig_{C2}-like domains) (Fig. 1). The extracellular domains of all CEACAMs are heavily glycosylated (Lucka, Fernando et al. 2005), only in the amino-terminal Ig_V-like domain one β-sheet is non-glycosylated, regulating homophilic intercellular binding. In addition, this non-glycosylated region serves also as target for pathogen binding for CEACAM1, 3, 6 and CEA (Watt, Teixeira et al. 2001; Tan, Zelus et al. 2002; Gray-Owen and Blumberg 2006).

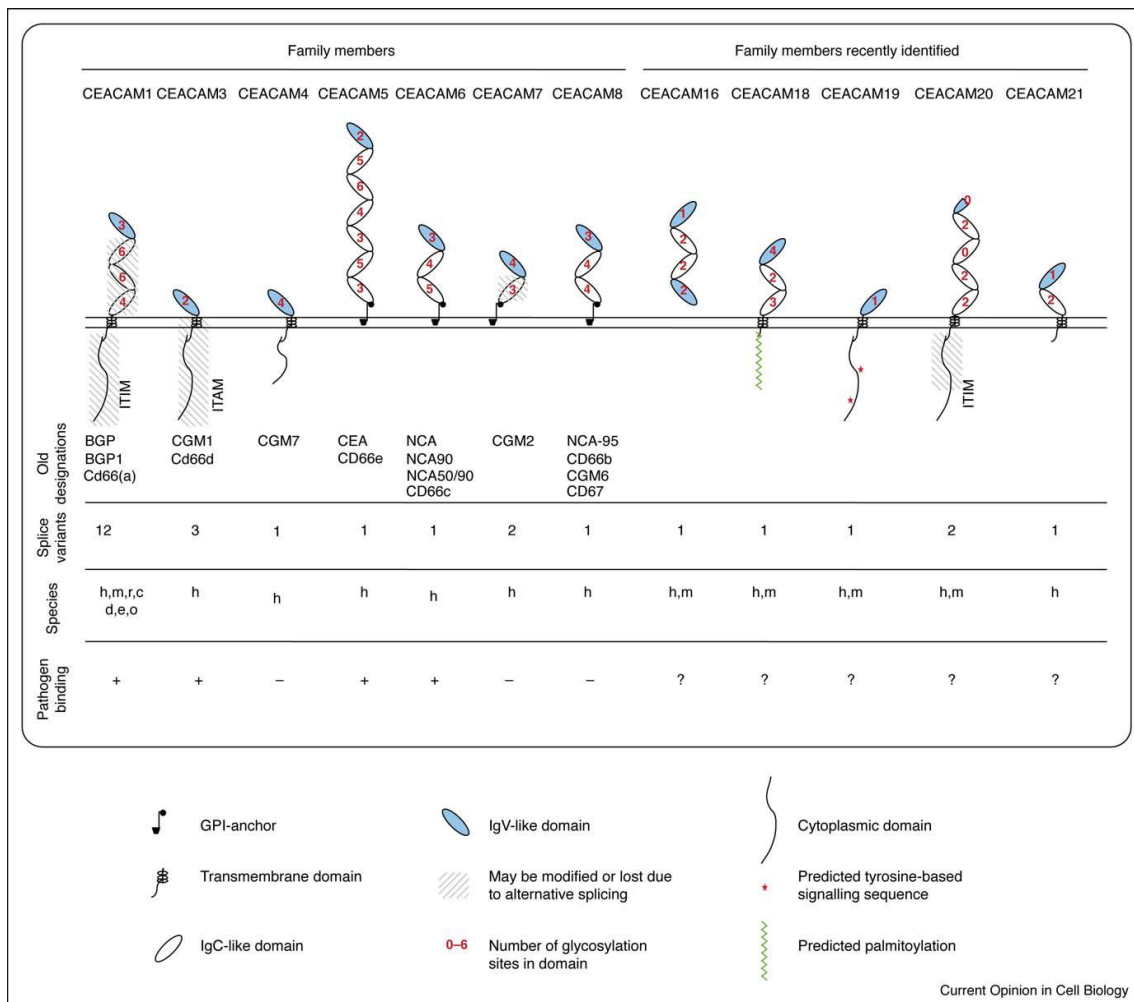


Fig. 1 Overview of the human CEACAM family. Schematic representation of major isoforms of the different CEACAM family members. Species abbreviations: c, cow; d, dog; e, elephant; h, human; m, mouse; o, opossum; r, rat (Kuespert, Pils et al. 2006).

Immunoglobulin-like domains have the widest representation of any protein domain in humans, being encoded in 765 genes (Lander, Linton et al. 2001). They contain around 100 amino acids and have a characteristic sandwich fold, built up of two beta-sheets containing antiparallel strands (Poljak, Amzel et al. 1973). Moreover, they can be divided in two categories, based on function and size: Variable domain (V-domain) and constant domain (C-domain). In general, they play a major role in cell-cell recognition and adhesion (Williams and Barclay 1988). In immunoglobulins, the V-domain contains the antigen-binding properties and the C-domain mediates the effector functions. C-domains are shorter because of lacking the C' and C'' strands. Later, immunoglobulin superfamily domains were found with sequence patterns more similar to V-domains but more the size of C-domains and were termed C2 (constant type II) domains (Williams and Barclay 1988; Barclay 2003). Interaction partners of immunoglobulin-like domains are mainly other immunoglobulin-like domains, but sometimes they also associate with other domain types like fibronectin type III, cytokine receptor domains, EGF domains, C-type lectin and complement control protein domains (Barclay 2003). The structure of CEACAMs with the one aminoterminal Ig_V-like domain followed by one or more Ig_{C2}-like domains is very typical for membrane proteins (Thomson, Krupey et al. 1969; Litman, Hawke et al. 2001). CEACAMs mediate cell-cell adhesion by homotypic binding to themselves or heterotypic binding to another member of the CEACAM family, also mediated by the CC'-FG-face of the Ig_V-like domain (Benchimol, Fuks et al. 1989). CEACAMs can either be associated with cell surface via a transmembrane domain or via a glycosylphosphatidylinositol (GPI) anchor. One exception is the soluble CEACAM16, which contains on each end one Ig_V-like domain (Kuespert, Pils et al. 2006). The tissue expression differs within the CEACAM family. Whereas CEACAM1 has the widest tissue distribution and is found on epithelial cells, endothelial cells, lymphocytes and myeloid cells, CEACAM3, 4 and 8 are exclusively expressed on granulocytes. CEA

and CEACAM7 are only expressed on epithelial cells, and CEACAM6 was found on both epithelial cells and granulocytes (Gray-Owen and Blumberg 2006). CEA is one of the most widely used tumor markers worldwide because of the discovery of higher levels in serum of patients with colorectal carcinoma and other types of cancer (Gold and Freedman 1965; Thomson, Krupey et al. 1969). In tumors CEA is often overexpressed (Kim, Kaye et al. 1992) and is involved in development of the tumors and metastasis (Screaton, Penn et al. 1997; Screaton, DeMarte et al. 2000). In this dissertation I am mainly interested in the CEACAMs which are bound by pathogens, especially CEACAM1 and CEACAM3.

3.1.2 CEACAM1

Carcinoembryonic antigen- related cell adhesion molecule 1 (CEACAM1) is a widely expressed glycoprotein of the CEACAM family. There are 11 different splice variants known in humans, which differ with regard to number of the extracellular Ig_{C2}-like domains (0 to 3), membrane anchorage and/or the length of the cytoplasmic tail (Gray-Owen and Blumberg 2006). The most expressed isoforms are CEACAM1-4L and CEACAM1-4S, with four extracellular domains, a transmembrane domain and either the long form of the cytoplasmic domain containing two immunoreceptor-tyrosine based inhibitory motifs (ITIM) or the short cytoplasmic domain missing the ITIM motifs but containing sequences for calmodulin, tropomyosin and globular actin binding (Gray-Owen and Blumberg 2006). CEACAM1 is important during a broad spectrum of cellular processes related to cellular activation and progression (Hammarstrom 1999) and is mostly found as *cis*-homodimer (Hunter, Sawa et al. 1996), which is necessary for recruitment of signaling molecules like Src family kinases and tyrosine phosphatase Src homology (SH2-) containing protein tyrosine phosphatase (SHP1 and SHP2) (Huber, Izzi et al. 1999) (Brummer, Neumaier et al. 1995; Skubitz, Campbell et al.

1995) via its ITIM- like region (Chen, Zimmermann et al. 2001). CEACAM1 also build up *trans*-homophilic (CEACAM1-CEACAM1) and *trans*-heterophilic (CEACAM1-CEA or CEACAM6) interactions for increased cell-adhesion via reciprocal interaction of the CC' loop in the aminoterminal Ig_v -like domains (Watt, Teixeira et al. 2001; Stern, Markel et al. 2005; Gray-Owen and Blumberg 2006).

Besides the role in cell-adhesion, CEACAM1 regulates the activity of immune cells. Because of the ITIM motif in the cytoplasmic domain, CEACAM1-4L is seen as a negative regulator of T cells and B cells, regulated by activation of SHP1, which inhibits JUN N-terminal kinase and extracellular-signal regulated kinase1 (ERK1) phosphorylation downstream of T-cell receptor and B-cell receptor (Chen, Zimmermann et al. 2001; Chen, Iijima et al. 2004). CEACAM1-4S was described to stimulate T-cell activity, so the relative expression on immune cells might provide an ability to fine-tune the inhibitory properties (Chen, Iijima et al. 2004; Gray-Owen and Blumberg 2006). However, for T-cells, neutrophils and natural killer (NK) cells surface expression of CEACAM1 has to be induced by activation signals, like interleukin-2, 7 or 15 (Moller, Kammerer et al. 1996; Kammerer, Hahn et al. 1998). On NK cells, activation of CEACAM1 has shown to inhibit the cytotoxic activity, explaining the poor prognosis for survival if CEACAM1 is expressed by primary melanoma lesions (Markel, Lieberman et al. 2002; Thies, Moll et al. 2002; Stern, Markel et al. 2005). For neutrophils it is difficult to investigate the function of CEACAM1 because of the co-expression of CEACAM3, 4, 6 and 8, but it seems that CEACAM1-dependent intercellular binding confers survival signals to prevent neutrophil apoptosis and triggers oxidative burst (Gray-Owen, Lorenzen et al. 1997; Singer, Klaile et al. 2005). Moreover, CEACAM1 expression in endothelial cells is increased after stimulation with vascular endothelial growth factor (VEGF) and blocking of CEACAM1 activity leads to reduced VEGF-induced endothelial tube formation (Ergun, Kilik et al. 2000; Chen, Chen et al. 2005). In addi-

tion, soluble CEACAM1 stimulates proliferation, chemotaxis and capillary-like tube formation of endothelial cells *in vitro* (Ergun, Kilik et al. 2000). So CEACAM1 is a critical regulator of neovascularization, discovered by overexpression of CEACAM1-4L in endothelial cells leading to promotion of vessel growth and vessel integrity *in vitro* and *in vivo* (Horst, Ito et al. 2006). Also, expression of CD105 (endoglin), a critical factor for angiogenesis, is stimulated via activation of CEACAM1 (Muenzner, Rohde et al. 2005).

Furthermore, CEACAM1 is involved in regulation of insulin action, because of its role as direct substrate of the insulin receptor, leading to tyrosine phosphorylation of CEACAM1 after activation of the insulin receptor. CEACAM1 acts as negative regulator of the mitogenic action, because of promotion of endocytosis of insulin receptor – insulin complex and degradation of insulin in lysosomes independent from the aminoterminal domain of CEACAM1 (Najjar 2002). There was also a reduced PI3K/Akt stimulation in response to insulin demonstrate, regulated by direct interaction of CEACAM1 to the SH2 domain of another substrate of the insulin receptor, namely Shc to compete with Shc binding to insulin receptor (Poy, Ruch et al. 2002).

Altogether, CEACAM1 is important in regulation of activity and proliferation of immune cells, regulation of insulin activity, neovascularization and cell adherence.

3.1.3 CEACAM3

Another member of the CEACAM family is CEACAM3 which is exclusively expressed on granulocytes and harbours one Ig_V-like domain at the amino terminus, directly followed by the transmembrane domain and the cytoplasmic tail (Gray-Owen and Blumberg 2006). In contrast to CEACAM1, Ig_{C2}-like domains are lacking in the extracellular part and in the cytoplasmic tail an immunoreceptor tyrosine based activation motif (ITAM)-like sequence is expressed instead of the immunoreceptor tyrosine based

inhibitory motif (ITIM)-like sequence. Furthermore, CEACAM3 is not engaged in cell-cell adhesion with other CEACAM family members, but promotes rapid internalization and efficient intracellular destruction of CEACAM-targeting pathogens in granulocytes (Schmitter, Agerer et al. 2004), leading to the suggestion that CEACAM3 is an innate immune receptor targeted against human-specific pathogens. cDNA cloning from sequences encoding CEACAM aminoterminal domains in different species appears that there are no orthologues of CEACAM3 in other species, suggesting that human CEACAM3 is a rather recent invention in evolution (Zhou, Zhang et al. 2001).

3.1.4 Endocytosis of pathogens via CEACAMs

As mentioned before, several human-restricted pathogens like *Neisseria gonorrhoeae*, *N. meningitidis*, *N. lactamica*, *N. subflava*, *Moraxella catarrhalis*, *Haemophilus influenzae* and *Escherichia coli* express distinct afimbrial adhesins to associate with human CEACAM1, 3, 6 and CEA via the non-glycosylated CC'-FG-face of the Ig_V-like triggering internalization and in some cases transcytosis of the pathogens (Fig. 2) (Sauter, Rutherford et al. 1993; Chen and Gotschlich 1996; Virji, Makepeace et al. 1996; Hill, Toleman et al. 2001; Toleman, Aho et al. 2001; Gray-Owen and Blumberg 2006). CEACAM1 engagement by pathogens has several advantages, based on the cellular physiological functions of this receptor. Activation of CEACAM1 by binding of *N. gonorrhoeae* triggers expression of CD105 (endoglin) in epithelial cells, leading to an increased extracellular matrix adhesion by activation of integrins.

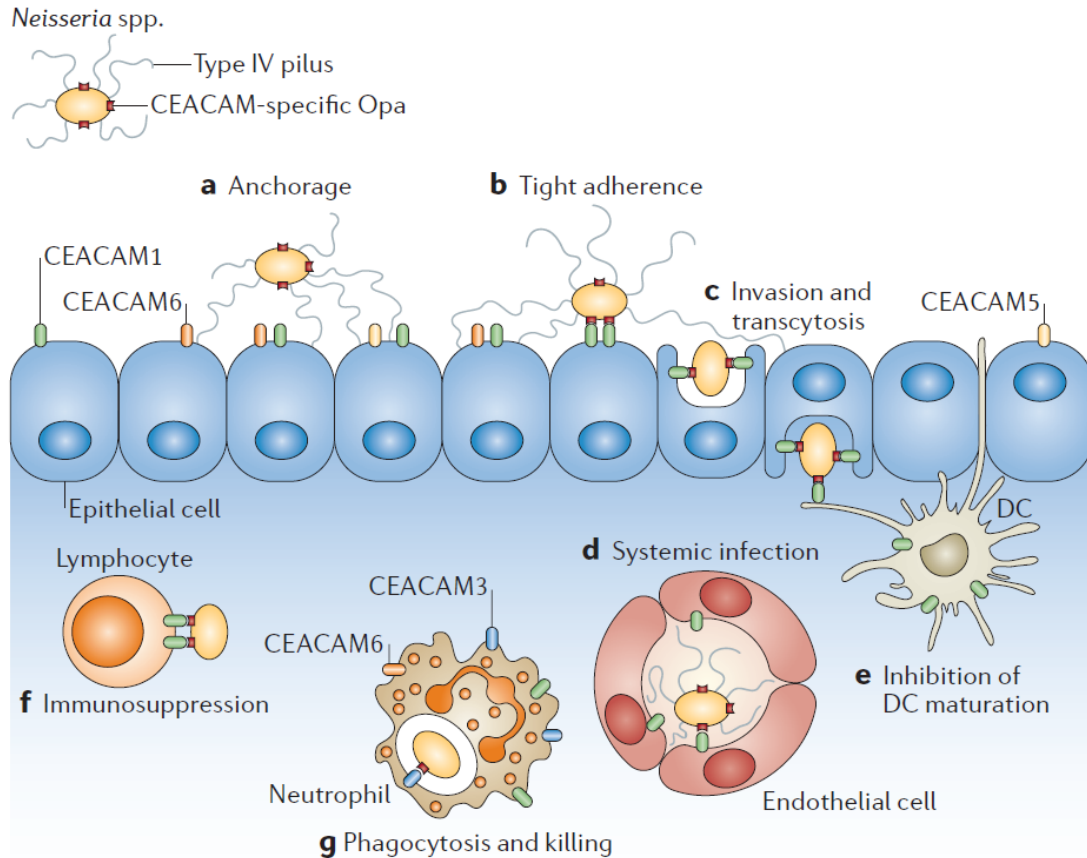


Fig. 2 CEACAM receptor binding by pathogenic *Neisseria* spp (Gray-Owen and Blumberg 2006). (a) Primary anchorage to surface of epithelial cells is mediated by type IV pili, followed by binding of the bacterial colony opacity-associated (Opa) proteins to CEACAM receptors (b). This interaction leads to transcellular transcytosis of bacteria or invasion into the cells (c). In rare cases, *Neisseria* enter into the blood stream (d). Opa-CEACAM interaction leads to inhibited maturation of dendritic cells (e) and suppresses lymphocyte responses (f). Binding of *Neisseria* to CEACAM3 leads to phagocytosis and killing of the bacteria (g).

A protective mechanism of the mucosa in the host is the rapid shedding of the epithelial cells (exfoliation) to prevent colonization, and upregulation of CD105 by engagement of CEACAM1 followed by integrin activation is a clear advantage for the gonococci to facilitate mucosal colonization (Muenzner, Rohde et al. 2005; Muenzner, Bachmann et al. 2010). Furthermore, interaction of bacteria and CEACAM1 can negatively modulate T-cell responses (Gray-Owen and Blumberg 2006; Lee, Ostrowski et al. 2008) and can increase the expression of pro-inflammatory cytokines like TNF- α as well as expression of the receptor itself (Gray-Owen, Lorenzen et al. 1997; Muenzner, Dehio et al. 2000; Muenzner, Naumann et al. 2001)

One of the main topics in my group is to investigate internalization of pathogens via CEACAM receptors, and also other groups are working in this field (Leusch, Drzeniek et al. 1991; Dveksler, Pensiero et al. 1993; Billker, Popp et al. 2000; Hill, Toleman et al. 2001; Billker, Popp et al. 2002; Hauck and Meyer 2003; Hill, Edwards et al. 2005; Korotkova, Cota et al. 2006; Kuespert, Pils et al. 2006; Sarantis and Gray-Owen 2007; Schmitter, Pils et al. 2007; Villullas, Hill et al. 2007; Pils, Gerrard et al. 2008; Kuespert and Hauck 2009; Kuespert, Roth et al. 2010; Buntru, Kopp et al. 2011; Buntru, Roth et al. 2012). Despite CEACAM1 and CEACAM3 are part of the same family and bind to pathogens via their N-domains it became clear, that cellular mechanism in bacterial uptake via CEACAM3 differs completely from bacterial uptake via epithelial CEACAM1 or the other epithelial CEACAMs (CEA, CEACAM6). A few years ago, it could be shown that the cytoplasmic domain of CEACAM1 is not involved in bacterial uptake and also the actin cytoskeleton plays only a minor role (Muenzner, Bachmann et al. 2008). Uptake of Afa/Dr expressing *Escherichia coli* via epithelial CEACAMs is dependent on microtubule (Guignot, Hudault et al. 2009) and also our group linked microtubule rearrangements to efficient uptake of *Neisseria gonorrhoeae* via epithelial CEACAMs (unpublished data). Furthermore, Caveolin1 seems to be involved in bacterial uptake via CEACAM; however Dynamin and Src kinases are not required, excluding the classical caveolae endocytotic pathway as mechanism for CEACAM1-mediated internalization (unpublished data). Though, internalization via epithelial CEACAMs requires cholesterol and sphingolipid-rich membrane microdomains (lipid rafts) and this could also be shown for uptake of murine hepatitis virus (Thorp and Gallagher 2004; Schmitter, Pils et al. 2007; Muenzner, Bachmann et al. 2008). Lipid raft localization and sensitivity to cholesterol-chelators like methyl- β -cyclodextrin during pathogen uptake is regulated via the transmembrane domain of CEACAM (Muenzner, Bachmann et al. 2008). Lipid rafts are defined as small (10 – 200 nm) heterogenous membrane micro-

domains highly enriched in cholesterol, phospholipids and sphingolipids, such as gangliosides and sphingomyelin (Simons and Ikonen 1997; Pike 2009). These domains facilitate the clustering of highly acylated proteins and glycosylphosphatidylinositol (GPI) anchored proteins (Hancock 2006; Parton, Hanzal-Bayer et al. 2006). In general, lipid rafts are involved in many signaling pathways like insulin signaling, immune cell response, neuronal signaling or fatty acid transport (Smart, Graf et al. 1999; Simons and Toomre 2000; Simons and Ehehalt 2002). In addition, it is known that membrane microdomains are important for adherence and internalization of several pathogens like *E.coli*, *Mycobacterium spp.*, *Chlamydia spp.*, *HIV*, *simian virus 40* and *polyoma virus* (Stang, Kartenbeck et al. 1997; Liao, Cimasky et al. 2001; Manes, del Real et al. 2003).

The cellular mechanism of CEACAM3-mediated uptake is much more investigated as for CEACAM1 (Buntru, Roth et al. 2012). Engagement of CEACAM3 via pathogenic *Neisseria* leads to an opsonin-independent phagocytosis. The first step is phosphorylation of two tyrosine residues within the ITAM-like sequence, Tyr230 and 241, regulated by Src family kinases like Hck or Fgr. The guanine nucleotide exchange factor (GEF) Vav can directly bind via its SH2 domain to phosphorylated Tyr230, in contrast to Fcγ receptors, where Vav is indirect linked to the receptor via an interaction with ITAM-bound Syc or the adaptor protein Slp-76 (Deckert, Tartare-Deckert et al. 1996; Tuosto, Michel et al. 1996; Rouard, Tamasdan et al. 1999). Another associated protein of CEACAM3 is the adaptor protein Nck, which connects the ITAM-like sequence with the WAVE complex, triggering local f-actin based lamellipodial structures (Pils, Kopp et al. 2012). Association and activation of Vav leads to stimulation of GTPase Rac, but not Cdc42, resulting in formation of actin-based lamellipodia regulated by Nck, and followed by uptake and elimination of pathogens by granulocytes (McCaw, Schneider et al. 2003; McCaw, Liao et al. 2004; Schmitter, Agerer et al. 2004; Schmitter, Pils et al.

2007; Pils, Gerrard et al. 2008). A shortcut is generated by direct association of CEACAM3 with an upstream and a downstream effector of Rac (Vav and Nck), leading to highly efficient lamellipodia formation and phagocytosis of pathogens, independent from PI3K activity, in contrast to Fc γ receptor-mediated uptake of pathogens, although the phosphorylated Tyr230 of CEACAM3 binds directly to the N-terminal SH2 domain of class I PI3K subunit p55 (Buntru, Kopp et al. 2011). Recently, it could be shown that the PI3K activity together with Syk is necessary for efficient oxidative burst (Sarantis and Gray-Owen 2007; Buntru, Kopp et al. 2011).

Taken together, engagement of epithelial CEACAM receptors comprise advantages for efficient colonization by increase of cellular adherence of the epithelial cells in the mucosa and modulation of the immune system, but probably as evolutionary process in regard to host-pathogen adaption, CEACAM3 function as opsonin-independent innate immune receptor for highly efficient phagocytosis of pathogens (Pils, Gerrard et al. 2008). Despite CEACAM1 and CEACAM3 contain to the same protein family, the bacterial uptake is regulated via entirely distinct cellular processes, mainly caused by tissue expression and the cytoplasmic ITAM-like sequence of CEACAM3.

3.2 CEACAM-binding pathogens

3.2.1 Neisseria: Pathogenicity, Epidemiology

The gram-negative bacteria *Neisseria gonorrhoeae* and *Neisseria meningitides* are the agents of epidemic gonorrhoeae and meningitides. They are strictly human-specific pathogens and settle in human mucosal niches. *N. meningitidis* are facultative commensals because of the colonization of the nasopharynx of 3 – 30% of healthy individuals. Especially in immune-deficient patients serious blood and brain infections, called septicaemia and meningitis can occur by traverse of the mucosal and blood-brain barrier

(Nassif 1999; Merz and So 2000). The disease is spreaded by aerosol droplets and is a worldwide endemic problem, especially in Sub-Saharan Africa. In two age groups meningococcal disease is prevalent in particular: children under 1 year or young adults between 15-19 years. *N.meningitidis* are encapsulated with 13 distinct capsular structures which are responsible for classification in different serotypes. There are at least 12 *N.meningitidis* serotypes known (L1 – L12). In respiratory tract, the pathogen gets finally non-encapsulated through numerous genetic mechanisms. The capsule is important to prevent the pathogen from antibody and complement deposition, is anti-opsonic and anti-phagocytic and promotes survival in blood (Achtman 1995; Virji 2009).

In contrast, *N.gonorrhoeae* is sexually transmitted and infects the urogenital tract, inducing local inflammation and a range of clinical manifestations. It can also infect the rectum, throat and conjunctiva of the eye. In female patients, the disease is often asymptomatic, whereas most male patients develop an intense inflammatory response. *N.gonorrhoeae* do not express capsules, but there are sialic acids added at the lipopolysaccharide, which can impart capsule-like properties for more resistance against antibodies and complement system (Virji 2009; Marrazzo, Handsfield et al. 2010). Both pathogens have evolved mechanism to evade human immune system by modulating their surface antigen make up with remarkable speed, one reason that there are no vaccines available until today. The polysaccharide capsule and / or the lipopolysaccharide (LPS) on bacterial surface protect the pathogens from host immune system; they also mimic host-cell surface structures to avoid antibody response. Phase variation is also very important for the pathogens to escape from the host immune system and is regulated via repetitive sequences of nucleotides within or upstream of genes, leading to slipped strand mispairing (SSM). This results in translational control of gene expression, by switching on and off in a reversible manner or transcriptional control of gene expression (for *Opc*, only present in *N.meningitidis*). Another important escape mecha-

nism is the invasion in non-phagocytic mammalian cells mediated by pili and Opa proteins on bacterial surface and traversing of polarized epithelial cells without disrupting barrier function (Pujol, Eugene et al. 1997; Merz and So 2000).

3.2.2 Neisseria: Interactions with host cells

The major adhesins of pathogenic *Neisseria* are the type IV pili, Opa and in case of *N.meningitidis* Opc, minor adhesins are the adhesion penetration protein (App) and for *N.meningitidis* *Neisseria* hia homologue A (NhhA), haemagglutinin/haemolysin-related protein A (HrpA), *Neisserial* adhesin A (NadA) and meningococcal serine protease (MspA) (Fig. 3).

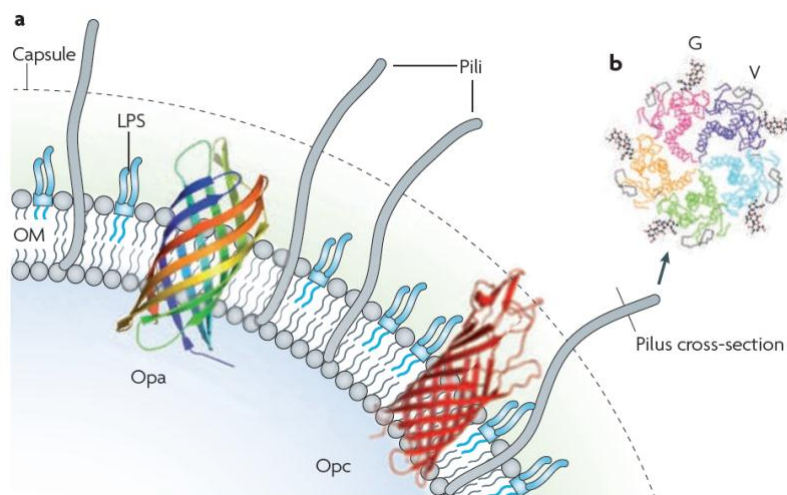


Fig. 3 Major outer membrane proteins of *Neisseria meningitidis* influencing bacterial interaction with host cells (Virji 2009). (a) Pili are the most prominent adhesins of *Neisseria*. In addition, Opa and Opc mediate interactions with specific host cell receptors. Lipopolysaccharide can interact with various cellular receptors. (b) Cross-section of a pilus fibre showing that variable domains (V) and glycans (G) as well as other substitutions located externally, whereas the constant domains are internal.

In the first hours, gonococci and meningococci attach as microcolonies of 10 – 100 diplococci, mediated by pili interactions with host cell microvilli or filopodia directed to the pathogens (Ward and Watt 1972; Ward, Watt et al. 1974; Nassif, Pujol et al. 1999; Merz and So 2000). The pili contains numerous PilE as major pilin, arranged in a helical configuration, together with several subunits like PilC, PilV and PilX for func-

tion modulation. Pili undergo posttranslational modifications like glycosylation, are important for the first host-pathogen contact to facilitate penetration of the negatively charged barrier at the interface and are also responsible for *Neisseria* motility (Merz and So 2000; Virji 2009). Binding of pili to host cells is mediated probably via CD46, C4 binding protein (C4BP) and complement receptor 3 (CR3) (Blom, Rytönen et al. 2001; Kallstrom, Blackmer Gill et al. 2001; Virji 2009). Later in the infection, the pili dissolve and the bacteria intimately associate via their opacity proteins with the host cell, leading to engulfment of bacteria. The opacity proteins, Opa and Opc (in meningococci) impart opacity to colonies that express the proteins. Opa proteins form eight-stranded transmembrane β -barrel structures in the outer membrane of the bacterium with four relatively variable surface-exposed loops. The pathogens can alternate between phase on and off of distinct *opa* genes for antigenic variation and in addition, homologous recombination can further increase the repertoire (Stern, Brown et al. 1986). Gonococci strains typically express 11 *opa* loci, whereas meningococci have 4 -5 *opa* loci (Dehio, Gray-Owen et al. 1998). The *opa* genes contain 5' tandem repeats, which are responsible for high-frequency variable expression (Stern, Brown et al. 1986). The cellular receptor for some of the Opa proteins (Opa_{HSPG}) with many positively charged amino acid residues and Opc is heparan sulfate proteoglycans (HSPG), which interact with many extracellular matrix proteins initiating a complex array of molecular interactions between pathogen and cell (Chen and Gotschlich 1996; Duensing and van Putten 1997; Virji 2009). The bacteria can also bind directly to vitronectin, which interacts with $\alpha_v\beta_5$ or $\alpha_v\beta_3$ integrins on host cells (Dehio, Gomez-Duarte et al. 1998), promoting adhesion and bacterial uptake into mammalian cells. Over 90% of *N.meningitidis* and *N.gonorrhoeae* bind to CEACAM receptors, mainly CEACAM1, but also CEACAM3, CEA and CEACAM6 at the aminoterminal, largely conserved immunoglobulin variable-like domain, leading to internalization of the bacteria into human cells (Chen and Gotschlich 1996; Virji,

Watt et al. 1996; Gray-Owen, Dehio et al. 1997). The N-terminal region consists of nine antiparallel β strands, arranged in two sheets, the ABED and the C''C'CFG interface. Binding of Opa_{CEA} occurs in the CC'FG sheet, dependent on interaction via the hydrophobic amino acids Tyr34 and Ile91 (Bos, Kuroki et al. 1998; Popp, Dehio et al. 1999; Virji, Evans et al. 1999). This interaction is strongly species specific and is probably the reason for the human specificity of *Neisseria* infections (Voges, Bachmann et al. 2010).

3.2.3 Other CEACAM-binding pathogens

Besides the described interaction of Opa_{CEA} proteins with human CEACAM receptors for efficient internalization into human cells, other pathogens also evolved surface proteins for engagement with CEACAM receptors. *Moraxella catarrhalis* is one of these pathogens, which is commonly associated with upper and lower respiratory tract infections like otitis media, sinusitis and chronic obstructive pulmonary disease. The bacterium is present in up to 75% of healthy individuals (Conners, Hill et al. 2008). Interaction between CEACAMs and *M.catarrhalis* is mediated via the bacterial surface protein UspA1 (an autotransporter protein) (Hill and Virji 2003; Hill, Edwards et al. 2005). With the head region, UspA1 can bind to laminin and fibronectin (Tan, Nordstrom et al. 2005). The CEACAM binding region is away from the head group, almost in the middle of the stalk in a left-handed trimeric coiled-coil region (Hill, Edwards et al. 2005). The interaction between CEACAM and UspA1 depends on bending of the stalk region to get the both proteins in closer contact (Conners, Hill et al. 2008).

Another CEACAM-binding pathogen is *Haemophilus influenzae*, also a commensal of the human mucosa in the respiratory tract, causing localized and systemic infections. The major groups are divided in capsulated and acapsulated strains. The CEACAM-interacting protein on the pathogen is the heat-modifiable, antigenically variable 37 kDa

protein P5, which binds also to the N-terminal C''C'CFG interface, leading to bacterial adhesion and invasion into human cells (Virji, Evans et al. 2000; Hill, Toleman et al. 2001).

In addition, enterobacteria like Afa/Dr expressing *Escherichia coli* and *Salmonella* can bind to CEACAM1 (Leusch, Drzeniek et al. 1991; Servin 2005) and for murine CEACAM1a isoforms the binding and internalization of mouse hepatitis virus (MHV-A59) was described (Dveksler, Dieffenbach et al. 1993). Altogether, several distinct pathogens have evolved over time different surface proteins for binding to human CEACAM receptors for stronger adhesion to the mucosa and preventing from the immune system caused by internalization into cells and transcytosis into the stroma.

3.3 Phosphatidylinositolphosphates

3.3.1 Membrane structure & different phosphorylation states

Membranes are important to form a barrier between each cell and its environment and to partition the cytoplasm of eukaryotes into compartments. Most of lipids in the bilayer are phospholipids with the hydrophilic phosphate end and the hydrophobic lipid tail region. One of the minor constituents in the inner leaflet of the plasma membrane is phosphatidylinositol (~10%), a negatively charged phospholipid (Fig. 4).

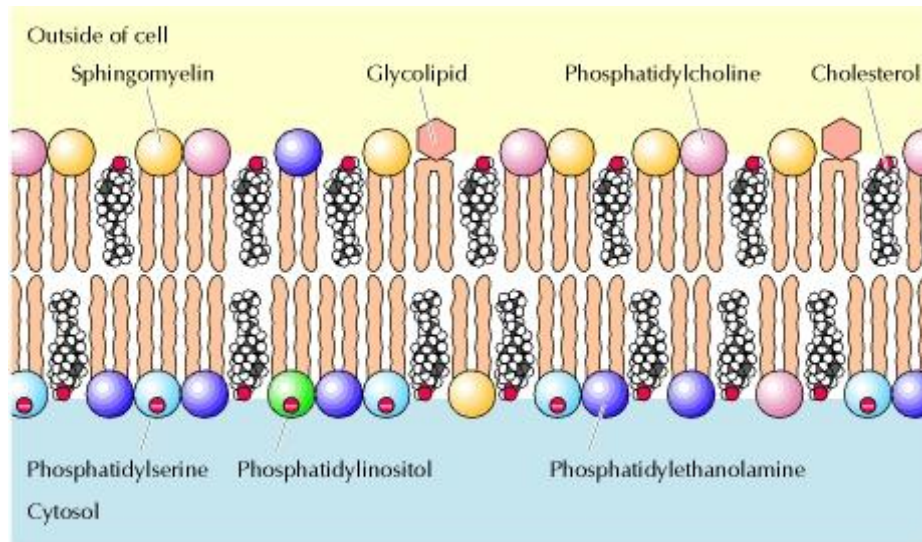


Fig. 4 Schematic representation of the lipid bilayer structure of cellular membranes. Most of the lipids are phospholipids with hydrophilic phosphate end and hydrophobic lipid tail. Major components on the extracellular side are sphingomyelin and phosphatidylcholine. On the cytosolic side mainly phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine are present. Cholesterol is distributed in both sides.

Source: <http://biologystation.wordpress.com/2011/02/23/plasma-membrane/>.

Phosphoinositides comprise the polar *myo*-inositol hexahydroxycyclohexane head group attached via a phosphoester bond to *sn*-1-stearoyl,2-arachidonoyl-diacylglycerol. There are seven different phosphoinositides species known, which differ in reversible phosphorylation of the three positions D-3, D-4 and D-5 at the inositol head group (Fig. 5) (Toker 2002). Phosphorylation of the three positions at the inositol head group is regulated via many different phosphoinositide kinases, phosphatases and phospholipases, which are specific for one position and in most cases also for one substrate, respectively. One example is the class I PI3Kinase, using phosphatidylinositol-4,5 phosphat (PI(4,5)P) as substrate and phosphorylate the 3' position to get PI(3,4,5)P (Vanhaesebroeck, Leever et al. 1997; Leever, Vanhaesebroeck et al. 1999; Hawkins, Anderson et al. 2006).

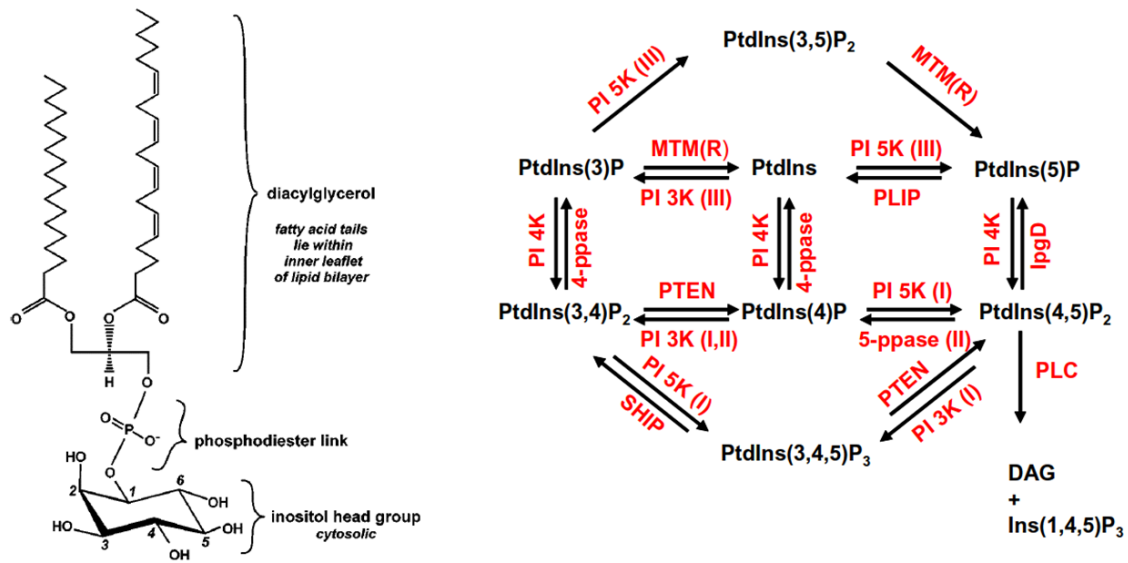


Fig. 5 Structure and regulation of the phosphorylation of phosphoinositides in mammalian cells. Phosphoinositides contain of the diacylglycerol tail, consisting of fatty acids laying in the inner leaflet of the lipid bilayer, linked via a phosphodiester to the inositol head group in the cytoplasm. The inositol head group of phosphoinositide (PtdIns) can be phosphorylated at 3-, 4-, and 5- position. Phosphorylation and dephosphorylation processes are regulated by different kinases and phosphatases (red) (Vanhaesebroeck, Leever et al. 2001; Blero, Payraestre et al. 2007).

The precursor of all phosphoinositides is phosphatidylinositol, which is a substrate for PI4-kinases, generating PI(4)P, which in turn function as substrate for PI5-kinases to generate PI(4,5)P. PI(4,5)P can be catalysed by phospholipase to generate the second messengers Ins-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG) or can be used as substrate for PI3Kinase as mentioned before. Other classes of PI3Kinases can use PI(4)P as substrate to generate PI(3,4)P or the precursor phosphoinositide itself to generate PI(3)P, which in turn can be phosphorylated at 5' position from PI5-kinases to get PI(3,5)P (Ishihara, Shibasaki et al. 1998; Vanhaesebroeck and Waterfield 1999; Toker 2002; Czech 2003; Oude Weernink, Schmidt et al. 2004; Vicinanza, D'Angelo et al. 2008; Botelho 2009; van den Bout and Divecha 2009). Besides phosphoinositide-kinases and lipases also phosphatases can regulate the phosphorylation state of phosphoinositides. One of the most investigated phosphatase is the 3'phosphatase PTEN because of its function as tumor suppressor. The substrate of PTEN is PI(3,4,5)P, lead-

ing to generation of PI(4,5)P (Stambolic, Suzuki et al. 1998; Tamura, Gu et al. 1999; Hawkins, Anderson et al. 2006). SHIP-2, an 5' phosphatase, also uses PI(3,4,5)P as substrate, generating PI(3,4)P (Astle, Seaton et al. 2006; Ooms, Horan et al. 2009). Another 5' phosphatase is PIPP, which uses PI(4,5)P and PI(3,4,5)P as substrates to generate PI(4)P and PI(3,4)P (Gurung, Tan et al. 2003; Astle, Seaton et al. 2006). So in general, the regulation of phosphoinositides in all phosphorylation states is strictly regulated by many kinases, phosphatases and lipases, and the different phosphoinositides-phosphates have their own cell localization and function.

3.3.2 Distribution and binding partners of phosphoinositides in mammalian cells

Phosphatidylinositols are generally localized at different membranes in the cell, anchored via the long fatty acid tail into the inner leaflet of membranes (Fig. 5). The distinct localization of specific phosphoinositides-phosphatases and kinases determine the enrichment of their phosphoinositide products at specific membrane compartments. PI(4,5)P, the most abundant state of phosphoinositides, and PI(3,4,5)P are localized at the plasma membrane, PI(3)P and PI(3,5)P on early and late endosomes, PI(4)P at the Golgi complex and PI(5)P is localized at the nucleus (Pizarro-Cerda and Cossart 2004; Vicinanza, D'Angelo et al. 2008). The localization and the interaction with different proteins determine the functions of the phosphoinositides in cellular signaling. Because of the fact, that phosphoinositides are minor constituents of cell membranes, they are not important for structural functions, but they initiate signaling by specific interactions with a large number of proteins, regulated by specific protein domains. In the last 20 years, it became clear that in almost all cellular processes phosphoinositides are involved in a regulatory way. Interaction of proteins with phosphoinositides can lead to different consequences. One of the most important functions is the relocalization of the

target protein from one area in the cell to another. The interaction can also lead to conformational changes in the protein, followed by modification of the activity. At least, the protein-phosphoinositide interaction can result in phosphorylation or dephosphorylation of the phosphoinositides itself or in protection of the phosphoinositide from phospholipase-catalyzed hydrolysis (Toker 2002). These effects can be various combined, leading to initiation of downstream signaling cascades involved in cell growth and proliferation, apoptosis, differentiation, secretion and vesicle trafficking, motility and intracellular metabolism. The interaction between phosphoinositides and proteins are mediated by phosphoinositide recognition domains. The most abundant domain is the pleckstrin-homology domain (PH) which is present in many cellular proteins involved in intracellular signaling or as constituents of the cytoskeleton. Individual PH domains possess specificities for phosphoinositides phosphorylated at different sites within the inositol ring, meaning that some bind PI(4,5)P but not PI(3,4,5)P (for example PLC, PLD and dynamin) and others the other way around (for example PDK-1, PKB, Btk). Other known interaction domains with phosphoinositides are the PX domain, the Fab1 YOTB Vac1 EEA1 (FYVE) zinc finger domain, the Tubby domain and the Epsin N-terminal homology (ENTH) domain (Blero, Payrastre et al. 2007; Lemon, Klepac-Ceraj et al. 2010). The phosphoinositides-binding proteins belong to many different categories, including enzymes (protein kinases, phospholipases), ion channels, scaffold proteins and trafficking regulators like membrane coats, microtubule and actin cytoskeleton proteins, GEFs and GAPs and tethering factors (Vicinanza, D'Angelo et al. 2008). Thus, cellular functions of phosphoinositides depend on localization inside the cell and the interacting proteins, binding via their different phosphoinositide recognition domains.

3.3.3 Phosphoinositides and endocytosis

Phosphoinositides are involved in regulation of almost all cellular signaling processes in mammalian cells, depending on the interaction partners and localization. For example, PI(5)P is localized in the nucleus and can regulate p53 acetylation in response to DNA damage by interaction with inhibitor of growth protein-2 (ING2) (Gozani, Karuman et al. 2003). PI(4)P is a regulator of the vesicle trafficking between Golgi complex and endoplasmatic reticulum via recruitment of numerous cytosolic proteins (Godi, Di Campli et al. 2004). Furthermore, PI(3)P is known to regulate constitutive membrane traffic (Wurmser and Emr 1998), whereas PI(3,5)P mediates trafficking between late endosomes, multivesicular bodies and the vacuole, which is important for degradation of proteins (Michell, Heath et al. 2006). Endocytotic processes are critical for eukaryotic cells, especially for the regulation of proteins and lipid composition in the plasma membrane. In mammalian cells, various distinct endocytotic pathways co-exist, and although lot of work was done in the last years, many details and whole pathways are not understood until now. The two major pathways are the clathrin-dependent and the non-clathrin dependent processes (Fig. 6). The best characterized endocytotic pathway is the clathrin-mediated endocytosis. Engagement of the receptors via their ligands lead to recruitment of adaptor protein 2 (AP-2) (or others) to the membrane. It could be shown, that recruitment of AP-2 and a lot of other endocytotic adaptors like Epsin, DAB2 and CALM are PI(4,5)P dependent (Zoncu, Perera et al. 2007). AP-2 builds a heterotetrameric complex and binds to clathrin, which then polymerizes into a lattice pulling the plasma membrane inside (Liu and Shapiro 2003).

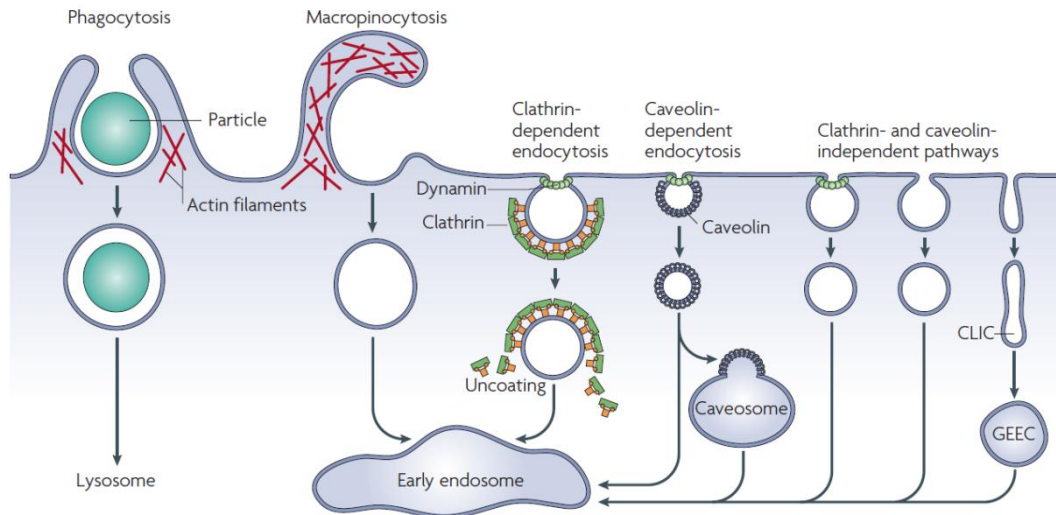


Fig. 6: Different endocytotic pathways. Large particles can be internalized via phagocytosis, whereas fluids are taken up via macropinocytosis. Both processes are strongly actin dependent. The coat protein clathrin and the GTPase dynamin are the major components of clathrin-dependent endocytosis, whereas caveolin-dependent endocytosis also requires dynamin and caveolin instead of clathrin. There are numerous clathrin- and caveolin-independent pathways described, for example CLIC (clathrin- and dynamin-independent carriers) which generates tubular intermediates. Some of the clathrin- and caveolin-independent pathways require dynamin, others are dynamin-independent. The first step in some pathways are intermediate compartments like caveosome or glycosyl phosphatidylinositol-anchored protein enriched early endosomal compartments (GEEC), ending in early endosomes (Mayor and Pagano 2007).

Clathrin itself does not bind to PI(4,5)P. The PI(4,5)P – protein and protein – protein network stabilize the clathrin-based coat (Czech 2003). Also PI(4,5)P phosphatases are necessary for efficient uptake via clathrin-dependent endocytosis, like synaptojanin-1, because of the inhibition of the GTPase dynamin by PI(4,5)P after binding. Dynamin-2 is important for separation of the vesicle from the membrane (Cremona, Di Paolo et al. 1999; Czech 2003). Clathrin-independent pathways are divided in dynamin-dependent and dynamin-independent pathways and also in Cdc42, RhoA and ARF6-regulated endocytotic pathways. All clathrin-independent pathways are linked to sphingolipid- and cholesterol-enriched membrane microdomains, and all of them are actin cytoskeleton dependent, which connects the role of phosphoinositides and small GTPases to endocytotic processes. Altogether, little is known about the mechanism of clathrin-independent endocytosis. Some of the clathrin-independent processes generate distinct early endo-

somal compartments, for example GPI-AP-enriched early endosomal compartments (GEECs), clathrin- and dynamin-independent carriers (CLICs) or the caveosome. However, all pathways end up in early endosomes linked to the markers Rab5 and early endosomal antigen-1 (EEA1) (Mayor and Pagano 2007). Early endosomes are also highly enriched in PI(3)P, which is constitutively present in cells, produced by type III PI3K and important in vesicle trafficking because of its interaction with proteins involved in endosome fusion (EEA-1), endosomal sorting (Hrs, ESCRT, SNX1, SNX2) or endosome dynamics (KIF-16B) (Toker 2002; Vicinanza, D'Angelo et al. 2008).

PI(4,5)P is in general important for actin cytoskeleton regulation by binding and impairing of actin-severing proteins like gelsolin and cofilin/ADF (Toker 1998), counteracting the solubilisation of the actin network. PI(4,5)P also facilitate actin polymerization by activating N-WASp (neuronal Wiskott-Aldrich syndrome protein) and Arp2/3 (actin-related protein 2/3)-mediated actin branching (Toker 1998; Logan and Mandato 2006). Another regulatory mechanism is the interaction with proteins responsible for actin filament uncapping, like Profilin and CapZ (McLaughlin, Wang et al. 2002; Logan and Mandato 2006). These mechanisms are important for polymerization and stabilization of actin filaments in the mammalian cell. In addition, PI(4,5)P modulates the function of several actin-crosslinking and regulatory proteins critical for generation of stress fibers, meshworks and membrane attachment (Fraley, Tran et al. 2003). Another function of PI(4,5)P is to induce conformational changes of vinculin, talin and ezrin (myosin/moesin (ERM) family proteins to promote the anchorage of the actin cytoskeleton to the plasma membrane via integrins in the focal adhesion side (Sechi and Wehland 2000). PI(4,5)P can be hydrolysed by phospholipase (PLC), generating the two second messengers inositol-1,4,5trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ can bind to calcium channels at the endoplasmatic reticulum leading to opening and calcium influx into the cytoplasm, additionally extracellular calcium channels gets open (SOC chan-

nels). Ca^{2+} is an important second messenger, mediated through calmodulin binding and regulation of kinases, synthases and transcription factors like myosin regulatory light chain kinase (MLCK). Activated MLCK phosphorylate the myosin regulatory light chain (MLC). Furthermore, DAG binds and activates protein kinase C (PKC) which also phosphorylates and activates MCL. This activation promotes the assembly of myosin II filaments, which is important for myosin-based contractility (Iwasaki, Murata-Hori et al. 2001; Varlamova, Spektor et al. 2001; McLaughlin, Wang et al. 2002). The plasma levels of PI(4,5)P are relative constant, but there are some situations which lead to acutely increased or decreased level. One example is activation of Phospholipase D (PLD) (mainly by binding of PI(4,5)P itself), leading to activation of phosphoinositides 4P5-kinase via phosphatidic acid, product of PLD and generation of elevated levels of PI(4,5)P (Czech 2000). Also the small GTPase ARF6 activates the phosphoinositides 4P5-kinase for generation of PI(4,5)P, regulated by PI(3,4,5)P and the GEF GRP1(Honda, Nakamoto et al. 1999). Decreased level of PI(4,5)P occur after activation of phospholipase C, which leads to hydrolysis of the phosphoinositide and after activation of class I PI3K, leading to phosphorylation at 3' position and generation of PI(3,4,5)P (Czech 2000).

PI(3,4,5)P is the product of the class I PI3Kinase after phosphorylation of PI(4,5)P. In unstimulated cells, almost no PI(3,4,5)P can be detected. Class I PI3K are activated by extracellular ligands like growth factors, many inflammatory stimuli, neurotransmitter, antigens and hormones, simultaneously with other, mostly much stronger, signal transduction events. Many different proteins can interact with PI(3,4,5)P via their PH domains, leading to regulation of growth, survival and proliferation via Ser/Thr kinases; calcium mobilisation and gene transcription via Tyr kinases; cytoskeletal organisation and membrane trafficking via guanine nucleotide exchange factors GEFs and GTP-accelerating proteins (GAPs) and signaling efficiency via adaptor proteins and scaffolds

(Hawkins, Anderson et al. 2006). Binding of PI(3,4,5)P to the threonine kinase PDK-1 leads to an increased ability to phosphorylate Akt/protein kinase B (PKB), which can also bind via its PH domain to PI(3,4,5)P. Activated Akt/PKB phosphorylate and activate the Glycogen synthase kinase 3 (GSK-3) which is important for regulation of cyclin D (cell cycle entry). Also the proapoptotic protein Bad is a direct target of Akt/PKB, leading to increased cell survival. Protein synthesis is promoted via Akt/PKB interaction with mTor and p70S6K and other targets of Akt/PKB are also linked to regulation of growth, differentiation, proliferation, metabolism and anti-apoptotic functions, like glucose transporter 4, NF- κ B and many more (Manning and Cantley 2007). Another important pathway is recruitment and binding of bruton's tyrosine kinase (BTK) to PI(3,4,5)P, leading to phosphorylation and activation of BTK via Src family kinases or PDK-1 related enzymes, followed by phosphorylation and activation of phospholipase C γ (PLC γ). PLC γ hydrolyze PI(4,5)P to IP₃ and DAG like described before, which is an important step in endocytosis for efficient separation of the vesicle from the membrane (Leevers, Vanhaesebroeck et al. 1999). PH domains are found in all GEFs specific for Rho family and in some GEFs specific for the ARF family, and binding of PI(3,4,5)P regulates the activity of these GEFs leading to more catalytic exchange from GDP to GTP of the target small GTPases ARF and Rho/Rac GTPases which are key regulators of cytoskeletal and membrane delivery events (Leevers, Vanhaesebroeck et al. 1999; Vanhaesebroeck, Leevers et al. 2001; Hawkins, Anderson et al. 2006). The class I PI3K, generating PI(3,4,5)P could be linked directly to early endosome fusion in vitro, dependent on the small GTPase Rab5 (Li, D'Souza-Schorey et al. 1995). Furthermore, PI(3,4,5)P can directly associate and activate Rac1 and, to a lesser extent, RhoA, but not Cdc42, catalyzing the GDP-GTP exchange and promoting the recruitment. The interaction of PI(3,4,5)P with GEFs of the Rho family influence not only the actin cytoskeleton, but also the microtubules, which is later described in more detail. In general,

PI(3,4,5)P seems to have more a regulatory role rather than a direct role in interaction with endocytosis, by regulating the activity of the small GTPases and diminishing of PI(4,5)P levels. In the last years it could be demonstrated from diverse groups all over the world, that a lot of pathogens using the phosphoinositide machinery to internalize efficient into mammalian cells, mostly involving one of the natural cellular endocytotic processes.

3.3.4 Manipulation of phosphatidylinositols by pathogens

Invasive pathogens have evolved mechanism to invade into cells, which are normally non-phagocytic, based on the cellular endocytotic processes. To internalize in an efficient way or to establish their replicative niches, the phosphoinositides metabolism is a popular target. Enteropathogenic *Yersenia* species are known to translocate through intestinal epithelium, regulated by interaction of the bacterial outer membrane protein invasion with host β 1-integrin. Uptake of the pathogen occurs in a Rac1 or RhoG-dependent manner, connected to recruitment and activation of phosphoinositides -5 kinase I α and generation of PI(4,5)P, regulated via the small GTPase ARF6. Recruitment of the proteins and generation of PI(4,5)P are necessary for the control of actin dynamics, which are required for bacterial uptake (Isberg, Hamburger et al. 2000; Isberg and Barnes 2001; Mohammadi and Isberg 2009). Another example is *Listeria monocytogenes*, which interact via the bacterial proteins Internalin A and Internalin B with the host proteins E-cadherin and the hepatocyte growth factor receptor c-Met for internalization in a PI3K dependent manner. PI3K activity is important for activation of the small GTPases Rac1 and Cdc42 for actin rearrangements and also important for PLC γ activation to hydrolyze PI(4,5)P at site of infection (Hamon, Bierne et al. 2006). Also modulation of cell survival via activation of PI3K followed by activation of Akt/PKB could be described (Mansell, Khelef et al. 2001). *Escherichia coli* (UPEC) can invade

via their surface protein FimH in epithelial cells in a PI3K, caveolin-1 and Rac-1 dependent manner. PI3K and Focal adhesion kinase (FAK) build a complex and modulate vinculin, tensin, talin or α -actinin, which then allow efficient uptake (Martinez, Mulvey et al. 2000; Duncan, Li et al. 2004). Some pathogens translocate bacterial phosphoinositide phosphatases through a type III secretion system directly into host cells, one example is *Shigella flexneri*. The virulence factor IpgD behaves as phosphoinositides-4phosphatase with PI(4,5)P as substrate, leading to decreased levels and reduced interaction between plasma membrane and actin cytoskeleton caused by less interaction between integrins, talin and vinculin in focal adhesion sites (Niebuhr, Giuriato et al. 2002). Another example is *Salmonella enterica* with its IpgD homologue SigD/SopB, leading to formation of membrane ruffles and Cdc42-dependent actin rearrangements in an unknown manner, required for efficient entry of the pathogen into mammalian cells (Zhou, Chen et al. 2001). SigD/SopB activity is also important for rapid disappearance of PI(4,5)P from the plasma membrane before fission and formation of the pathogen-containing vesicles (Terebiznik, Vieira et al. 2002). At last, some pathogens interact with phosphoinositide kinases, like enteropathogenic *Escherichia coli* (EPEC), which also translocate effectors into the cytoplasm of cells via the type III secretion system. These effectors induce formation of actin-rich protrusions, where the bacteria reside, the inhibition of the PI3K activity inside the cells has been shown to be the mechanism to inhibit the phagocytosis (Celli, Deng et al. 2000; Celli, Olivier et al. 2001).

To sum up, phosphoinositides are very important as regulators of the actin cytoskeleton and also of microtubules via a lot of different ways like direct interaction with actin cytoskeleton factors, interaction with focal adhesion sites and activation of small GTPases. Also the survival of cells is strongly dependent on phosphoinositides and they are required for vesicular trafficking. For efficient infection of the host, many pathogens

target phosphoinositides for internalization, survival, trafficking and inhibition of phagocytosis.

3.4 GTPases

3.4.1 Overview: Families and general mechanism of GTPases

Small GTPases function as molecular switches in signal transduction by alternating binding of the nucleotides guanosine-diphosphate (GDP) or guanosine-triphosphate (GTP). There are 10 families of GTPases with multiple isoforms described until now, created by gene duplication and divergence. The four major classes of GTPases are elongation factors, small GTPases of the RAS superfamily, trimeric G-proteins and dynamin-related GTPases. Within this dissertation it will be concentrated on the RAS protein superfamily, which is divided in five major subfamilies: Ras, Rho, Rab, Arf/Sar and Ran (Takai, Sasaki et al. 2001). They contain only a GTP-binding domain of about 200 residues folded into a β -sheet of six-strand sandwiches between five α -helices. GTP is bound in a flat groove, anchored by hydrogen bonds between the protein and the nucleotide including the di- or triphosphate and an additional Mg^{2+} ion. A typical G-protein is active after binding of GTP, which causes three conformational changes in the GTPase (switch-I, -II and -III), leading to the binding of diverse effector proteins in these switch loops. Most of them are anchored to cellular membranes by covalently attached lipids and almost all signal transduction pathways are regulated by members of the RAS protein superfamily (Colicelli 2004; Rojas, Fuentes et al. 2012).

GDP dissociates with a half-time of 10 hours, so GTPases accumulate in the inactive state (GDP-bound). Exchange of GDP to GTP can only occur after dissociation of GDP, and Mg^{2+} is required for binding of the nucleotide to the GTPase (Zhang, Zhang et al. 2000). Because of high cytosolic concentrations of free Mg^{2+} , preventing spontaneous

guanine nucleotide exchange from GDP to GTP, the cycling between these two activation states is catalyzed by guanine exchange factors (GEFs) and GTP-accelerating proteins (GAPs) (Fig. 7). After stimulation of the GEFs via different upstream signals, GEFs catalyze the release of bound GDP by deforming the part of the nucleotide binding site, which results in dissociation of GDP in a time scale from seconds to minutes instead of 10 hours, followed by rapid binding of GTP. Also GEFs stabilize the nucleotide-free small GTPase until GTP is bound to the small GTPase (Cherfils and Chardin 1999). GTPases have intrinsic GTP hydrolysis activity from GTP to GDP, leading to determination of the effector protein binding by GTPases, but with a half-time of about 4 hours, which is in contrast to the observed fast “on” and “off” signaling. The superfamily of GTPase-binding proteins increase the intrinsic GTPase activity dramatically (GTPase-accelerating proteins, GAPs) by contributing a positively charged arginine side chain to stabilize the negative charges on the oxygen bridging the β - and γ -phosphate leading to a rapid dissociation of the γ -phosphate from the GTPase-GDP-P complex (Fig. 7) (Boguski and McCormick 1993). Some members of the Rho and Rab family are also controlled by GDP dissociation inhibitors (GDIs) (Fig. 7). Three different functions have been described: They inhibit dissociation of GDP from Rho proteins which leads to decreased activation potential of the GTPase by GEFs and remaining in the inactive state. Another function is to modulate the cycling of RhoGTPases between cytosol and membrane by forming high affinity complexes in pocket form around the geranylgeranyl membrane-targeting moiety at the C-terminus of some RhoGTPases (Rojas, Fuentes et al. 2012). After release of the GTPase from GDIs, GTPases can insert into the membrane and are activated by membrane-associated GEFs. Extraction from membrane (possibly stimulated by GTP hydrolysis) and re-association with GDIs is reported for recycling of GTPase into the cytosol (DerMardirossian and Bokoch 2005).

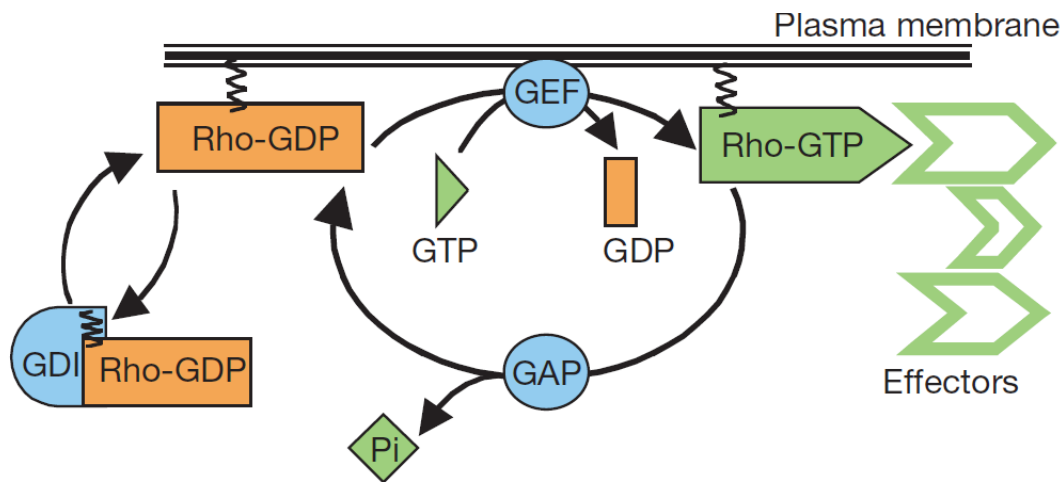


Fig. 7: Schematic overview of the activation cycle exemplified by the family of RhoGTPases. GEFs catalyse the exchange between GDP and GTP, leading to effector binding (green). GAPs hydrolyze GTP to GDP, to stop the interaction between GTP-bound GTPase and the different effectors. GDIs control the balance of membrane bound and cytosolic GTPases by binding and protection of the geranylgeranyl moiety (Etienne-Manneville and Hall 2002).

3.4.2 Functions of the different Ras superfamily members

The largest family within the RAS protein superfamily is represented by the Rab family, whose 63 members are involved in vesicular cargo trafficking by regulating of the intracellular vesicular transport and trafficking of proteins between different organelles via endocytotic and secretory pathways (Zerial and Stenmark 1993; Stenmark and Olkkonen 2001). In the Ran family, only one member is expressed in all eukaryotes, with the exception of plants (Sazer and Dasso 2000). Ran regulates nuclear transport in interphase cells by acting as molecular switch for the importin (karyopherin) family of cargo transporters (Poon and Jans 2005; Clarke and Zhang 2008). Ras is the prototypical small GTPase and is highly connected to human tumor pathogenesis because of its function to transmit signals from growth factor receptor tyrosine kinases to transcription factors, controlling cellular proliferation (Karnoub and Weinberg 2008). The ADP-ribosylation factor (ARF) family of proteins is involved in vesicular trafficking pathways and in humans, 15 members are known. They are the most divergent proteins within the Ras superfamily of small GTPases and are localized to plasma membrane and

the membranes of the secretory, endosomal and lysosomal pathways (D'Souza-Schorey and Chavrier 2006; Donaldson 2008). Because of the important role of RhoG in this dissertation, the Rho subfamily is explained in more detail. The human Rho family contains 23 isoforms, including RhoA, B, and C, Rac1, 2 and 3, Cdc42, RhoD, Rnd1,2, RhoE/Rnd3, RhoG TC10, TCL, RhoH/TTF, Chp, Wrch-1, Rif, RhoBTB1 and 2, RhoJ, Miro-1 and 2 and they are all found in all eukaryotic cells (Aspenstrom, Fransson et al. 2004). They act as molecular switches like the other GTPases described before, except of the three Rnd proteins and RhoH with no GTPase activity. Over 50 effectors are known for Rho, Rac and Cdc42 GTPases, most of them serine/threonine kinases, tyrosine kinases, lipid kinases, lipases, oxidases and scaffold proteins.

3.4.2.1 Regulation of the actin cytoskeleton

Rac and Cdc42 are known to regulate the actin cytoskeleton via indirect activation of actin-related proteins (Arp) 2/3, which serves as nucleation site for new actin filaments. Activity of Arp2/3 is regulated via members of the Wiskott-Aldrich syndrome protein (WASP) family, Cdc42 can directly bind to N-WASP, whereas Rac interacts with members of the WAVE family (Miki, Suetsugu et al. 1998) leading to activation of Arp 2/3 and initiation of growth of new actin filaments. Rho on the other hand stimulates actin polymerization by indirect interaction via formins, which are associated with the fast-growing end of actin filaments (Watanabe, Madaule et al. 1997). The main effector for Rho is the Rho-associated protein kinase (ROCK), which induce formation of stress fibers and focal adhesions by phosphorylation of myosin light chain (MLC) which leads to increased actin binding. Furthermore, activity of ADF/Cofilin, which severs actin filaments, is inhibited by Rac and Cdc42, mediated through binding and activation of PAK kinases followed by activation of ADF phosphorylating LIM kinases which finally

leads to phosphorylation and inhibition of ADF/Cofilin (Svitkina and Borisy 1999). LIM kinases can be also activated by ROCK.

3.4.2.2 Regulation of the microtubules

Besides the effects of members of the Rho family on the actin cytoskeleton, which is important for CEACAM3-mediated uptake of gonococci, there are also effects known on the microtubules, which is more important for CEACAM1-mediated uptake of pathogens. Activity of members of the Op18 (oncoprotein 18)/stathmin family is controlled by phosphorylation of four key residues, one of them (Ser16) is mediated by Cdc42/Rac-dependent activation of PAK after stimulation with extracellular stimuli. Op18/stathmin is known to interact with microtubule plus end to destabilize microtubules by sequestering tubulin dimers. Rho is known to promote formation of stabilized microtubules in migrating fibroblasts, mediated by guanine nucleotide exchange factor mDia, but the pathway is unknown until now. Furthermore, activity of a lot of microtubule plus-end binding proteins is controlled via RhoGTPases, for example CLIP170 or EB1 interacting with each other and a complex of other proteins and stabilize the plus ends of microtubule. EB1 and CLIP170 can both bind to mDia (Jaffe and Hall 2005). By binding to the CLIP170, IQGAP1 captures growing microtubules at the leading edge of migrating fibroblasts for cell polarization.

3.4.2.3 Regulation of enzyme activity

Other important effectors of RhoGTPases with regard to their role in endocytotic processes are the enzymes, which are involved in lipid metabolism, for example phosphoinositides 4P 5-kinase, PI3K, diacylglycerol kinase, phospholipase D and phospholipase C. RhoG and Cdc42 are known to bind directly to p85 subunit of PI3K (Zheng, Bagrodia et al. 1994; Murga, Zohar et al. 2002), which leads to generation of PI(3,4,5)P. Rac and Rho bind and activate PIP5-kinase, which increases the amount of PI(4,5)P,

furthermore they can activate phospholipase D which increase amounts of phosphatidic acid and choline. Summing up, members of the Rho protein family are mainly involved in actin and microtubule remodeling and can also be linked to regulation of apoptosis and gene regulation.

3.4.3 RhoG – functions and effectors

During this dissertation it could be shown, that the small GTPase (Ras homology Growth-related) RhoG, which is a small (~21kDa) monomeric GTP-binding protein and contains to the Rac subfamily of the Ras superfamily of small G proteins, plays an important role during CEACAM1-mediated uptake of gonococci. Expression of RhoG in mammals is widespread and shares a subset of binding partners with Rac, Cdc42 and RhoU/V. Upstream and downstream signaling of RhoG is poorly described until now. Only a few GEFs have been described for RhoG and no cellular RhoG GAPs are known. Intercellular adhesion molecule (ICAM-1), syndecan-4 and ephrin type-A receptor 2 (EphA2) are cell surface receptors modulating activity of RhoG (van Buul, Allingham et al. 2007; Elfenbein, Rhodes et al. 2009; Hiramoto-Yamaki, Takeuchi et al. 2010). The interaction between RhoG and Engulfment and Cell Motility 2 (ELMO2) is very important for regulating of cell polarity and migration. GTP-loaded RhoG binds and activate ELMO2, which can also be associated with scaffold protein integrin-linked kinase (ILK). ILK is linked to cytoplasmic domain of β 1-integrin, and ELMO2 serves as a bridge between RhoG and ILK. This complex induces polarization and ELMO2 can also bind to members of the Dedicator of cytokinesis (DOCK) family, which can activate Rac1, another member of the Rac subfamily, promoting lamellipodium formation and cell migration (Katoh and Negishi 2003; Ho and Dagnino 2011). So one of the important functions of RhoG is to operate as a key upstream regulator of Rac1 activity, and this interaction is responsible for the most intracellular functions of RhoG like pro-

motion of cell migration, neurite outgrowth, gene expression in lymphocytes and stimulation of phagocytosis and macropinocytosis (Brugnera, Haney et al. 2002; Katoh and Negishi 2003; Katoh, Hiramoto et al. 2006). Dynamic membrane protrusion forming a cup in leukocytes depends on activation of intercellular adhesion molecule-1 (ICAM1), followed by activation of RhoG-specific GEF SGEF leading to GTP-loading of RhoG is one of the most well-known pathway for RhoG activation (van Buul, Allingham et al. 2007). Recently, another important pathway was found: Activation of RhoG by its newly discovered guanine nucleotide exchange factor Ephexin4 leads to increased migration after stimulation of the upstream receptor tyrosine kinase EphA2, which was also linked to activation of EGF-receptor via EGF (Hiramoto-Yamaki, Takeuchi et al. 2010). The same group could show, that activation of RhoG by Ephexin4 causes increased resistance against the programmed cell death anoikis by direct binding of RhoG-GTP to p85 subunit of classI PI3K to activate the PI3K, followed of signal transduction by AKT (PKB) (Harada, Hiramoto-Yamaki et al. 2011). The connection between RhoG and PI3K for increased survival of cells was also published in 2002, where they could show that Rac1 and RhoG activates PI3K and AKT independent from activation of c-Jun N-terminal kinase (JNK) and NF- κ B (Murga, Zohar et al. 2002), which are Rac1 effectors and important for regulation of apoptosis and immune response. There is a linkage to endocytotic processes in mammalian cells mainly by syndecan-4, a protein which modulates the function of integrins in cell- matrix adhesion, associates with protein kinase C α (PKC α), triggers RhoG activation mediating the endocytotic signal of dynamin- and caveolin-dependent integrin uptake, important for tissue repair (Bass, Williamson et al. 2011). RhoG was linked earlier to caveolar endocytosis (Prieto-Sanchez, Berenjeno et al. 2006), where colocalization of RhoG could be shown in caveolar endocytosis. This process is dynamin2 dependent, but until now, other involved signaling molecules are not known. As conclusion, most functions of RhoG (especially on the actin cytoskele-

ton) are mediated by its ability to activate Rac1, but there are other pathways, which are independent from Rac1 activation, like EphA2- and EGF-receptor internalization.

3.4.4 Manipulation of small GTPases by pathogens

There are some reports about different pathogens using small GTPases to invade in mammalian cells, mostly mediated by the influence on the cytoskeleton of activated Rac1 via RhoG and Dock. Members of the family *Salmonella enterica*, gram-negative pathogenic bacteria, express different proteins to modulate RhoGTPases during the invasion. Bacterial proteins SopE and SopE2 can catalyze nucleotide exchange of Rac1 and Cdc42 (Hardt, Chen et al. 1998), whereas SopB/SigD operates as phosphoinositide phosphatase to stimulate Rho-dependent actin remodeling leading to activation of RhoG-specific GEF SGEF. The signaling components are not known (Norris, Wilson et al. 1998; Patel and Galan 2006). Another RhoGTPase-interacting pathogen is *Escherichia coli*, which translocates through the type III secretion system the mammalian RhoGEF inhibitor EspH into mammalian cells to inactivate present RhoGEFs and induce at the same time the EspH insensitive bacterial RhoGEFs EspM2 and EspT into the cell. This leads to neutralizing of EspH-induced focal adhesion disassembly, cell attachment and caspase-3 activation, important for invasion and survival of the pathogens (Wong, Clements et al. 2012). Also *Shigella flexneri* express and translocate the bacterial protein IpgB1 into mammalian cells through the type III secretion system, which is a bacterial mimic of RhoG, binding to GEF ELMO-DOCK180 complex to activate Rac1. Activation of Rac1 leads to membrane ruffling of the cells followed by effective internalization of *Shigella* (Handa, Suzuki et al. 2007). Members of the pathogenic bacteria family *Yersenia* also contain a type III secretion system to translocate different effectors in the mammalian cell, like YopT, a cysteine protease which inactivates RhoA, Cdc42 and Rac1 by removing their C-terminal isoprenoid moiety (Shao,

Merritt et al. 2002). The protein Invasin triggers outside-in signaling via β 1-integrins, which activates RhoA, Rac1 and Cdc42 activation leading to cytoskeletal reorganization, IL-8 release and bacterial internalization (Wiedemann, Linder et al. 2001; Grassl, Kracht et al. 2003). The serine/threonine kinase YopO/YpkA inhibits activity of Rac1 and RhoA via its guanine nucleotide dissociation inhibitor (GDI) function for RhoGTPases (Prehna, Ivanov et al. 2006), and YopE mimics a GAP for inactivating of RhoGTPases Rac1, RhoA and Cdc42 (Black, Marie-Cardine et al. 2000). Also RhoG is modulated directly by *Yersenia*: Invasin could be shown as activator of RhoG whereas YopE is a specific inhibitor of RhoG activity (Roppenser, Roder et al. 2009). Also for *Neisseria gonorrhoeae* involvement of RhoGTPases by mediating uptake via CEACAM receptors was described, especially for CEACAM3, where activity of Rac1 and Cdc42 but not of RhoA is important for actin remodeling during uptake process (Billker, Popp et al. 2002). To conclude, different invasive pathogens use small GTPases of the Rho family to internalize into mammalian cells, mainly based on the functions in actin cytoskeleton remodeling via the GTPases.

3.5 Erythropoetin-producing hepatoma (Eph) receptor tyrosine kinases

3.5.1 Family members and structure of Eph receptors

Nearly two decades ago, Erythropoetin-producing hepatoma (Eph) receptor tyrosine kinases were discovered. They comprise the largest family of tyrosine kinases encoded in the human genome. Nine EphA (EphA1-8 and EphA10) and five EphB (EphA1-4 and EphB6) receptors are known in humans, and eight different ligands are described, which are based on their structural features and binding affinity for EphA or EphB receptors divided in GPI-anchored ephrinA (A1-5) and ephrinB (B1-3) (Pasquale 2005;

Pitulescu and Adams 2010). The extracellular part of Eph receptors contains a globular ligand-binding domain, followed by a cysteine-rich region and two fibronectin-type III repeats. The cytoplasmic part consists of a juxtamembrane region with several conserved tyrosine residues followed by a tyrosine kinase domain, a sterile α motif (SAM) protein-protein interaction domain and a C-terminal PDZ-binding motif (Fig. 8).

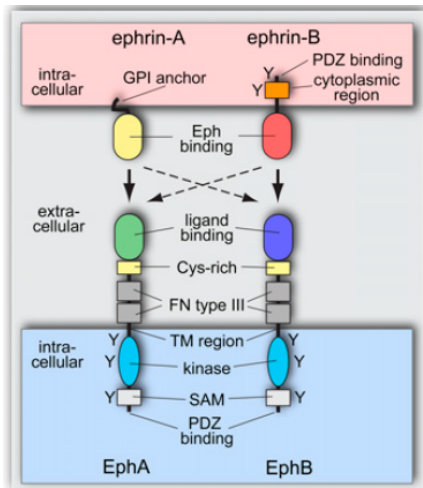


Fig. 8: Eph/Ephrin structure. Domain organization of Eph receptors and Ephrin ligands. Cystein (Cys) rich, fibronectin (FN) type III, SAM domains, transmembrane (TM) region and tyrosine (Y) phosphorylation sites are indicated. Binding of ephrins to Eph receptors are shown with arrows, crosstalk is possible (dashed arrows)(Pitulescu and Adams 2010)

3.5.2 Signaling of Eph receptors

Eph-ephrin binding leads to bidirectional signal transduction into both, the receptor cells (“forward signaling”) and the ligand cell (“reverse signaling”). Eph/ephrin interactions are very important for tissue assembly and in maintaining tissue homeostasis, especially for embryonic development including axon guidance, cell migration and segmentation. In adult organism the interaction is critical for long-term potentiation, angiogenesis, stem cell differentiation and cancer. In many cases Eph-ephrin interactions generate repulsive signals, which are important for example to guide growing neuronal axons and migrating cells to their targets. Binding of monomeric Eph receptor and ephrin leads to conformational changes and recruitment of further Eph/ephrin complexes (dimerization, building of tetramers, aggregation into large clusters), followed by transphosphorylation of the cytoplasmic domains of the Eph receptors, together with the often associated Src-family kinases. Apart from homooligomerization, heterooligomeri-

zation between EphA and EphB receptors is also described (Janes, Griesshaber et al. 2011). The interactions with proteins containing a src-homology-2 domain (SH2) are phosphorylation-dependent, whereas interactions with PDZ-domain containing proteins and guanine exchange factors (GEFs) are independent of the phosphorylation of the Eph receptor kinase domain. Phosphotyrosin phosphatases and ubiquitin ligase Cbl can be recruited to activated Eph receptors to terminate the signal (Pasquale 2005). In addition, terminating of the signal can be regulated by proteolytic cleavage of the Ephrin via metalloprotease ADAM10. Another possibility to disrupt the signals between Eph receptors and ephrin ligands is endocytosis of the intact receptor-ligand complex and associated cytoplasmic proteins together with surrounding plasma membrane (termed *trans* endocytosis) into the Eph- or the Ephrin-expressing cell (Pitulescu and Adams 2010). Endocytosis requires actin polymerization and activation of the small GTPase Rac1 (Marston, Dickinson et al. 2003). Internalization of Ephrin-B1 is linked to the classical clathrin-dependent endocytosis. But in contrast, during uptake of EphB receptors from cell surface, caveolae seems to be involved (Vihanto, Vindis et al. 2006). Eph/Ephrin signaling is coupled to activity of small GTPases, mainly Rac1, Cdc42 and Rho, which are important for actin cytoskeleton rearrangements. Endocytosis of Eph receptors (especially EphA2) seems to be PI3K dependent, because it could be shown, that SHIP2, a 5'phosphatase of PI(3,4,5)P is recruited to activated EphA2 after ligand stimulation via its SAM domain. Overexpression of SHIP2 inhibits endocytosis, whereas suppression of SHIP2 promotes ligand-induced receptor internalization and is Rac1/actin cytoskeleton dependent (Zhuang, Hunter et al. 2007). Another important function of Eph receptors in the human body is the linkage to angiogenesis and developing of lymphatic vessels. CEACAM1 is also known to regulate angiogenesis, so this could be a cellular linkage between Eph receptors and CEACAMs, independent of bacterial uptake.

3.5.3 EphA2

The cellular functions of EphA2 are not well understood, but based on studies in tumor-based models, roles in regulation of cell growth, survival, migration and angiogenesis are suggested. EphA2 is abundantly overexpressed in several solid tumors, but limited in normal tissues to those having a high proportion of dividing epithelial cells like lung, skin, and small intestine. EphA2 is an exception within the family, because its kinase activity is not dependent on activation via ligand binding followed by phosphorylation of the conserved activation loop tyrosines. It could be shown, that E-cadherin, which is an adhesion protein important for cell-cell interactions, is required for correct phosphorylation and cell localization (Zantek, Azimi et al. 1999). EphA2 expression is directly regulated by the Ras-MAPK pathway, for example after stimulation of the mammalian cell with EGF, whereas EphrinA1 (the ephA2 ligand) expression is inhibited by the same pathway. Stimulation of EphA2 via Ephrin-A1 leads to phosphorylation of EphA2, internalization and association with the adaptor protein c-Cbl, followed by degradation of EphA2. Ligand-induced phosphorylation of EphA2 leads to interaction and complex formation with the adaptor molecules SHC in a direct way and indirect interaction by SHC to growth factor receptor-bound protein2 (GRB2) leading to activation of MAP/ERK pathway and thereby negatively regulates attachment to the extracellular matrix (Pratt and Kinch 2002). Furthermore, EphA2 associates with focal adhesion kinase (FAK), and activation of EphA2 leads to disruption of this complex, followed by reduced integrin-mediated cell adhesion to extracellular matrix (Miao, Burnett et al. 2000). EphA2 could be linked directly to PI3K, because of binding to class I PI3K p85 subunit by phosphorylated EphA2, which is very important for angiogenesis (Wykosky and Debinski 2008). Another linkage to PI3K activity was described by the group of Katoh in the last years: activation of EphA2 receptors leads to recruitment of the guanine nucleotide exchange factor Ephexin4, which catalyze the GTP-binding of the small

GTPase RhoG. GTP-RhoG binds directly to p85 subunit of class I PI3K, leading to suppression of apoptotic processes in cells (suppression of anoikis) (Katoh and Negishi 2003; Katoh, Hiramoto et al. 2006; Hiramoto-Yamaki, Takeuchi et al. 2010; Harada, Hiramoto-Yamaki et al. 2011).

3.5.4 Manipulation of Eph receptors by pathogens

In the last years, different entry mechanism of pathogens could be linked to EphA2 receptor, for example the Kaposi's sarcoma associated herpesvirus (KSHV), which activates EphA2 receptor during invasion via integrins to amplify KSHV-induced Src- and PI3K signals in lipid rafts (Chakraborty, Veettil et al. 2012). Also for effective entry of the hepatitis C (HCV) virus into mammalian cells EphA2 was identified as host co-factor, by regulating of CD81-claudin-1 co-receptor association and viral-glycoprotein-dependent membrane fusion during clathrin-dependent internalization of the virus (Lupberger, Zeisel et al. 2011). Further examples for Eph receptor-pathogen interactions are the direct binding of EphrinB2 to paramyxoviridae Nipah and Hendra virus (Bonaparte, Dimitrov et al. 2005; Negrete, Levroney et al. 2005) and regulation of the T-cell immune response by binding and activation of EphA receptors via unknown bacterial factors of *Mycobacterium tuberculosis* (Khounlotham, Subbian et al. 2009). It is also known that *M. tuberculosis* induces EphA1 and EphA3 expression in monocytic cells, and with particular interest for this dissertation it was published that infection of epithelial cells with pathogenic *Neisseria gonorrhoeae* leads to induction of expression of EphA2 and EphA3 (Plant, Asp et al. 2004). This supports our findings of the role of EphA2 during CEACAM1-mediated uptake of *Neisseria gonorrhoeae*.

4 AIMS OF THE STUDY

CEACAM receptors serve as entry portal for pathogens like *Neisseria gonorrhoeae*, a wide spread sexually transmitted disease and *Neisseria meningitides*, responsible for endemic outbreaks of meningitis with 10% mortality. The antibiotic multi-resistant strains are a major problem, so studying this pathogen-host interaction is very important for development of urgently required new therapies.

In the first chapter we investigated, whether the human specificity of these pathogens is based on the species-specific binding to their receptors. We used the N-terminal Ig_V-like domains of different mammalian species and compared the binding of pathogens to the Ig_V-like domain of human CEACAM1.

In the second chapter we wanted to get more insight in the host cell proteins and mechanism during bacterial uptake via epithelial CEACAMs, especially CEACAM1. Uptake of gonococci via CEACAM3 into granulocytes is elucidated in a wide manner. It is regulated mainly by Src kinases, the guanine nucleotide exchange factor Vav, the small GTPase Rac1, leading to massive actin cytoskeleton rearrangements. In contrast, for CEACAM1 (or other epithelial CEACAMs) almost nothing is known about the mechanistically uptake and the involved proteins. It became clear, that the actin cytoskeleton is only partially involved into bacterial uptake and lipid rafts play an important role. We investigated the role of the PI3K and which part of CEACAM1 is responsible for PI3K activation. In the third chapter we further analyzed signaling molecules involved in CEACAM1-mediated uptake of pathogens, especially the role of small GTPases and the guanine nucleotide exchange factors, furthermore we revealed a possible co-receptor for epithelial CEACAMs which is responsible for signal transmitting into mammalian cells.

5 CHAPTER I

CEACAM1 recognition by bacterial pathogens is species- specific

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5.1 Abstract

Background: Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), an immunoglobulin (Ig)-related glycoprotein, serves as cellular receptor for a variety of Gram-negative bacterial pathogens associated with the human mucosa. In particular, *Neisseria gonorrhoeae*, *N. meningitidis*, *Moraxella catarrhalis*, and *Haemophilus influenzae* possess well-characterized CEACAM1-binding adhesins. CEACAM1 is typically involved in cell-cell attachment, epithelial differentiation, neovascularisation and regulation of T-cell proliferation, and is one of the few CEACAM family members with homologues in different mammalian lineages. However, it is unknown whether bacterial adhesins of human pathogens can recognize CEACAM1 orthologues from other mammals.

Results: Sequence comparisons of the amino-terminal Ig-variable-like domain of CEACAM1 reveal that the highest sequence divergence between human, murine, canine and bovine orthologues is found in the β -strands comprising the bacteria-binding CC'FG-face of the Ig-fold. Using GFP-tagged, soluble amino-terminal domains of CEACAM1, we demonstrate that bacterial pathogens selectively associate with human, but not other mammalian CEACAM1 orthologues. Whereas full-length human CEACAM1 can mediate internalization of *Neisseria gonorrhoeae* in transfected cells, murine CEACAM1 fails to support bacterial internalization, demonstrating that the sequence divergence of CEACAM1 orthologues has functional consequences with regard to bacterial recognition and cellular invasion.

Conclusions: Our results establish the selective interaction of several human-restricted bacterial pathogens with human CEACAM1 and suggest that co-evolution of microbial adhesins with their corresponding receptors on mammalian cells contributes to the limited host range of these highly adapted infectious agents.

5.2 Introduction

The immunoglobulin (Ig) superfamily contains a large number of receptors that serve as cell adhesion molecules (CAMs) mediating homotypic cell-cell-adhesion in multicellular animals. One group of mammalian IgCAMs is named according to the carcinoembryonic antigen (CEA), a tumor marker used in the surveillance of colon cancer (Hammarstrom 1999). Interestingly, CEA is only known from primates, where it is expressed by mucosal epithelial cells. Similar to CEA, most other CEA-related CAMs (CEACAMs) are restricted to specific mammalian lineages, and only a few CEACAMs, such as CEACAM1 or CEACAM16-20, have orthologues in distantly related mammals (Zebhauser, Kammerer et al. 2005; Kammerer, Popp et al. 2007; Kammerer and Zimmermann 2010). Accordingly, sequence comparisons based on published genome data have provided evidence that CEACAMs have independently diversified in each mammalian order (Kammerer, Popp et al. 2004; Kammerer, Popp et al. 2007).

In humans, CEACAM1 is the target of several Gram-negative commensal and pathogenic bacteria that inhabit the nasopharyngeal, intestinal, or urogenital mucosa. In particular, *Neisseria gonorrhoeae*, *N. lactamica*, *N. meningitidis*, *N. subflava*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Escherichia coli* strains have been found to associate with the protein core or carbohydrate structures of this glycoprotein (Sauter, Rutherford et al. 1993; Chen and Gotschlich 1996; Virji, Makepeace et al. 1996; Hill, Toleman et al. 2001; Toleman, Aho et al. 2001; Hill and Virji 2003). These bacterial species utilize distinct surface proteins (adhesins) to engage CEACAMs. For example, the neisserial colony opacity associated (Opa) proteins allow gonococci and meningococci to bind several CEACAM family members including CEACAM1, CEA, and CEACAM6, which are expressed on the apical surface of mucosal epithelial cells. Opa proteins are integral outer membrane proteins with 8 transmembrane β -strands and 4

small extracellular loops, with the central loops participating in CEACAM recognition (Bos, Kao et al. 2002). Opa-like proteins with a similar β -barrel structure are also found in commensal *Neisseria* species and can mediate the association with CEACAM1 (Toleman, Aho et al. 2001). In addition, several typeable and non-typeable strains of *Haemophilus influenzae*, a species that shares the mucosal habitat and lifestyle of *Neisseria*, can engage CEACAM1 via their outer membrane protein P5 (Hill, Toleman et al. 2001). Another inhabitant of the human oro-pharyngeal mucosa, *Moraxella catarrhalis*, can bind via the UspA1 surface protein to the N-terminal domain of CEACAMs (Hill and Virji 2003). UspA1 belongs to the family of trimeric autotransporter or oligomeric coiled-coil adhesin (Oca) family. The prototype of the Oca family is the adhesin YadA of enteropathogenic *Yersiniae* that has a lollipop structure with a head group, an extended coiled-coil stalk region and a membrane anchor domain (Hoiczky, Roggenkamp et al. 2000). The mature trimeric UspA1 with a size of about 250 – 300 kDa protrudes up to 60 nm from the bacterial surface and is therefore completely distinct from membrane-embedded neisserial Opa proteins or the *Haemophilus* protein P5 (Hoiczky, Roggenkamp et al. 2000). Surprisingly, CEACAM recognition by the *Moraxella* UspA1 is mediated by a short sequence within the stalk region requiring a bend conformation of the UspA1 extracellular domain to accommodate CEACAM1 binding (Conners, Hill et al. 2008). *Moraxella* strains lacking this peptide sequence within their stalk region fail to bind to CEACAMs (Brooks, Sedillo et al. 2008).

The striking convergent evolution of structurally distinct adhesive proteins to engage CEACAM1 suggests that this binding is important during the life cycle of these bacteria. As all CEACAM-binding bacteria greatly differ in their pathogenic potential, but share the same ecological niche, it is highly likely that CEACAM-binding promotes colonization of the mucosa. Indeed, in vitro experiments have suggested that CEACAM-binding is not only a means to firmly attach to the host cell surface, but also sup-

presses the detachment of infected epithelial cells (Muenzner, Rohde et al. 2005). CEACAM-targeting bacterial adhesins might therefore represent colonization factors that promote the ability of bacteria to establish a firm foothold in their ecological niche. Whether this specialization is also a determinant of the host range of these bacterial pathogens is not known. Though bacterial species expressing CEACAM-binding adhesive proteins are in most cases human-specific, and have no other natural host organism, it has not been experimentally tested whether their adhesins selectively recognize human CEACAMs or can also bind to orthologues from other mammalian species.

In the present study, we analysed the binding of CEACAM1 orthologues from several mammals to bacterial pathogens with distinct adhesive proteins. In particular, we tested Opa protein-expressing *N. gonorrhoeae* and *N. meningitidis* as well as UspA1-expressing *M. catarrhalis* for their ability to recognize CEACAM1 homologues of human, murine, canine or bovine origin. Biochemical binding studies clearly demonstrate that these bacteria selectively interact with human CEACAM1. Furthermore, analyses of bacterial internalization show that the observed amino acid changes in the amino-terminal domain of mammalian CEACAM1 orthologues have clear-cut functional consequences. Accordingly, our data not only demonstrate that bacterial adhesins have co-evolved with the receptor molecules of their mammalian host, but also support the view that the diversification of CEACAMs in mammalian lineages is a pathogen-driven process.

5.3 Material & Methods

Amino acid sequence alignment

For the amino acid sequence alignment of the N-terminal domains of CEACAM1 following sequences were used: human CEACAM1 (hCEA1, NM_001712), murine CEACAM1a (mCEA1, BC016891), canine CEACAM1 (cCEA1, NM_001097557.1), bovine CEACAM1 (bCEA1, AY345129), bovine CEACAM1 isoform b (bCEA1b, AY487418). The alignment was performed using CLUSTALW.

Cell culture and transfection

The human embryonic kidney cell line 293T (293 cells) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum at 37°C in 5% CO₂ and subcultured every second to third day. 293T cells were transfected by calcium-phosphate coprecipitation using 5 - 8 µg of plasmid DNA for each 10 cm culture dish.

Bacteria and infection

Opa₅₂-expressing (Opa_{CEA}), non-piliated *N. gonorrhoeae* MS11-B2.1 (strain N309), and non-piliated, non-opaque gonococci MS11-B2.1 (strain N302) were kindly provided by T.F. Meyer (Max-Planck Institut für Infektionsbiologie, Berlin, Germany) and were cultured as described previously (Schmitter, Agerer et al. 2004). Opa-expressing, non-encapsulated *N. meningitidis* (SiaD mutant of strain MC58) was obtained from Matthias Frosch (Institut für Hygiene und Mikrobiologie, Universität Würzburg, Germany). *M. catarrhalis* strain ATCC 25238 was obtained from DSMZ (Braunschweig, Germany). Both *Moraxella* and *Neisseriae* were grown on GC agar plates (Difco BRL, Paisley, UK) supplemented with vitamins at 37°C, 5% CO₂ and subcultured daily. For infection, bacteria were suspended in DMEM and the optical density of the suspension was used

to estimate the number of the microorganisms according to a standard curve generated for each strain.

Recombinant plasmid constructs

Mammalian expression plasmids encoding GFP-tagged human CEACAM1-4L (hCEACAM1-4L), human CEACAM1-4S, and the amino-terminal domain of human CEACAM1 (hCEA1-N) were described previously (Kuespert, Weibel et al. 2007; Muenzner, Bachmann et al. 2008). Murine CEACAM1-4S was constructed by amplifying the full-length cDNA of murine CEACAM1-4S (clone BF584691; ImaGenes, Berlin, Germany) with primers mCEACAM1-sense 5'-GAAGTTATCAGTCGACATGGAGCTGGCCTCAGCAC-3' and mCEACAM1-anti 5'-ATGGTCTAGAAAGCTTCCGCCAGACTTCCTGG-3'. The amino-terminal domain of murine CEACAM1 was amplified with primers mCEACAM1-sense and mCEACAM1-N-anti 5'-ATGGTCTAGAAAGCTTGGGTGTACATGAAATCGC-3'. The N-terminal domains of bovine CEACAM1 isoforms a and b as well as canine CEACAM1 were amplified from full-length cDNA using primers bovine CEACAM1abN for 5'-GAAGTTATCAGTCGACATGGGGACCCCCTCAG-3', bovine CEACAM1aN rev 5'-ATGGTCTAGAAAGCTTGGGAGTATGTGGAGGTGTCCAG-3', bovine CEACAM1bN rev 5'-ATGGTCTAGAAAGCTTTGGAGTACGTGGAGGTGTCC-3', canine CEACAM1N for 5'-GAAGTTATCAGTCGACATGGAGCCCCCCTCG-3' and canine CEACAM1N rev 5'-ATGGTCTAGAAAGCTTGGGAATACTTGGAGCTGTCC-3'. All the resulting PCR fragments were cloned into pDNR-Dual using the In-Fusion PCR Cloning Kit (Clontech, Mountain View, CA) and transferred by Cre-mediated recombination into pLPS-3'EGFP (Clontech) resulting in GFP fused to the carboxy-terminus of the expressed

proteins. Full-length human CEACAM1-4S and murine CEACAM1-4S were also transferred from pDNR-Dual into pLPS3'mCerulean resulting in mCerulean fused to the carboxy-terminus of the expressed proteins. pLPS3'mCerulean was generated by replacing the GFP coding sequence in pLPS3'EGFP with the cDNA encoding mCerulean (Rizzo, Springer et al. 2004) generously provided by D.W. Piston (Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee, USA).

Cell lysis and Western blotting

Cell lysis and Western blotting were performed as described (Schmitter, Agerer et al. 2004) using a rabbit polyclonal antibody against His-tagged GFP (produced at the animal core facility; University of Konstanz) or a monoclonal antibody against Opa proteins (clone 4B2/C11; generous gift of Marc Achtman, MPI für Infektionsbiologie, Berlin, Germany). Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA).

Binding studies of the different pathogens

Binding studies of the different pathogens with the soluble N-terminal domains of human, murine, bovine and canine CEACAM1 were performed as described (Kuespert, Weibel et al. 2007). Briefly, 4×10^7 bacteria were added to CEACAM1-N-domain-containing cell culture supernatants in a total volume of 1 ml and incubated for 30 min. After four washing steps, the samples were analysed on a LSR II flow cytometer (BD Bioscience, Heidelberg, Germany) by gating on the bacteria (based on forward and sideward scatter) and measuring bacteria-associated GFP fluorescence. In each case, 10,000 events per sample were obtained.

Gentamicin protection assay

Gentamicin protection assays were conducted as described (Schmitter, Agerer et al. 2004). Briefly, 5×10^5 293 cells were seeded in 24-well plates coated with 10 $\mu\text{g/ml}$ poly-L-lysine. Cells were infected with 30 bacteria/cell (MOI 30) for two hours. Then, the medium was replaced with DMEM containing 50 $\mu\text{g/ml}$ gentamicin. After 45 min of incubation in gentamicin-containing medium, cells were lysed by the addition of 1% saponin in PBS for 10 min. Suitable dilutions were plated in triplicates on GC agar to determine the number of recovered viable bacteria.

Flow cytometry invasion assay

Bacterial uptake by transfected 293 cells was analysed by flow cytometry as described (Pils, Schmitter et al. 2006). Prior to infection, bacteria were labelled with 0.2 $\mu\text{g/ml}$ 5-(6)-carboxyfluorescein-succinylester (fluorescein; Invitrogen-Molecular Probes, Karlsruhe, Germany) in PBS at 37°C for 30 min. Cells were infected with labelled bacteria at an MOI of 30 for 2 h. After infection, cells were washed with PBS and the samples were analysed on a LSR II flow cytometer (BD Bioscience) by gating on the cells based on forward and sideward scatter. Cell-associated fluorescein fluorescence was measured in the presence of 2 mg/ml trypan blue to quench fluorescence of extracellular bacteria and to selectively detect the fluorescence derived from intracellular bacteria. The percentage of fluorescein-positive cells was multiplied by the mean fluorescence intensity of the sample to obtain an estimate of the total number of internalized bacteria (uptake index). In each sample 10,000 cells were counted.

Immunofluorescence staining

293 cells transfected with the indicated constructs were seeded onto poly-L-lysine- and fibronectin-coated (10 $\mu\text{g/ml}$ and 4 $\mu\text{g/ml}$, respectively, in PBS) coverslips in 24-well

plates. Cells were infected for 2 h with 5-(and-6)-carboxytetramethylrhodamine-succinimidyl- and biotin-labelled Opa_{CEA}-expressing *N. gonorrhoeae* at an MOI of 20 essentially as described (Agerer, Waeckerle et al. 2004). To discriminate between extracellular and intracellular bacteria, infected samples were fixed with 4% paraformaldehyde in PBS and washed three times with PBS, prior to incubation in blocking buffer (PBS, 10% FCS) for 15 min. Extracellular bacteria were stained with AlexaFluor647-streptavidin (Invitrogen, Karlsruhe, Germany) diluted 1:100 in blocking buffer for 1 h. Following three washes, samples were embedded in mounting medium (Dako, Glastrup, DK).

Samples were viewed with a TCS SP5 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) using a 63x, 1.3 NA Plan Neofluar oil-immersion objective. Fluorescence signals of triple-labelled specimens were serially recorded to avoid bleed-through. Images were digitally processed with NIH ImageJ and merged to yield pseudo-coloured pictures.

5.4 Results

Mammalian CEACAM1 orthologues show conserved as well as divergent regions in their amino-terminal domains

The amino-terminal domain of CEACAM1 is a target for bacterial pathogens (Leusch, Drzeniek et al. 1991; Chen and Gotschlich 1996; Virji, Makepeace et al. 1996; Virji, Evans et al. 2000; Hill and Virji 2003). In particular, the non-glycosylated CC'C''FG-face of the immunoglobulin fold is the binding interface recognized by microorganisms (Villullas, Hill et al. 2007). To analyse if this potential evolutionary pressure by pathogens is reflected in sequence variation within this domain, we aligned and compared the published sequences of the amino-terminal immunoglobulin variable (Ig_v)-like domain of human, murine, bovine and canine CEACAM1 (Fig. 9A). Indeed, sequence differences between the mammalian species are most prominent in β -strands forming the CC'C''FG-face, whereas the glycosylated AA'BDE-face of the immunoglobulin-fold has a higher amino acid sequence identity (Fig. 9B). To test if these sequence differences result in an altered functionality with regard to pathogen binding, we generated several constructs that allowed us to test the association of CEACAM amino-terminal Ig_v-like domains with various pathogens and to analyse their ability to mediate bacterial internalization by mammalian cells (Fig. 9C). Accordingly, we expressed Ig_v-like amino-terminal domains derived from human, bovine, murine, or canine CEACAM1 as secreted GFP fusion proteins in human 293 cells, a cell line that does not express any CEACAM family members endogenously (Fig. 9D). Importantly, GFP-tagged fusion proteins were found in cell culture supernatants of transfected cells and were expressed at similar levels as detected by Western blotting with GFP antibodies (Fig. 9D).

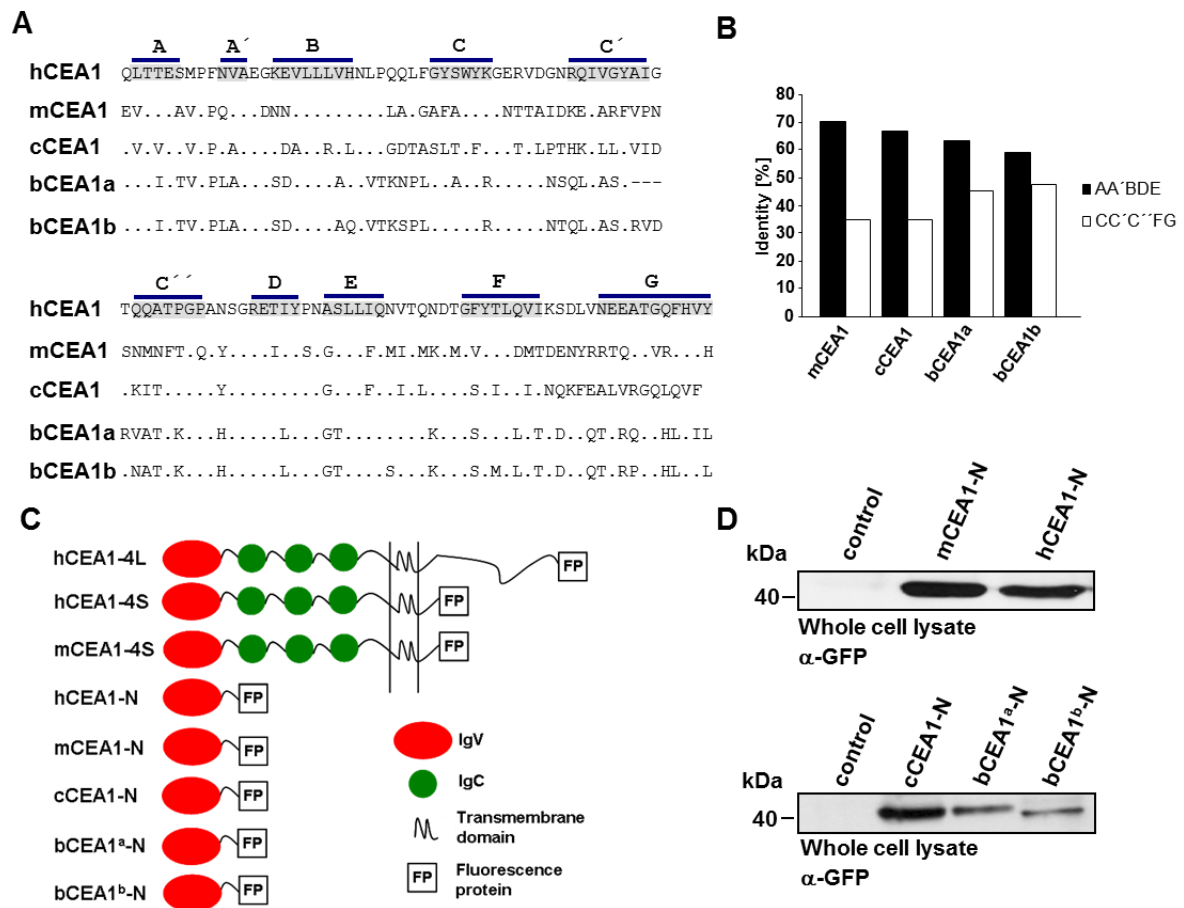


Fig. 9 Amino acid sequence alignment and expression of soluble CEACAM1 proteins of different mammals

(A) Amino acid sequence alignment of the N-terminal domains of human, murine, bovine and canine CEACAM1 proteins. The following sequences were used: human CEACAM1 (hCEA1, NM_001712), murine CEACAM1a (mCEA1, BC016891), canine CEACAM1 (cCEA1, NM_001097557.1), bovine CEACAM1 (bCEA1, AY345129), bovine CEACAM1 isoform b (bCEA1b, AY487418). Amino acids identical to the human CEACAM1 sequence are indicated by dots. The characteristic beta-strands of the Ig variable-like domain are marked by blue lines and letters above the human sequence. (B) Amino acid identity between different mammalian CEACAM1 orthologues. Percent identity compared to the human sequence is given for amino acid residues comprising the beta strands of either the AA'BDE-face or the CC'C''FG-face of the immunoglobulin fold. (C) Schematic illustration of the proteins used in this study. Human CEACAM1-4L isoform containing a long cytoplasmic domain (hCEA1-4L), the human CEACAM1 isoform containing a short cytoplasmic domain (hCEA1-4S), and the corresponding murine isoform (mCEA1-4S) were expressed as GFP or cerulean fusion proteins. Amino-terminal Igv-like domains of CEACAM1 from human (hCEA1), mouse (mCEA1), dog (cCEA1), or cattle (isoform a, bCEA1a; isoform b, bCEA1b) were expressed in human cells as soluble GFP-fusion proteins.

Binding of *Neisseria gonorrhoeae* to the amino-terminal domain of CEACAM1 is human specific

The soluble GFP-tagged amino-terminal domains of CEACAM1 orthologues were incubated with isogenic strains of the human pathogen *N. gonorrhoeae*. The bacterial strains used either expressed a specific Opa protein, which is known to bind human CEACAM1 and other human CEACAMs (Ngo Opa_{CEA}), or they did not express any Opa protein (Ngo Opa⁻). Opa expression by the gonococci was confirmed by Western blotting with a monoclonal antibody against neisserial Opa proteins (Fig. 10A). Following incubation with the amino-terminal CEACAM1 domains from different mammalian species, the samples were washed, and the bacteria-associated fluorescence was measured by flow cytometry. Clearly, the non-opaque bacteria (Ngo Opa⁻) did not reveal a positive signal in the GFP channel for any tested protein, confirming that Opa proteins are the sole neisserial factor necessary for CEACAM recognition (Fig. 10B). In contrast to the non-opaque gonococci, the Opa_{CEA}-expressing bacteria clearly associated with the isolated amino-terminal Ig_v-like domain of human CEACAM1 (Fig. 10B). Most importantly, Opa_{CEA}-positive gonococci did not associate with the Ig_v-like domains of murine, canine or bovine origin (Fig. 10B). These results demonstrate that the association of *Neisseria gonorrhoeae* with CEACAM1 is limited to the human orthologue of this protein and suggests that CEACAM1 recognition is species-specific.

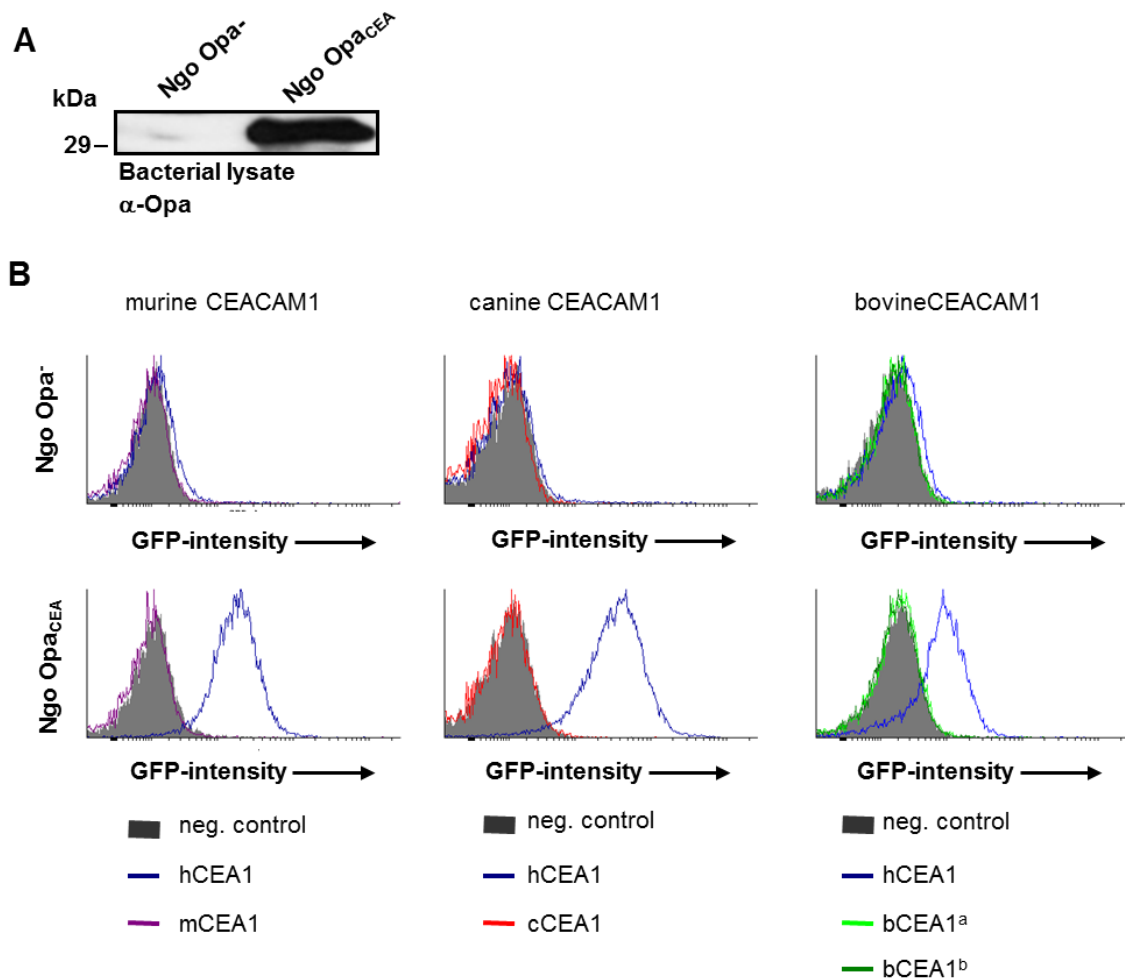


Fig. 10 OpaCEA protein expressing *Neisseria gonorrhoeae* selectively binds to human CEACAM1 A) *Neisseria gonorrhoeae* MS11 strains lacking Opa protein expression (Ngo Opa⁻) or expressing a CEACAM-binding Opa protein (Ngo Opa^{CEA}) were lysed and the Opa protein expression was determined by Western blotting with a monoclonal anti-Opa antibody (clone 4B12/C11). (B) Culture supernatants containing soluble GFP-tagged amino-terminal domains of the indicated mammalian CEACAMs or a control culture supernatant from GFP-transfected cells (neg. control) were incubated with OpaCEA protein-expressing *N. gonorrhoeae* (Ngo Opa^{CEA}) or the non-opaque strain (Ngo Opa⁻). After washing, bacteria were analysed by flow cytometry and the bacteria-associated GFP-fluorescence was determined. Only human CEACAM1 (hCEA1) binds to Ngo Opa^{CEA}.

Binding of *Moraxella catarrhalis* and *Neisseria meningitidis* to the amino-terminal domain of CEACAM1 is species-specific

To extend these findings to other pathogens known to engage the amino-terminal domain of CEACAM1 we employed *N. meningitidis* MC58, a serogroup B strain, and *M. catarrhalis* ATCC 25238 in CEACAM binding assays. Accordingly, the bacteria were incubated with supernatants containing GFP-tagged amino-terminal Igv-like domains of

distinct mammalian CEACAM1 orthologues, and after washing, the bacteria were analyzed by flow cytometry for associated GFP-fluorescence. Similar to what we had observed with *N. gonorrhoeae*, both bacterial species did not associate with the amino-terminal Ig_v-like domains of bovine, murine, or canine origin (Fig. 11).

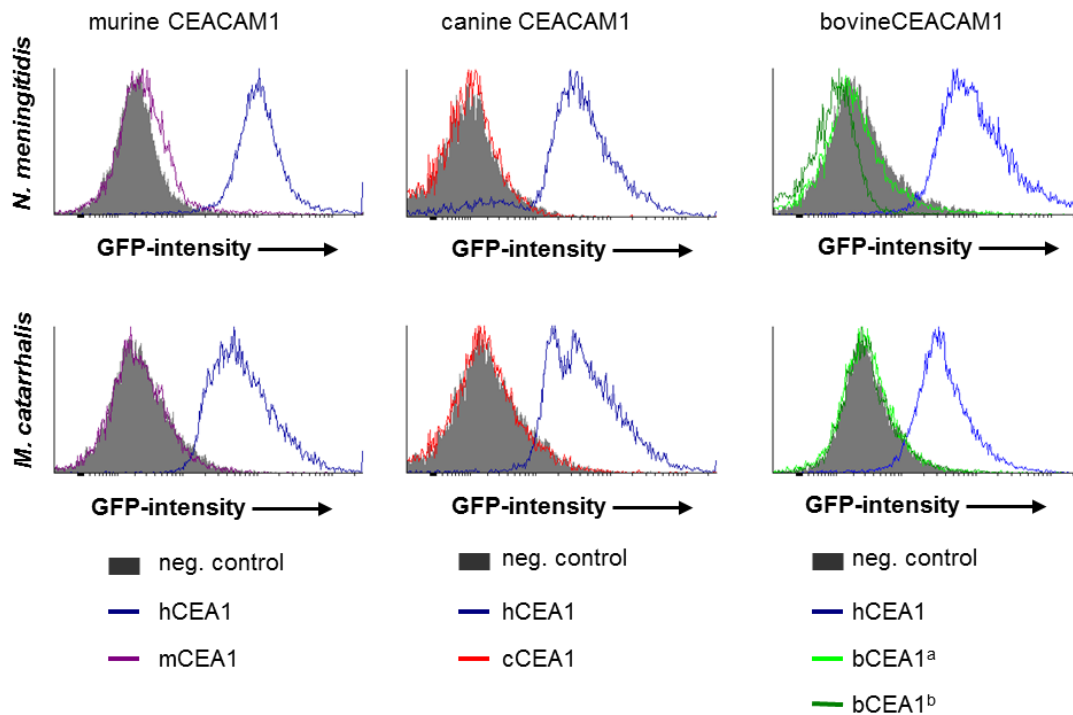


Fig. 11 Binding of *Neisseria meningitidis* and *Moraxella catarrhalis* to CEACAM1 orthologues

Culture supernatants containing soluble GFP-tagged amino-terminal domains of the indicated mammalian CEACAMs or a control culture supernatant from GFP-transfected cells (neg. control) were incubated with OpaCEA protein-expressing *N. meningitidis* or UspA1-expressing *M. catarrhalis*. After washing, bacteria were analysed by flow cytometry and the bacteria-associated GFP-fluorescence was determined. Only human CEACAM1 (hCEA1) binds to the pathogenic bacteria.

In contrast, the human CEACAM1 N-terminal domain was strongly associated with both, *N. meningitidis* as well as *M. catarrhalis* (Fig. 11). These results demonstrate that several Gram-negative human pathogens selectively recognize the amino-terminal Ig_v-like domain of human CEACAM1 and do not bind to the same region of orthologue proteins from various mammals.

Human, but not murine CEACAM1 mediates internalization of *Neisseria gonorrhoeae*

As the major isoforms of CEACAM1 contain four extracellular Ig domains, we wondered whether other determinants outside of the amino-terminal Ig_v-like domain might influence the association with microorganisms across species boundaries. Therefore, full length murine CEACAM1-4S (encompassing four extracellular domains and the short (S) cytoplasmic domain) or human CEACAM1-4S as well as human CEACAM1-4L were expressed in 293 cells. GFP- or Cerulean-tagged human CEACAM1-4L and CEACAM1-4S, as well as murine CEACAM1-4S were expressed at comparable levels as shown by Western blotting with a polyclonal antibody against GFP, which recognizes also Cerulean (Fig. 12A).

CEACAM engagement by Opa_{CEA}-expressing *N. gonorrhoeae* was evaluated through functional analysis of bacterial uptake by the transfected cells. In a first set of experiments, we used an antibiotic protection assay that is based on recovery of viable intracellular bacteria after treatment of the infected cells with gentamicin, an antibiotic that kills extracellular bacteria. In the case of non-opaque gonococci, only very low numbers of bacteria were recovered from murine or human CEACAM1-4S expressing cells similar to the numbers isolated from control transfected cells (Fig. 12B). In contrast, upon infection with Opa_{CEA}-expressing *N. gonorrhoeae*, 50 – 100 times more bacteria were recovered from cells expressing human CEACAM1 (Fig. 12B). Similar to what has been observed before (Muenzner, Bachmann et al. 2008), both the short and the long isoform of human CEACAM1-4 were able to mediate efficient uptake of the pathogens (Fig. 12B). Importantly, murine CEACAM1-4S was not able to mediate internalization of Opa_{CEA}-expressing *N. gonorrhoeae* consistent with the lack of bacterial binding to the Ig_v-like amino-terminal domain of murine CEACAM1 (Fig. 12B).

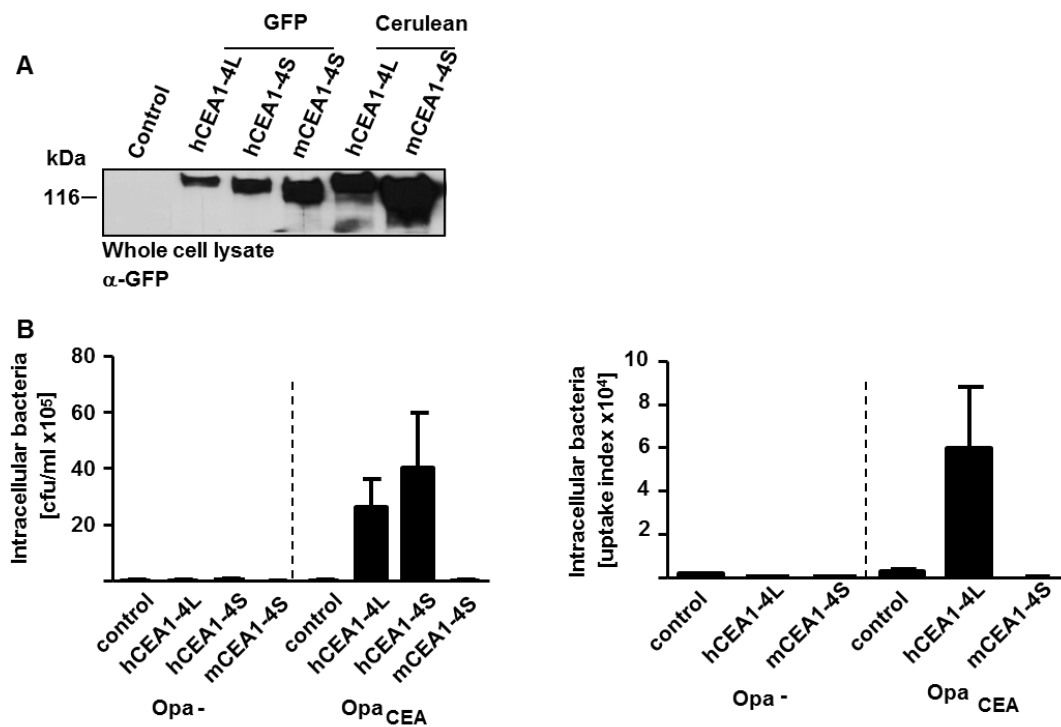


Fig. 12 Internalization of OpaCEA-expressing *Neisseria gonorrhoeae* is only mediated by human CEACAM1

(A) 293 cells were transfected with constructs encoding the indicated human or murine CEACAM1 isoforms fused to GFP or Cerulean. Cells transfected with a GFP-encoding vector served as control. After 48h, cells were lysed and the expression was determined by Western blotting with a polyclonal anti-GFP antibody.

(B) Cells transfected as in A) were infected with Opa-negative (Ngo Opa-) or OpaCEA-expressing *N. gonorrhoeae* (Ngo OpaCEA) at an MOI of 30 for 2h. The number of viable intracellular bacteria was determined by gentamicin protection assay. Bars represent mean values \pm SEM of three independent experiments done in triplicate. For statistical analysis, samples were compared against control transfected cells by one-tailed Mann-Whitney U-test; *, $p < 0.001$. (C) 293 cells were transfected with constructs encoding human CEACAM1 isoform containing a short cytoplasmic domain (hCEA1), the corresponding murine isoform (mCEA1) or an empty control vector. Cells were infected with fluorescein-labelled Opa-negative (Ngo Opa-) or OpaCEA-expressing *N. gonorrhoeae* (Ngo OpaCEA) at an MOI of 30 for 2h. The uptake index was determined by flow cytometry as described in Material and Methods. Bars represent mean values \pm SEM of three independent experiments.

To further confirm that full length murine CEACAM1-4S does not mediate bacterial internalization, we analysed transfected cells upon infection with fluorescein-labeled bacteria by an established flow cytometry method (Pils, Schmitter et al. 2006). Addition of trypan blue quenches the fluorescence emitted by extracellular bacteria, resulting in cell-associated fluorescence signals derived exclusively from intracellular bacteria. In

line with the results of the antibiotic protection assay, non-opaque *N. gonorrhoeae* was not internalized, whereas Opa_{CEA}-expressing bacteria were taken up by cells transfected with human CEACAM1-4S (Fig. 12C). Moreover, cells expressing murine CEACAM1-4S did not harbor intracellular bacteria, further corroborating the notion that Opa_{CEA} proteins of *N. gonorrhoeae* do not functionally engage CEACAM1 orthologues of other mammalian species (Fig. 12C).

Microscopic determination of *Neisseria gonorrhoeae* internalization via CEACAM1

To finally demonstrate the selective binding and internalization of Opa_{CEA}-expressing *N. gonorrhoeae* by human, but not murine CEACAM1, we analysed infected samples with confocal fluorescence microscopy. Therefore, 293 cells were transfected with GFP (negative control), human CEACAM1-4S-GFP, or murine CEACAM1-4S-GFP. Prior to infection, bacteria were labeled with rhodamine and biotin as a pre-requisite to allow the differential visualization of intracellular and extracellular bacteria (Agerer, Waeckerle et al. 2004). Cells infected for 2 h with rhodamine/biotin-labeled bacteria were fixed and the extracellular bacteria were selectively marked with AlexaFluor647-streptavidin, which does not have access to intracellular bacteria. In GFP-expressing cells, bacteria were rarely found associated with cells (Fig. 13). Moreover, in all cases these microbes were located outside the GFP-expressing cells as evidenced by their rhodamine and AlexaFluor647 labeling (Fig. 13, arrowhead). In contrast, cells expressing human CEACAM1 contained numerous intracellular bacteria that co-localized with the GFP-tagged receptor in intracellular vesicles (Fig. 13, arrow). The absence of the AlexaFluor647 label clearly confirms the intracellular localization of these bacteria (Fig. 13, arrow). Similar to the situation in GFP-transfected cells, 293 cells expressing murine CEACAM1 showed only very few cell-associated bacteria and no intracellular

bacteria were detected (Fig. 13, arrowhead). Though both human as well as murine CEACAM1-4S-GFP localized on the cell surface, only human CEACAM1 is recruited to the cell associated bacteria and is co-internalized with Opa_{CEA}-expressing gonococci (Fig. 13).

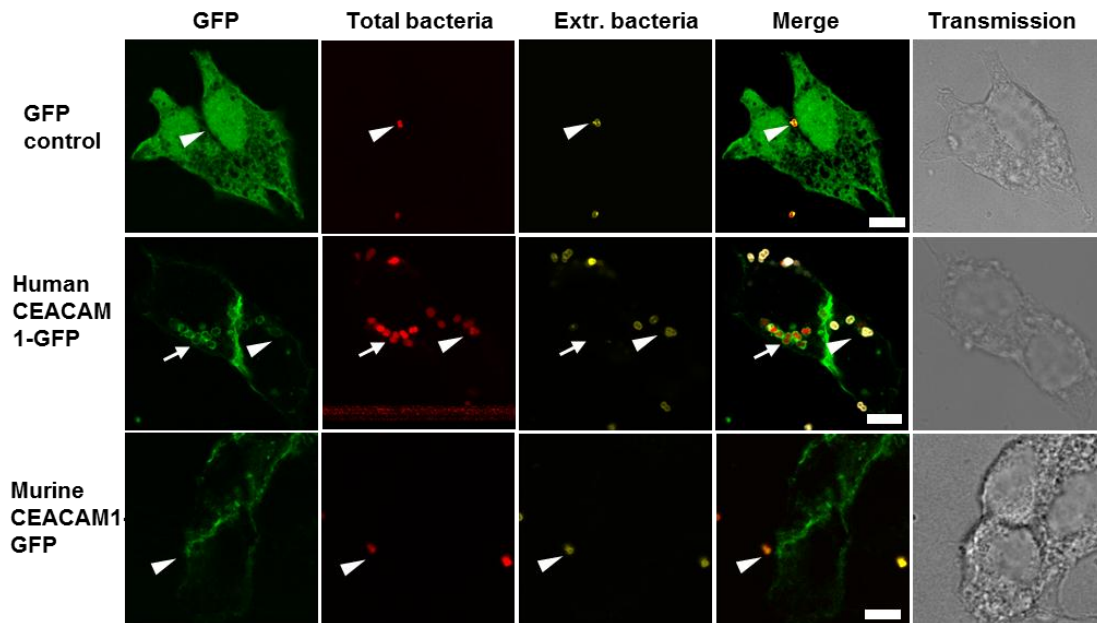


Fig. 13 Microscopic verification of *N. gonorrhoeae* uptake via human CEACAM1

293 cells were transfected with constructs encoding GFP, human CEACAM1-4S-GFP, or murine CEACAM1-4S-GFP as indicated. Cells were infected for 2 h with biotin- and rhodamine-labelled non-opaque (Ngo Opa-) or Opa_{CEA}-expressing *N. gonorrhoeae* (Ngo Opa_{CEA}). Infected cells were fixed, but not permeabilized, and samples were stained with AlexaFluor647-streptavidin to label extracellular bacteria (Extr. bacteria). Intracellular bacteria (small arrow) are marked by their selective rhodamine labelling, whereas extracellular bacteria (arrowheads) are stained with both rhodamine and AlexaFluor647. Bars represent 5 μ m.

Together, these microscopic investigations provide further evidence, that only the human CEACAM1 orthologue is a target for the Opa protein adhesins of *N. gonorrhoeae* and is able to mediate the binding and uptake into eukaryotic cells.

5.5 Discussion

Members of the CEACAM family serve as receptors for a variety of Gram-negative bacteria that live on mucosal surfaces of the human body. In an example of convergent evolution these microbes have evolved distinct CEACAM-binding adhesins that seem to promote the colonization of the mucosa. Here we provide evidence that CEACAM-binding adhesins from pathogenic *Neisseriae* and *Moraxella catarrhalis* display a high selectivity for human CEACAMs and do not associate with orthologues from non-primate mammalian species. Accordingly, the amino acid sequence divergence, that is particularly high in the CC'C''FG-face of the amino-terminal Ig_v-like domain of mammalian CEACAM1 orthologues, results in functional differences with regard to bacterial binding. CEACAM-binding bacterial species, which specifically colonize and infect humans, only recognize human CEACAM1 suggesting that the microbial adhesive proteins have co-evolved with their host receptor.

It has been observed earlier, that CEACAM1 orthologues from different mammalian species display high sequence diversity (Kammerer, Popp et al. 2004; Kammerer and Zimmermann 2010). Starting from a primordial CEACAM1-like gene, CEACAMs seem to have undergone independent duplication and diversification events in different mammalian lineages resulting in an expanded family of closely related surface molecules (Frangmyr, Israelsson et al. 2000; Zebhauser, Kammerer et al. 2005). Therefore, even within a mammalian order such as the primates it is difficult to assign orthologue genes except for CEACAM1 (Zhou, Zhang et al. 2001). As several members of the CEACAM family are exploited by viral and bacterial pathogens, it has been suggested that the driving force behind the rapid diversification of CEACAMs in different mammalian lineages might be the selective pressure by pathogens (Hammarstrom and Baranov 2001; Kammerer, Popp et al. 2007).

An additional example of CEACAM1 recognition by pathogens is found in rodents, where the mouse hepatitis virus strain A59 (MHV-A59), belonging to the coronavirus complex, binds via its spike protein to murine CEACAM1 (Dveksler, Dieffenbach et al. 1993; Dveksler, Pensiero et al. 1993). Of the two CEACAM1 alleles present in the mouse population, MHV-A59 selectively recognizes CEACAM1a and only marginally binds to the CEACAM1b allele (Zelus, Wessner et al. 1998). Therefore, inbred mouse lines that carry the CEACAM1a allele are susceptible, whereas lines carrying the CEACAM1b allele or CEACAM1-deficient mice are resistant to MHV-A59 (Hemmila, Turbide et al. 2004). However, despite this selectivity for the murine CEACAM1a allele, it has been shown that several MHV strains, including A59 and MHV-2, can utilize human CEACAM1 as well as CEA to infect eukaryotic cells in vitro (Chen, Asanaka et al. 1997).

In contrast to this promiscuity of host receptor utilization, our results highlight the specificity of bacterial adhesins for human CEACAMs. Consistent with the strict selectivity of these pathogens for humans as natural host organisms, they only associate with human CEACAM1. Accordingly, the bacteria can efficiently invade only cells that express the human orthologue of CEACAM1, but not the murine orthologue. It is interesting to note, that additional pathogenicity factors of these bacteria show a similar exquisite specialisation for human molecules. For example, the neisserial IgA1 protease (Plaut, Gilbert et al. 1975) only cleaves human IgA1 molecules, but not IgA molecules from other mammalian species. Similarly, the transferrin-binding protein, that is critical for iron acquisition in the human host, can utilize only transferrin from human sources or from closely related apes such as chimpanzee (Lee and Schryvers 1988; Gray-Owen and Schryvers 1993). Gonococci are also able to escape from host complement attack by recruiting complement component 4b-binding protein (C4bp) (Ram, Cullinane et al. 2001). This ability is again specific for human C4bp and even chimpanzee C4bp only

provides protection from complement for some, but not all gonococcal strains (Ngampasutadol, Ram et al. 2005).

Besides immune escape and nutrient acquisition, our results reveal another area, where these Gram-negative pathogens employ species-specific pathogenicity factors. Clearly, adhesion to the mucosal surface epithelium is the initial step in the colonization by CEACAM-binding bacteria, and the possession of adhesive proteins specifically targeting human CEACAMs might promote this step. However, at the same time this specialization could contribute to the limited host spectrum not only of pathogenic *Neisseriae*, but also of *M. catarrhalis* and *Haemophilus influenzae*.

Recognition of host surface structures is critical for many bacterial pathogens to establish a first foothold in their target organism. Whereas a high degree of specificity might allow intimate binding of the microorganisms to eukaryotic cells, it might at the same time limit the host range of the pathogen. Here we reveal a selective interaction between bacteria and the human form of the cell surface receptor CEACAM1 that correlates with the human-restricted pathogenicity of these microbes. Our analysis not only points to an ongoing pathogen-host co-evolution at the level of receptor-adhesin interaction, but further strengthens the idea that the Opa_{CEA} protein-mediated interaction with human CEACAMs might provide an access point for preventing or limiting infection.

5.6 Authors' contributions

CRH, MV, UG, and RK conceived of the study, MV and CRH designed the experiments, MV and VB performed the experiments, CRH and MV wrote the paper. All authors read and approved the final manuscript

5.7 Acknowledgments

We thank M. Frosch (Universität Würzburg, Germany) and T.F. Meyer (Max-Planck-Institute für Infektionsbiologie, Berlin, Germany) for the bacterial strains used in this study. We thank D.W. Piston (Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN) for Cerulean cDNA, S. Feindler-Boeckh and R. Hohenberger-Bregger for expert technical assistance. MV and CRH acknowledge the support by the Konstanz Research School-Chemical Biology. This study was supported by funds from the DFG (Ha2856/6-1) to C.R.H.

6 CHAPTER II

Extracellular Ig_{C2}-like
domains of CEACAMs mediate
PI3K sensitivity during uptake of
pathogens

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6.1 Abstract

Background. Several pathogenic bacteria utilize receptors of the CEACAM family to attach to human cells. Binding to different members of this receptor family can result in uptake of the bacteria. Uptake of *Neisseria gonorrhoeae*, a Gram-negative human pathogen, via CEACAMs found on epithelial cells, such as CEACAM1, CEA or CEACAM6, differs mechanistically from phagocytosis mediated by CEACAM3, a CEACAM family member expressed selectively by human granulocytes.

Principal Findings. We find that CEACAM1- as well as CEACAM3-mediated bacterial internalization are accompanied by a rapid increase in phosphatidylinositol-3,4,5 phosphate (PI(3,4,5)P) at the site of bacterial entry. However, pharmacological inhibition of phosphatidylinositol-3' kinase (PI3K) selectively affects CEACAM1-mediated uptake of *Neisseria gonorrhoeae*. Accordingly, overexpression of the PI(3,4,5)P phosphatase SHIP diminishes and expression of a constitutive active PI3K increases CEACAM1-mediated internalization of gonococci, without influencing uptake by CEACAM3. Furthermore, bacterial uptake by GPI-linked members of the CEACAM family (CEA and CEACAM6) and CEACAM1-mediated internalization of *N. meningitidis* by endothelial cells require PI3K activity. Sensitivity of CEACAM1-mediated uptake towards PI3K inhibition is independent of receptor localization in cholesterol-rich membrane microdomains and does not require the cytoplasmic or the transmembrane domain of CEACAM1. However, PI3K inhibitor sensitivity requires the Ig_{C2}-like domains of CEACAM1, which are also present in CEA and CEACAM6, but which are absent from CEACAM3. Accordingly, overexpression of CEACAM1 Ig_{C2}-like domains blocks CEACAM1-mediated internalization.

Conclusions. Our results provide novel mechanistic insight into CEACAM1-mediated endocytosis and suggest that epithelial CEACAMs associate in *cis* with other membrane

receptor(s) via their extracellular domains to trigger bacterial uptake in a PI3K dependent manner.

6.2 Introduction

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is a widely expressed glycoprotein of the CEACAM family, which in humans comprises 12 genes and a number of pseudogenes (Zebhauser, Kammerer et al. 2005; Kuespert, Pils et al. 2006). Similar to several other human CEACAMs, CEACAM1 is characterized by an amino-terminal immunoglobulin variable-like domain (Ig_V-like), followed by one to three immunoglobulin constant type2 (Ig_{C2})-like domains, which are defined by the reduced number of beta-strands in the Ig fold compared to Ig_V-like domains (Barclay 2003). CEACAM1 is involved in a broad spectrum of cellular processes ranging from tissue morphogenesis and apoptosis, to insulin homeostasis, angiogenesis, or regulation of T-cell activity (Kuespert, Pils et al. 2006). Another member of the CEACAM family is CEACAM3, which is exclusively expressed on granulocytes and harbours a single Ig_V-like domain followed by a transmembrane helix and a cytoplasmic domain (Pils, Gerrard et al. 2008). Besides CEACAM1 and CEACAM3, two additional members of the CEACAM family, namely CEA (the product of the *CEACAM5* gene) and CEACAM6, can serve as cellular receptors for a range of gram-negative bacteria (Chen and Gotschlich 1996; Virji, Watt et al. 1996; Chen, Grunert et al. 1997; Toleman, Aho et al. 2001; Hill and Virji 2003; Berger, Billker et al. 2004; Voges, Bachmann et al. 2010). In all these cases, bacteria engage the non-glycosylated face of the N-terminal Ig_V-like domain, which shares more than 90% sequence similarity between the four CEACAMs exploited as host receptors.

A common hallmark of CEACAM-binding bacteria, such as *Neisseria gonorrhoeae*, *N. meningitidis*, *N. lactamica* or *Moraxella catarrhalis*, is their ability to efficiently colo-

nize mucosal epithelia of the human body. Indeed, engagement of epithelial CEACAM family members, such as CEACAM1, CEA, or CEACAM6, allows the microorganisms to trigger increased matrix-adhesion of the infected host cells (Muenzner, Rohde et al. 2005). For *N. gonorrhoeae*, this process promotes mucosal colonization in vivo by preventing exfoliation of superficial epithelial cells from stratified tissues in the urogenital tract (Muenzner, Bachmann et al. 2010). Furthermore, the binding to CEACAMs can trigger bacterial engulfment by various cell types including both professional as well as non-professional phagocytes (Bos, Grunert et al. 1997; Chen, Grunert et al. 1997; Gray-Owen, Lorenzen et al. 1997; Hauck, Meyer et al. 1998; Muenzner, Dehio et al. 2000; Rowe, Griffiths et al. 2006). Interestingly, in polarized epithelia CEACAMs are generally expressed on the apical membrane and support not only internalization, but also transcytosis of CEACAM-binding bacteria through an intact cell layer (Wang, Gray-Owen et al. 1998; Wang, Meyer et al. 2007). How internalization and transcellular trafficking via epithelial CEACAMs is regulated on the molecular level is currently unclear.

In contrast to epithelial CEACAMs, the endocytotic function of CEACAM3, a bacteria-binding member of the family exclusively expressed on granulocytes, has been delineated in great detail (for review see (Buntru, Roth et al. 2012)). The cytoplasmic domain of CEACAM3 contains a sequence with similarity to the so-called immunoreceptor tyrosine-based activation motif (ITAM), which is phosphorylated by Src family protein tyrosine kinases upon bacterial engagement (Schmitter, Pils et al. 2007). The phosphorylated cytoplasmic domain of CEACAM3 coordinates the local assembly of a signaling complex, which is responsible for the efficient internalization of bound bacteria (McCaw, Schneider et al. 2003; Schmitter, Agerer et al. 2004; Buntru, Zimmermann et al. 2009). By directly associating with the SH2 domains of the guanine nucleotide exchange factor (GEF) Vav and the adaptor molecule Nck, CEACAM3 recruits an up-

stream stimulator of the small GTPase Rac (the GEF Vav) and a downstream effector of GTP-loaded Rac (the Nck-associated WAVE-complex) to trigger massive actin rearrangements required for CEACAM3-mediated phagocytosis (Schmitter, Pils et al. 2007; Pils, Kopp et al. 2012). The involvement of the CEACAM3 cytoplasmic domain and the strict requirement for dynamic actin rearrangements is clearly distinct from epithelial CEACAMs, which mediate endocytosis in the absence of a cytoplasmic domain. Indeed, epithelial CEACAMs either do not possess cytoplasmic domains (such as the GPI-anchored CEA and CEACAM6) or their endocytotic function is not affected by the deletion of the cytoplasmic domain (as is the case for CEACAM1) (Schmitter, Pils et al. 2007; Muenzner, Bachmann et al. 2008; Kuespert, Roth et al. 2011). Moreover, interference with actin polymerization only partially affects endocytosis via epithelial CEACAMs (McCaw, Liao et al. 2004; Schmitter, Pils et al. 2007). Interestingly, epithelial CEACAMs re-locate into sphingolipid-rich membrane microdomains upon clustering, and cholesterol-depletion affects their internalization, whereas CEACAM3-mediated uptake is not sensitive to cholesterol-chelators (Schmitter, Pils et al. 2007; Muenzner, Bachmann et al. 2008). Together, the previous studies suggest that internalization initiated by epithelial CEACAMs (CEACAM1, CEA and CEACAM6) is mechanistically distinct from CEACAM3-triggered phagocytosis, though they share the same ligands. Besides the distribution into membrane microdomains, which for CEACAM1 seems to depend on determinants in the transmembrane helix, no further downstream factors responsible for the distinct behaviour of epithelial CEACAMs during endocytosis have been described.

Recently, we made the surprising observation that phosphatidylinositol-3' kinase (PI3Ks) activity is not involved in CEACAM3-triggered phagocytosis by human granulocytes (Buntru, Kopp et al. 2011), despite the well-characterized role of phosphatidylinositol phosphates (PIPs) in numerous endocytic processes (Indik, Park et al. 1995;

Araki, Johnson et al. 1996). These findings prompted us to investigate the involvement of PI3K and PIPs in bacterial internalization via epithelial CEACAMs. We report here, that 3'-phosphorylated PIPs are not only enriched around bacterial uptake sites, but that blockade of PI3Ks severely reduces bacterial internalization via CEACAM1 and other epithelial CEACAMs. In line with a role of phosphatidylinositol 3,4,5-phosphate (PI(3,4,5)P), overexpression of class I PI3K increases, whereas expression of SHIP, which dephosphorylates PI(3,4,5)P, reduces CEACAM1-mediated uptake. Surprisingly, PI3K dependent endocytosis of CEACAM1 was not connected to cytoplasmic determinants of the receptor or to its location in membrane microdomains, but rather required the extracellular Ig_{C2}-like domains of CEACAM1. These data provide novel mechanistic insight into CEACAM1 endocytosis and suggest the existence of *cis*-interactions between epithelial CEACAMs and additional membrane proteins triggering PI3K-dependent uptake.

6.3 Material & Methods

Neisserial strains and growth conditions

Opa₅₂ protein-expressing, non-piliated *Neisseria gonorrhoeae* strain MS11 (Ngo Opa_{CEA}) and the isogenic non-opaque strain (Ngo Opa-) were kindly provided by Thomas F. Meyer (Max-Planck-Institut für Infektionsbiologie, Berlin, Germany). Opa_{CEA} protein-expressing, unencapsulated *Neisseria meningitidis* strain MC58 (Δ siaD, Δ lgtA) (Nm Opa_{CEA}) was obtained from Matthias Frosch (Institut für Hygiene und Mikrobiologie, Universität Würzburg, Germany). Both, *Neisseria meningitidis* and *N. gonorrhoeae* were grown as described before (Kuespert, Weibel et al. 2007) on GC agar plates (Difco BRL, Paisley, UK) supplemented with vitamins at 37°C, 5% CO₂. For infection, overnight grown bacteria were taken from GC agar plates, suspended in PBS, and colony forming units (cfu) were estimated by OD₅₅₀ readings according to a standard curve.

Epithelial and endothelial cell lines

Human embryonic kidney epithelial 293T cells (293 cells; ACC-635, DSMZ, Braunschweig, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. Human brain microvascular endothelial cells (HBMEC) (Slanina, König et al. 2010) were grown in endothelial cell medium (PAA, Pasching, Austria) supplemented with L-glutamine. All cells were grown in the absence of antibiotics at 37°C in 5% CO₂ and subcultured every 2-3 days.

Recombinant DNA constructs

Mammalian expression plasmids encoding HA-tagged versions of human CEACAM1-4L (CEACAM1), CEACAM1 lacking the complete cytoplasmic domain (CEACAM1- Δ CT), CEACAM3, CEA and CEACAM6 were described previously (Schmitter, Agerer

et al. 2004; Muenzner, Rohde et al. 2005). The mKate-tagged and mCerulean-tagged versions of CEACAM1, CEACAM1 Δ CT, and CEACAM3 were generated by amplifying the HA-tagged versions of CEACAM1, CEACAM1 Δ CT, or CEACAM3, respectively, with primers CEACAM1-IF sense 5'-GAAGTTATCAGTCGATACCATGGGGCACTTCTCAGCCCC-3' and HA-CEACAM-IF antisense 5'-ATGGTCTAGAAAGCTTGCAGCGTAATCTGGAACGTCATATGG-3', or with CEACAM3-IF-sense, 5'-GAAGTTATCAGTCGATACCATGGGGCCCCCCTCAGCC-3', and HA-CEACAM-IF-antisense. The resulting PCR fragments were cloned into pDNR-Dual using the In-Fusion PCR Cloning Kit (Clontech, Mountain View, CA) and transferred by Cre-mediated recombination into pLPS-3'mKate (Muenzner, Bachmann et al. 2010) or pLPS-3'mCerulean [11] resulting in mKate or mCerulean, respectively, fused to the carboxy-terminus of the expressed proteins. The different CEACAM1 deletion mutants lacking either three, two or one Ig_{C2}-like extracellular domains (CEACAM1-N, CEACAM1-NA1, CEACAM1-NA1B) and the CEACAM8/1 chimera were described previously (Kuespert, Roth et al. 2010). The CEACAM1/CEACAM3-TM chimera was constructed by amplifying CEACAM1 lacking the cytoplasmic domain with sense Chimera3cd-HA primer 5'-ATAATGGCCATAGTGGCGCTGGTGGCCGCAC-TGGTGTGTTTCCTGCTCCTTCATTTCTGGGAAATATCCCTATGACG-3' and rev-Chimera3ab primer 5'-ATAATGGCCACTCCGACCAGGACCCCGGTCACGATCCCAGGTGAGAGGCC-3' resulting in the CEACAM1 extracellular domains fused to the transmembrane domain of CEACAM3. The plasmids pEGFP-Btk-PH and pEGFP-PLC δ -PH were a kind gift from T. Balla (NIH, Bethesda, MD).

The cDNA of the enzymatic p110 subunit of PI3K was a gift from J. Downward (Cancer Research UK, London, UK). Full-length PI3K was amplified with primers PI3KCA-IF-sense-5'- GAAGTTATCAGTCGACCCTCCAAGACCATCATCAG-3' and PI3KCA-IF-anti -5'- ATGGTCTAGAAAGCTTAGGCGGCTCAGTTCAATGCATGCTG-3'. The resulting PCR fragment was cloned into pDNR-Dual using the In-Fusion PCR Cloning Kit (Clontech, Mountain View, CA) and transferred by Cre-mediated recombination into a modified pcDNA vector with loxP site 3' of the cerulean coding sequence (pcDNA Cerulean loxP). The cDNAs of murine SHIP1 and the phosphatase inactive mutant SHIP D675G were a kind gift from G. Krystal (British Columbia Cancer Agency, Vancouver, Canada). The phosphatase domains were cloned in pDNR-dual with primers SHIP-PD-IF sense-5' GAAGTTATCAGTCGACGAGCCAGAGCCTGAC-3' and SHIP-PD-IF antisense-5' ATGGTCTAGAAAGCTTAAGGGACCCTGCCAGAAGG-3' and transferred in pcDNA Cerulean loxP via Cre-mediated recombination.

The N-terminal SH2 domains of PI3KR3 and Hck were previously described (Buntru, Kopp et al. 2011). The C-terminal SH2 domain of SHP2 was constructed by amplification from human SHP2 cDNA (a gift from B.G. Neel, University of Toronto, Toronto, Canada) with primers PTPN11-C-SH2-IF-sense 5'- GAAGTTATCAGTCGACCCTCTGAACTGTGCAGATC-3' and PTPN11-C-SH2-IF-anti 5'-ATGGTCTAGAAAGCTTAATTTATACGAGTCGTGTTAAG-3'. The resulting PCR fragments were cloned into pDNR-Dual using the In-Fusion PCR Cloning Kit (Clontech, Mountain View, CA) and transferred by Cre-mediated recombination into pGEX4-T1 loxP [26]. The cloning and production of CEACAM1-GFP or GFP encoding lentiviral particles and transduction of cells was performed as described previously (Muenzner, Rohde et al. 2005).

Antibodies and reagents

Monoclonal antibody (mAb) against CEACAMs (clone D14HD11; recognizing CEACAM1, CEACAM3, CEA and CEACAM6) were purchased from Genovac (Freiburg, Germany), mAb against Flotillin2 (clone ESA) was from BD Biosciences (Heidelberg, Germany), mAbs against the HA-tag (clone 12CA5), against tubulin (clone E7), and against transferrin (clone G1/221/12) were purified from hybridoma cell supernatants. Rabbit polyclonal α -GFP antibody was custom made at our in-house Animal Research Facility (Universität Konstanz, Germany) and affinity purified. Secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Transfection of cells, cell lysis, GST pulldown assay and Western blot

293 cells were transfected by calcium phosphate precipitation using 5- 8 μ g of appropriate cDNA in each case. The transfection efficiency ranged between 30% and 70% as reported (Schmitter, Pils et al. 2007). Transfected 293 cells were employed in experiments 48h after transfection. Cell lysis, Western blotting, and GST pulldown assays were performed as described (Schmitter, Agerer et al. 2004; Buntru, Kopp et al. 2011).

Gentamicin protection assay

293 cells were seeded at 5×10^5 cells/well in 24-well plates or 2.5×10^5 cells/well in 48-well plates coated with poly-L-lysine (10 μ g/ml). A multiplicity of infection (MOI) of 30 bacteria per cell was routinely used, and after 2 hour of infection, extracellular bacteria were killed by 60 min incubation in 50 μ g/ml gentamicin in DMEM. In the case of endothelial cells, HBMECs were seeded at 2×10^5 cells/well in 24-well plates coated with poly-L-lysine (10 μ g/ml). A multiplicity of infection (MOI) of 40 bacteria was used, and after 3 hour of infection, extracellular bacteria were killed by 60 min incubation in 50 μ g/ml gentamicin in endothelial medium. Following gentamicin treatment, cells were

lysed with 1% saponin in PBS for 15 min. The samples were diluted in PBS, and the number of viable bacteria was determined by plating suitable dilutions on GC agar. For inhibition studies, cells were treated with the inhibitors wortmannin (200 nM), LY294002 (50 μ M) or methyl- β -cyclodextrin (500 μ M) 30 min prior to infection.

Bacterial adherence assay

Cells were seeded and infected as described for gentamicin protection assays. After the infection, the cells were gently washed three times, before they were lysed by addition of 1% saponin in PBS for 15 min. Total cell-associated bacteria were suspended by vigorous pipetting, and colony forming units were determined by plating of serial dilutions on GC agar.

Flow cytometry-based invasion assay

Bacterial uptake by 293 cells was analysed by flow cytometry as described (Pils, Schmitter et al. 2006). Prior to infection, bacteria were surface labelled with 0.2 μ g/ml 5-(6)-carboxyfluorescein-succinimidylester (fluorescein; Invitrogen-Molecular Probes, Karlsruhe, Germany) in PBS at 37°C for 30 min prior to infection. 1×10^6 cells were seeded in 6-well plates coated with poly-L-lysine (10 μ g/ml) and the next day, cells were infected with labelled bacteria at an MOI of 30 for 2 h. After infection, cells were washed with PBS and the samples were analysed on a LSR II flow cytometer (BD Bioscience) by gating on the cells based on forward and sideward scatter. Cell-associated fluorescein fluorescence was measured in the presence of 2 mg/ml trypan blue to quench fluorescence derived from extracellular bacteria and to selectively detect the fluorescence derived from intracellular bacteria. The percentage of fluorescein-positive cells within the transfected cell population was multiplied by the mean fluorescence

intensity of the sample to obtain an estimate of the total number of internalized bacteria (uptake index). In each sample at least 4,000 cells were counted.

Immunofluorescence staining

For microscopic analysis of 293 cells, 7×10^4 cells were seeded onto poly-L-lysine- and fibronectin-coated (10 and 4 $\mu\text{g/ml}$, respectively, in PBS) glass-coverslips in 24-well plates. For microscopic analysis of HBMEC cells, 3×10^4 cells were seeded onto poly-L-lysine- and fibronectin-coated (10 and 4 $\mu\text{g/ml}$, respectively, in PBS) glass-coverslips in 24-well plates. Cells were infected with Pacific blue-labeled (and in case of intra-/extracellular staining additionally biotinylated) bacteria for 3h at an MOI of 30 (HBMEC with meningococci) or for 1h at an MOI of 40 (293 cells with gonococci). Samples were washed once and fixed with 4% paraformaldehyde. After three washes with PBS, samples were incubated in blocking buffer (PBS, 10% fetal calf serum) for 5 min and then stained for extracellular bacteria with streptavidin-Cy3 (diluted 1:100 in blocking buffer). Following three washes, samples were embedded in mounting medium (Dako, Glostrup, Denmark). Samples were analysed with a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). Fluorescence signals of triple-labelled specimens were serially recorded with appropriate excitation and emission filters to avoid bleed-through. Images were digitally processed with ImageJ and merged to yield pseudo-coloured pictures.

Analysis of detergent-resistant membrane fractions

To analyse the association of proteins with detergent-resistant membrane (DRM) fractions, the flotation assay as described by Umlauf et al. (Umlauf, Mairhofer et al. 2006) was used with slight modifications. Briefly, 293 cells were transfected with HA-tagged recombinant constructs of CEACAM1 or CEACAM3 together with GFP-tagged flotil-

in2. Transfected cells were left untreated or CEACAMs were clustered as described [28]. In some cases, transfected cells were treated with 200 nM wortmannin prior to CEACAM. After washing with ice-cold PBS, cells were homogenized in ice-cold buffer containing 50 mM Tris, pH 7.4, 2 mM MgCl₂ and 8% sucrose and centrifuged for 15 min at 5700 g. The pellet of unbroken cells and organelles was discarded and the supernatant was adjusted to 4 mM EDTA, before centrifugation at 48 000 rpm for 2 h using an SW60 rotor (Beckman). The resulting pellet containing cellular membranes was subsequently solubilized at 4°C for 15 min in 280 µl of ice-cold TNE-T buffer (20 mM Tris-Cl, pH 8.0, 130 mM NaCl, 5 mM EDTA, 0.7% Triton X-100 and protease inhibitors (10 µg ml⁻¹ aprotinin and leupeptin, 1 µg ml⁻¹ pepstatin A and 1 mM phenylmethyl-sulfonyl fluoride). The lysate was adjusted to 50% sucrose by the addition of 490 µl of 80% sucrose in TNE-T and covered with 3 ml of 35% sucrose followed by 1.2 ml of 5% sucrose in the same buffer. After ultracentrifugation at 48 000 rpm in a SW 60 rotor (Beckman) for 18 h, eight fractions were collected from the top of the gradient and analysed by Western blotting. DRM components were recovered in the low-density fractions 2–4.

6.4 Results

Phosphatidylinositol 3,4,5-phosphate is generated during CEACAM1- and CEACAM3-mediated bacterial host cell contact

It is well documented, that PI(3,4,5)P constitutes a critical host factor during endocytosis of bacteria via distinct surface receptors (Brumell and Grinstein 2003). However, we observed previously that lipid kinases of the PI3K family, which generate PI(3,4,5)P, are not required for bacterial uptake via CEACAM3 (Buntru, Kopp et al. 2011). To investigate, if PI(3,4,5)P might be involved in CEACAM1-mediated internalization, we co-transfected 293 cells with constructs encoding GFP-tagged PH domain of Bruton's tyrosine kinase (Btk) together with either mKate-tagged CEACAM1 or mKate-tagged CEACAM3. The PH-domain of Btk can serve as an intracellular PI(3,4,5)P reporter, as it binds specifically to this membrane lipid (Balla and Varnai 2002). The transfected cells were either left uninfected or were infected for 60 min with Pacific Blue-labelled *N. gonorrhoeae* expressing a CEACAM-binding Opa protein (Ngo Opa_{CEA}). After infection, the samples were fixed and analyzed by confocal microscopy (Fig. 14A). Whereas the PH domain was evenly distributed in the membrane of uninfected cells, engagement of CEACAM3 as well as CEACAM1 by gonococci resulted in a massive recruitment of the Btk-PH domain to the site of bacteria-host cell contact (Fig. 14A). PI(3,4,5)P can be generated from PI(4,5)P by the action of class I PI3Ks. To address, if the class I PI3K substrate is present at the site of bacterial uptake, we co-transfected cells with CEACAM1-mKate or CEACAM3-mKate expression vectors together with a construct encoding the GFP-tagged PH-domain of PLC- δ , which binds specifically to PI(4,5)P (Balla and Varnai 2002). Similar to the recruitment of Btk-PH domain, bacterial engagement of either CEACAM resulted in a pronounced accumulation of the PLC- δ -PH domain (Fig. 14B).

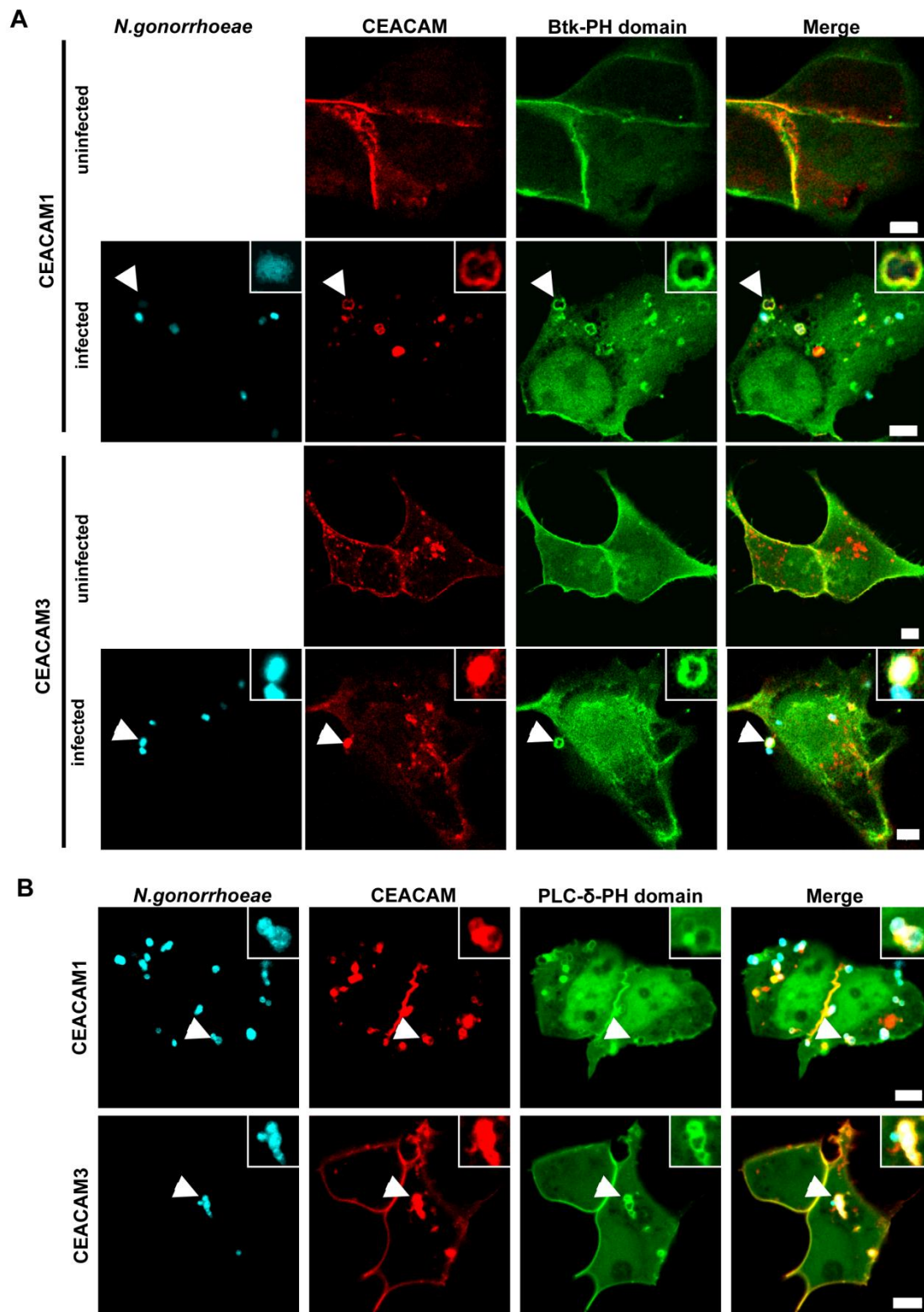


Fig. 14 PI(3,4,5)P and PI(4,5)P are generated during CEACAM1- and 3- mediated bacterial entry. (A) 293 cells were cotransfected with constructs encoding mKate-tagged CEACAM1-4L or CEACAM3 together with GFP-tagged Btk-PH domain (which binds specifically to PI(3,4,5)P). Cells were left uninfected or were infected with Pacific Blue labelled *Opa*_{CEA}-expressing *N. gonorrhoeae* for 60 min, fixed and analysed by confocal microscopy. Arrowheads highlight bacteria associated with CEACAMs and the Btk-PH domain. Insets show enlargement of the highlighted area. Bars represent 5µm. (B) Cells were cotransfected with CEACAM constructs as in (A) together with the GFP-tagged PLC-δ-PH domain (which specifically binds to

PI(4,5)P). Cells were infected and analyzed as in (A). Arrowheads highlight bacteria associated with CEACAMs and the PLC- δ -PH domain. Insets show enlargement of the highlighted area. Bars represent 5 μ m.

Together, these results indicate that both the substrate (PI(4,5)P) as well as the product (PI(3,4,5)P) of class I PI3Ks accumulate upon binding of bacteria to CEACAM1 or CEACAM3.

PI3K inhibition selectively interferes with uptake of Opa_{CEA}-expressing gonococci via CEACAM1

Since the PI(3,4,5)P level in unstimulated cells is generally low, the accumulation of this phospholipid around CEACAM-bound bacteria suggested the local activation of class I PI3Ks. Though PI3Ks are not involved in CEACAM3-mediated internalization, we wondered whether these enzymes play a role during CEACAM1-mediated uptake of bacteria. Therefore, we transfected cells with constructs encoding HA-tagged CEACAM1 or CEACAM3 or the empty vector and similar expression was verified by Western blotting with a monoclonal antibody against the HA-tag (Fig. 15A). Then, the activity of endogenous PI3K in CEACAM1- or CEACAM3 transfected cells was blocked by addition of the PI3K selective inhibitor LY294002, before cells were infected with Opa_{CEA}-expressing gonococci for 2 h. Following the infection, cells were washed and total cell associated bacteria were enumerated by plating dilutions on selective media (adherence assay; Fig. 15B). Parallel samples were treated with gentamicin for 1 hour to kill extracellular bacteria, and the intracellular bacteria were then released by mild detergent lysis of the eukaryotic cells (invasion assay; Fig. 15C). Importantly, cells transfected with the empty vector did only associate with low numbers of bacteria and did not contain viable intracellular bacteria (Fig. 15B and C). In contrast, transfection with either CEACAM1 or CEACAM3 allowed cells to associate with Opa_{CEA}-expressing *N.*

gonorrhoeae (Fig. 15B). Incubation with LY294002 did not decrease binding of the bacteria to CEACAMs and even slightly increased cell-associated bacteria in CEACAM1-expressing cells (Fig. 15B).

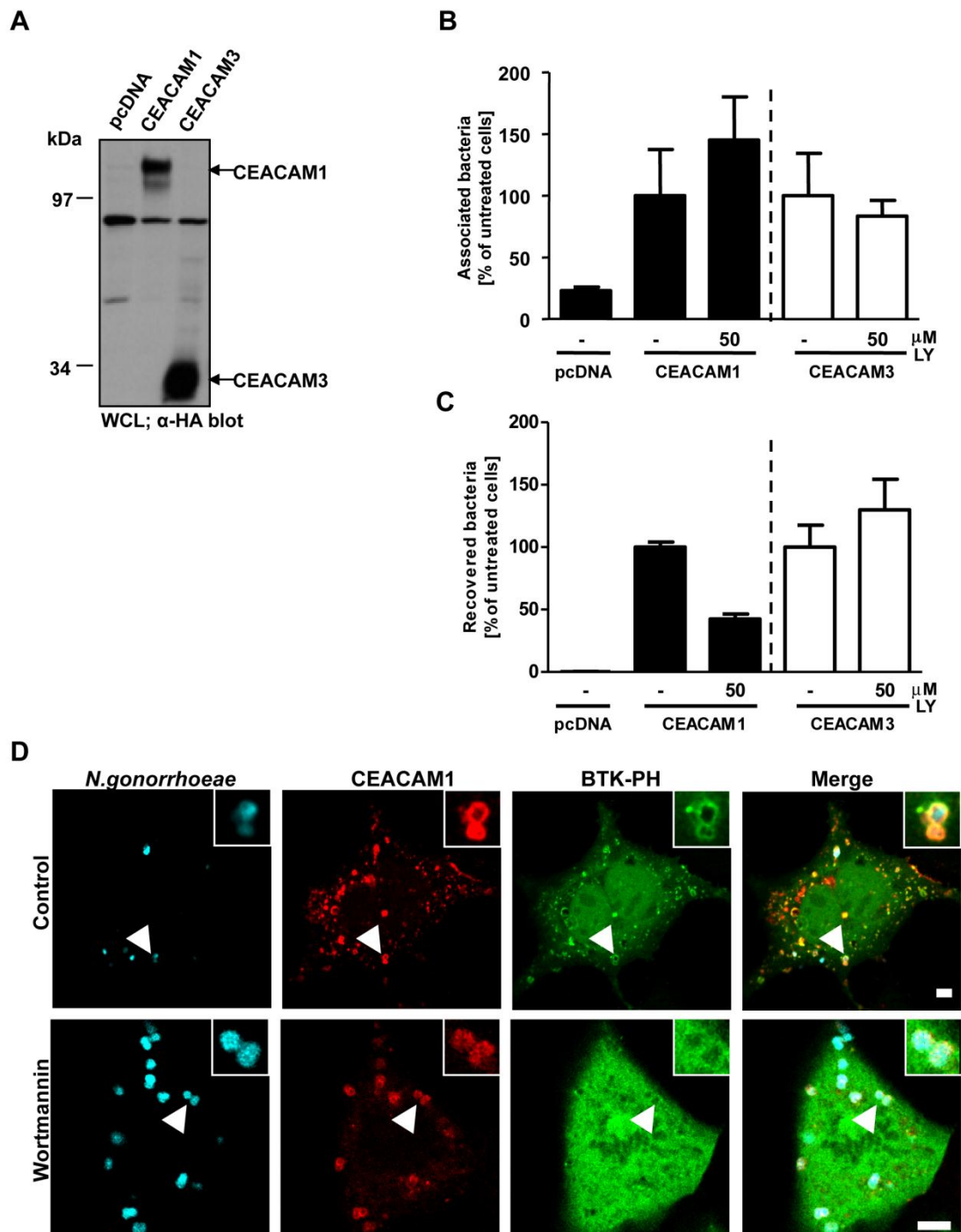


Fig. 15 Inhibition of PI3K activity decreases uptake of Opa_{CEA} -expressing gonococci via CEACAM1. (A) 293 cells were transfected with empty vector pcDNA, CEACAM1-4L or CEACAM3 WT. CEACAM expression was verified by Western blotting of whole cell lysates (WCL) using α -HA antibody. (B) Cells were transfected as in (A) and pretreated for 30 min with 50 μ M of the PI3K inhibitor LY294002 (LY). After infection for 2 h with Opa_{CEA} -

expressing gonococci, the number of total cell-associated bacteria was determined. Bars represent mean values \pm S.E.M of three independent experiments done in triplicate. Total cell associated bacteria are shown relative to cells expressing the respective receptor without PI3K inhibitor treatment. **(C)** Cells were transfected and infected as in **(B)**. Viable intracellular bacteria were determined in gentamicin protection assays. Bars represent mean values \pm S.E.M of three independent experiments done in triplicate. Recovered bacteria are shown relative to cells expressing the respective receptor without PI3K inhibitor treatment. **(D)** 293 cells were cotransfected with mKate-tagged CEACAM1 and the GFP-tagged Btk-PH domain. 30 min before infection with Pacific Blue labelled Opa_{CEA}-expressing gonococci, cells were treated with 200nM wortmannin or left untreated. Fixed samples were analyzed by confocal microscopy. Arrowheads highlight CEACAM- recruitment to cell-associated bacteria. Insets show enlargement of the highlighted area. Bars represent 5 μ m.

Whereas CEACAM3-mediated uptake was not reduced in the presence of the PI3K inhibitor, a finding that corroborates previous results (Buntru, Kopp et al. 2011), bacterial internalization via CEACAM1 was clearly diminished by LY294002 (Fig. 15C). Moreover, confocal microscopy demonstrated that a second PI3K inhibitor, wortmannin, abrogated the recruitment of the Btk-PH domain without interfering with bacteria binding to and clustering of CEACAM1 (Fig. 15D).

Together, these results suggest that PI(3,4,5)P is locally produced by PI3Ks in response to CEACAM1 engagement and interference with PI3K activity impairs CEACAM1-mediated internalization of bacteria.

CEACAM1-mediated endocytosis of meningococci by endothelial cells is also PI(3,4,5)P-dependent

CEACAM1 has also been shown to promote the internalization of *N. meningitidis* by endothelial cells (Muenzner, Dehio et al. 2000). To investigate, if the PI3K dependence of CEACAM1-mediated bacterial internalization is a general feature in different cell types, we studied uptake of *N. meningitidis* by human brain-derived microvascular endothelial cells (HBMEC). Upon exposure to pro-inflammatory cytokines, endothelial cells are known to strongly upregulate CEACAM1 expression, which then allows internalization of Opa_{CEA}-expressing meningococci (Muenzner, Dehio et al. 2000;

Muenzner, Naumann et al. 2001). In a first step, we transduced HBMEC with recombinant lentivirus encoding CEACAM1-GFP or GFP alone to generate stable cell lines. Expression of CEACAM1-GFP in the transduced cells was verified by FACS analysis (data not shown) and Western Blotting (Fig. 16A).

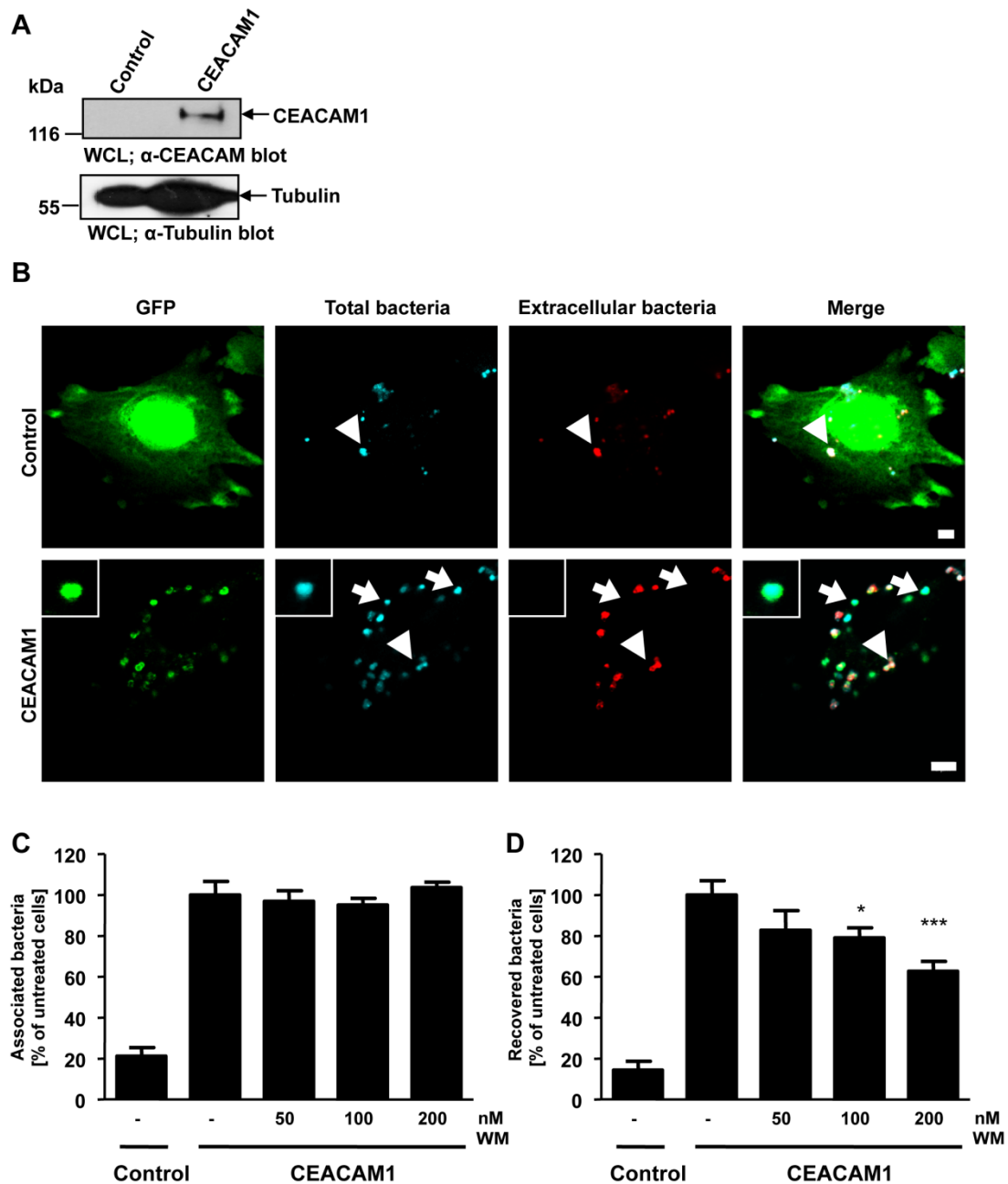


Fig. 16 CEACAM1-mediated uptake of *Opa*_{CEA}-expressing *N. meningitidis* by endothelial cells depends on PI3K activity. (A) Human brain microvascular endothelial cells (HBMEC) were transduced with GFP-encoding control lentivirus (control) or a CEACAM1-GFP-encoding lentivirus. CEACAM1 expression in transduced HBMEC is analysed by Western blotting of whole cell lysates (WCL) using monoclonal α -CEACAM antibody (upper panel) and equal loading of the samples was demonstrated by α -tubulin antibody (lower panel). (B) Stable CEA-

CAM1-GFP or control GFP expressing HBMECs were infected with biotin- and AlexaFluor647-labelled Opa_{CEA}-expressing meningococci for 60 min. Upon fixation and staining of extracellular bacteria with streptavidin-rhodamine, samples were analyzed by confocal microscopy. Arrows highlight intracellular bacteria, whereas arrowheads point to extracellular bacteria. Bars represent 5 μ m. **(C and D)** CEACAM1-GFP or GFP expressing HBMECs were pre-treated with the indicated concentrations of wortmannin for 30 min or left untreated. Cells were infected for 3 h with Opa_{CEA}-expressing meningococci and total cell-associated **(C)** or viable intracellular bacteria **(D)** were quantified. Bars represent mean values \pm S.E.M of three independent experiments done in triplicate (n = 9). Numbers are expressed relative to CEACAM1-GFP cells without PI3K inhibitor treatment. Significance was tested using an unpaired, two-sided Student's t-test; ***, p<0.001, *, p<0.05.

Next, HBMEC-GFP or HBMEC-CEACAM1-GFP were infected with Opa_{CEA}-expressing *N. meningitidis* for 1 h and the fixed samples were differentially stained for intracellular and extracellular bacteria. Confocal microscopy revealed that small numbers of Opa_{CEA}-expressing *N. meningitidis* adhered to GFP-expressing endothelial cells, but these bacteria were not internalized (Fig. 16B). In contrast, large numbers of meningococci were associated with CEACAM1-GFP expressed by HBMECs and a large proportion of the bacteria were internalized (Fig. 16B). Upon pre-treatment of CEACAM1-GFP expressing HBMECs with different concentrations of the PI3K inhibitor wortmannin, bacterial binding to the CEACAM1-GFP expressing endothelial cells was not affected (Fig. 16C). At the same time, the uptake of meningococci was decreased in a dose-dependent manner as measured by gentamicin protection assays (Fig. 16D) demonstrating that inhibition of PI(3,4,5)P generation affects CEACAM1-mediated endocytosis of bacteria in multiple cell types.

Overexpression of constitutive active PI3K increases and expression of SHIP decreases uptake of Opa_{CEA}-expressing gonococci via CEACAM1

To further investigate the role of PI(3,4,5)P during CEACAM-mediated endocytosis, cells were cotransfected with vectors encoding CEACAM1 or CEACAM3 together with the cerulean-tagged enzymatic subunit (p110) of PI3K or cerulean alone, respectively. Cells were infected with fluorescein-labeled Opa_{CEA}-expressing gonococci for 2 h and,

after washing, the infected cells were analysed by flow cytometry by gating on the cerulean-positive, transfected cells (Fig. 17A). To specifically detect internalized bacteria, signals from cell-associated extracellular bacteria were quenched by the addition of trypan blue (Pils, Schmitter et al. 2006) and the fluorescein signal from internalized bacteria was recorded. Whereas CEACAM3-expressing cells did not show an altered uptake of bacteria upon overexpression of PI3K, CEACAM1-mediated uptake of Opa_{CEA}-expressing gonococci was more than doubled in cells with elevated PI3K activity (Fig. 17B).

To further demonstrate that PI(3,4,5)P is critical for CEACAM1-mediated endocytosis, we overexpressed the PI(3,4,5)P-specific phosphatidylinositol-phosphatase SHIP, which dephosphorylates the 5'-position of PI(3,4,5)P to yield PI(3,4)P (Damen, Liu et al. 1996; Lioubin, Algate et al. 1996). The cerulean-tagged phosphatase domain of wildtype SHIP or an enzymatically inactive form of SHIP (SHIP D675G) were co-expressed with either HA-tagged CEACAM1 or HA-tagged CEACAM3, respectively. As a further control, cerulean alone was co-expressed with CEACAMs (control). Equivalent expression of cerulean-tagged SHIP constructs or cerulean was verified by flow cytometry (Fig. 17C). Next, cells were infected for 2 h with fluorescein-labeled Opa_{CEA}-expressing gonococci and samples were analysed by gating on cerulean-positive cells during flow cytometry. Cell-association and internalization of fluorescein-labelled bacteria was measured in cerulean-positive cells in the absence (cell-association; Fig. 17D) or presence (invasion; Fig. 17E) of trypan blue, which quenches fluorescein fluorescence derived from extracellular bacteria. Overexpression of the wildtype SHIP phosphatase domain reduced uptake of bacteria via CEACAM1 by about 50%, whereas CEACAM3-mediated uptake was not affected (Fig. 17E).

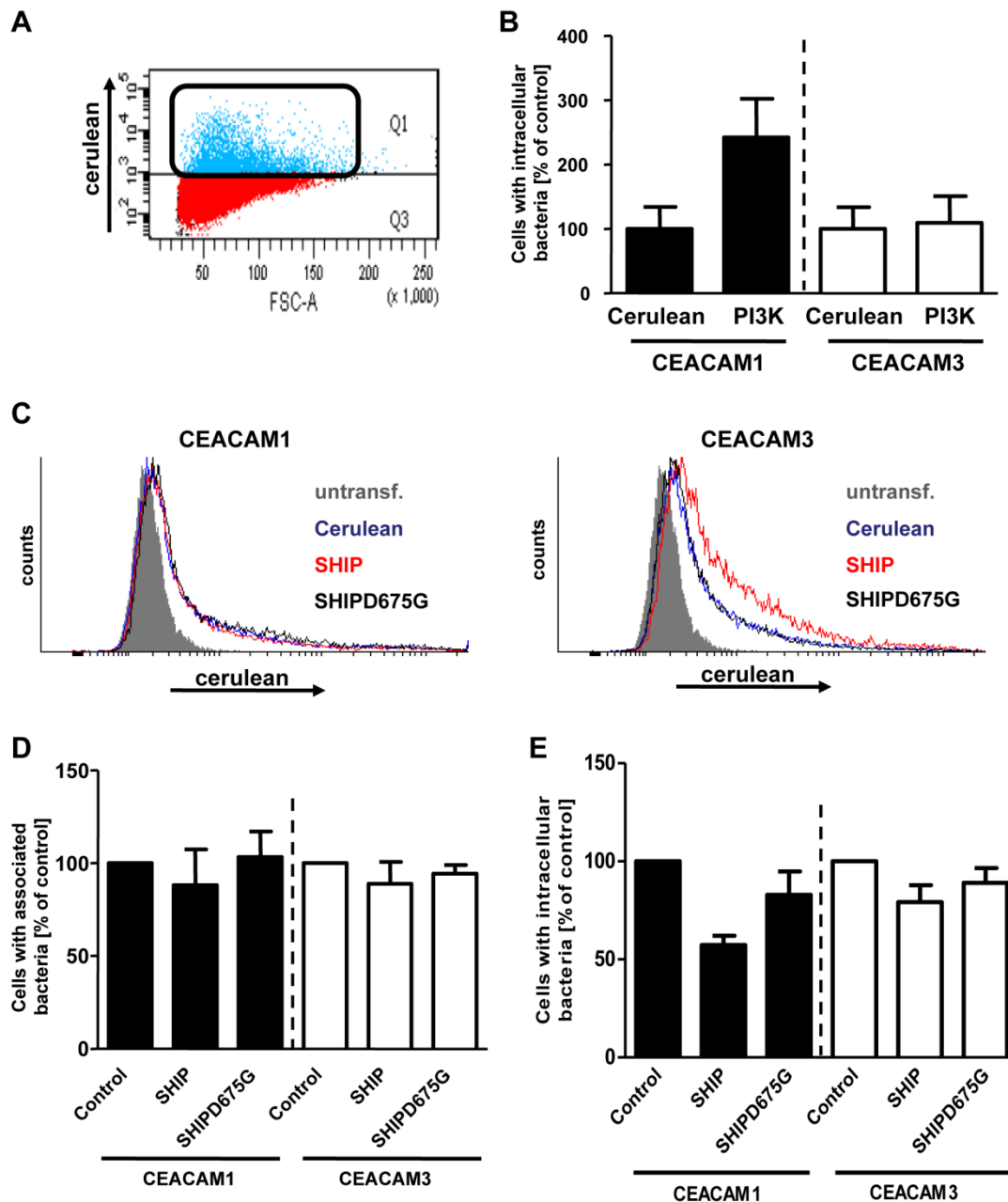


Fig. 17 Constitutive active PI3K increases, whereas expression of SHIP decreases uptake of Opa_{CEA} -expressing gonococci via CEACAM1. (A) 293 cells were cotransfected with constructs encoding CEACAM1-HA or CEACAM3-HA together with cerulean-tagged PI3K or cerulean alone. Cells were infected with fluorescein-labelled Opa_{CEA} -expressing gonococci for 2 h. Samples were analysed using a flow cytometer by gating on cerulean-positive cells. Dot blot shows a representative gate used to detect the cerulean-positive cell population. (B) The fluorescein signal derived from cerulean-positive cells in (A) was quantified in the presence of trypan blue, which quenches fluorescence derived from extracellular gonococci. Bars represent mean values \pm S.E.M of a representative experiment done in triplicate. (C - E) 293 cells were cotransfected with CEACAM1-HA or CEACAM3-HA together with cerulean, cerulean-tagged SHIP1 phosphatase domain (SHIP), or an inactive form of the SHIP1 phosphatase domain (SHIP D675G), respectively. (C) Expression of cerulean, SHIP1, or SHIP1 D675G was analysed by flow cytometry and histograms show representative samples. (D and E) Cells were infected with fluorescein-stained Opa_{CEA} -expressing gonococci for 2 h and the fluorescein signal derived from cerulean-positive cells was detected in the absence (D) or presence (E) of try-

pan blue. This allows quantification of total cell-associated bacteria (**D**) or internalized gonococci (**E**). Bars represent mean values \pm S.E.M of three independent experiments. Numbers are expressed relative to cells co-expressing cerulean and the respective receptor.

In contrast, overexpression of phosphatase-inactive SHIP D675G did not influence internalization via either of the receptors (Fig. 17E). Furthermore, binding of bacteria to CEACAM1- or CEACAM3-expressing cells was not altered upon co-expression of wildtype or phosphatase-inactive SHIP (Fig. 17D).

Thus, modulation of cellular PI(3,4,5)P levels selectively affects CEACAM1-mediated internalization. The results with overexpression of PI3K or SHIP are in agreement with the idea that PI(3,4,5)P is critical for efficient CEACAM1-mediated endocytosis of bacteria.

PI3K inhibition decreases bacterial uptake via CEACAM1 and CEACAM1 Δ CT

The cytoplasmic domain of CEACAM1, and in particular the cytoplasmic domain of the long isoform of CEACAM1, can accommodate multiple protein-protein interactions (Gray-Owen and Blumberg 2006). Indeed, upon tyrosine phosphorylation, SH2-domain mediated binding of several proteins, including the regulatory subunit of class I PI3K, to the CEACAM1 cytoplasmic domain can be observed (Fig. 18A). Similarly, a direct association of PI3K with CEACAM3 has been reported and this interaction is mediated by the SH2 domains of PI3K binding to the cytoplasmic domain of CEACAM3 (Buntru, Kopp et al. 2011)(Fig. 18B). However, a cytoplasmic domain is not required for efficient CEACAM1-mediated internalization of gonococci (Muenzner, Bachmann et al. 2008).

encoding the GFP-tagged Btk PH domain and mKate-tagged CEACAM1 lacking the cytoplasmic domain (CEACAM1 Δ CT). Upon infection with Pacific Blue-labelled CEACAM-binding *N. gonorrhoeae*, a strong recruitment of the Btk-PH domain was observed (Fig. 19A), demonstrating that PI(3,4,5)P generation in response to CEACAM1 engagement does not require the cytoplasmic domain of the receptor. To address the functional role of PI(3,4,5)P and of the CEACAM1 cytoplasmic domain, cells were transfected with constructs encoding HA-tagged CEACAM1 wildtype (CEACAM1), HA-tagged CEACAM1 Δ CT or the empty control vector (pcDNA). Similar expression levels of the receptors in the transfected cells were verified by Western blotting (Fig. 19B). The transfected cells were treated or not with the PI3K inhibitor wortmannin and infected with Opa_{CEA}-expressing gonococci for 2 h. Whereas total numbers of cell-associated bacteria were not altered by PI3K inhibition (Fig. 19C), wortmannin severely decreased uptake of pathogens via both CEACAM1 and CEACAM1 Δ CT (Fig. 19D). These results suggest that the CEACAM1 cytoplasmic domain is not essential for PI(3,4,5)P generation required for CEACAM1-mediated uptake.

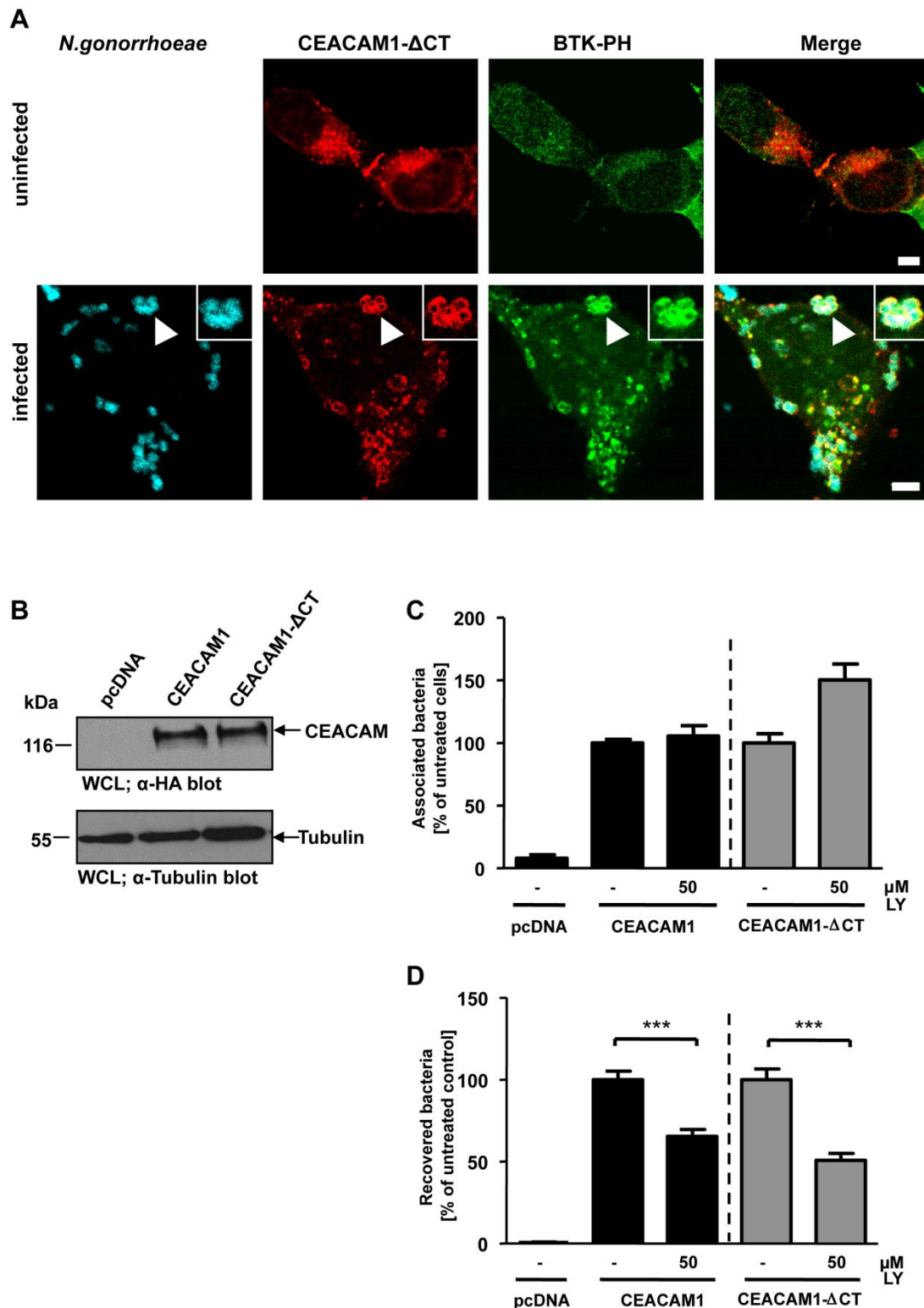


Fig. 19 PI3K inhibition decreases bacterial uptake via CEACAM1 and CEACAM1 Δ CT. (A) 293 cells were co-transfected with mKate-tagged CEACAM1 lacking the cytoplasmic domain (CEACAM1 Δ CT) together with GFP-tagged Btk-PH domain and infected or not with Pacific Blue labelled Opa_{CEA}-expressing gonococci for 60 min. After fixation, samples were analyzed by confocal microscopy. Arrowhead highlights CEACAM1 Δ CT clustering by gonococci and recruitment of Btk-PH domain. Insets show enlargement of the highlighted area. Bars represent 5 μ m. (B) 293 cells were transfected with the empty vector (pcDNA), HA-tagged CEACAM1 wildtype or CEACAM1 Δ CT. CEACAM expression was analysed in whole cell

lysates (WCLs) by Western blotting with α -HA antibody (upper panels) and equal loading of the samples was demonstrated by α -tubulin antibody (lower panel). **(C and D)** Cells transfected as in **(B)** were pretreated for 30 min with 50 μ M of PI3K inhibitor LY294002 (LY) or left untreated. Cells were infected for 2 h with Opa_{CEA}-expressing gonococci and total cell-associated **(C)** or viable intracellular bacteria **(D)** were quantified. Bars represent mean values \pm S.E.M of three independent experiments done in triplicate (n = 9). Numbers are expressed relative to cells expressing the respective receptor without PI3K inhibitor treatment. Significance was tested using an unpaired, two-sided Student's t-test; ***, p<0.001.

Inhibition of PI3K activity does not affect CEACAM1 relocalisation to membrane microdomains

A main difference between CEACAM1- and CEACAM3-mediated endocytosis concerns the membrane distribution of these receptors. Whereas CEACAM1 has been shown to translocate into membrane microdomains (lipid rafts) after receptor engagement, CEACAM3 remains in the non-raft membrane fraction (Muenzner, Bachmann et al. 2008). Interestingly, membrane microdomain association of CEACAM1 occurs also in the absence of a cytoplasmic domain (Muenzner, Bachmann et al. 2008). To investigate, whether the abundance of PI(3,4,5)P affects the relocalization of CEACAM1 into membrane microdomains, we analysed detergent-resistant membrane fractions of CEACAM-expressing cells in the presence or absence of a PI3K inhibitor. Before receptor clustering, CEACAM1 and CEACAM3 were found in a high-density membrane fraction similar to the non-lipid raft-associated transferrin receptor (TfR) (Fig. 20A). As observed before, receptor-clustering promoted a re-distribution of CEACAM1, but not CEACAM3, into the low-density membrane fractions, which were also enriched for the lipid raft marker protein flotillin2 (Fig. 20B). Importantly, pre-treatment of CEACAM1-expressing cells for 30 min with 200 nM wortmannin did not prohibit the re-distribution of CEACAM1 into membrane microdomains upon crosslinking (Fig. 20B). Similar expression of CEACAM1 and CEACAM3, as well as the membrane marker proteins TfR and flotillin2 in the employed cell lysates was verified by Western Blotting (Fig. 20C).

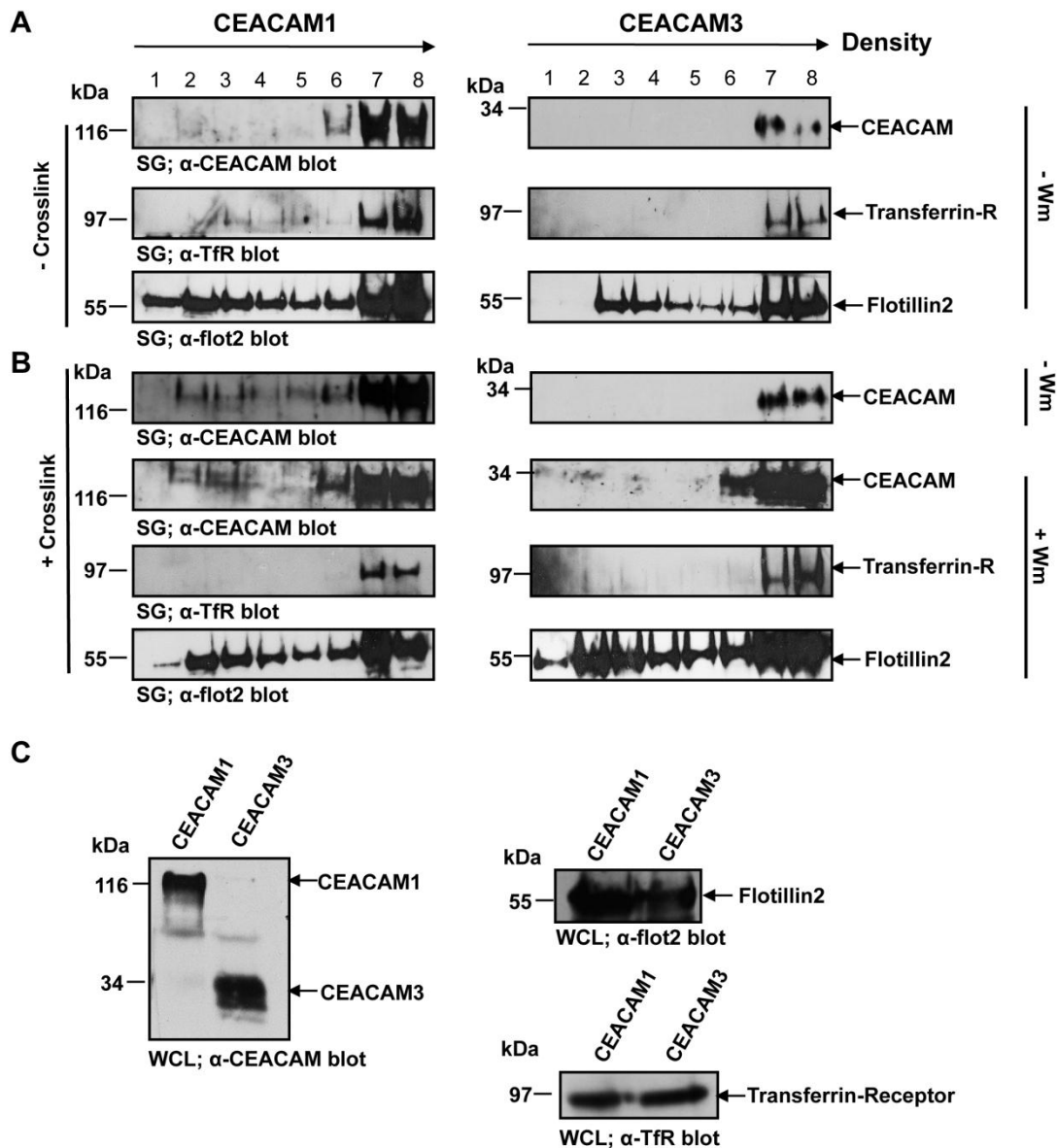


Fig. 20 Inhibition of PI3K activity does not affect CEACAM1 relocalisation to membrane microdomains. (A) 293 cells were co-transfected with HA-tagged CEACAM1 or CEACAM3 together with GFP-tagged flotillin2. Following cells homogenization, the membrane fraction was extracted with 0.7% Triton X-100-containing buffer and loaded on a discontinuous sucrose gradient (SG). After ultracentrifugation, eight distinct fractions were collected from top (low density: fraction 1) to bottom (high density: fraction 8) of the gradient and analyzed by Western blotting with antibodies against CEACAM, flotillin2 (flot2), or transferrin receptor (TfR). Detergent-resistant, low-density membrane components were identified mainly in fraction 2–4. (B) Cells were transfected as in (A) and CEACAMs were crosslinked using antibodies prior to homogenization. As indicated, some samples were treated for 30 min with 200nM wortmannin (+WM) before crosslinking. Membrane fractions and Western blot analysis was as in (A). (C) Expression of CEACAMs, TfR, or flot2 in whole cell lysates (WCLs) was determined by Western blotting with the indicated antibodies.

These results demonstrated that membrane microdomain localization of CEACAM1 is not influenced by the generation of PI(3,4,5)P and suggested that CEACAM1-initiated PI3K activity could be downstream of CEACAM1 re-distribution to lipid rafts.

Membrane microdomain localization is not required for CEACAM1-initiated PI(3,4,5)P generation

To test the idea, that membrane microdomain localization might be a pre-requisite for the CEACAM1-dependent generation of PI(3,4,5)P, we employed a chimeric receptor, CEACAM1/CEACAM3-TM, which encompasses the extracellular domain of CEACAM1 fused to the transmembrane domain (TM) of CEACAM3 (Fig. 21A). Expression of CEACAM1, CEACAM3 and the chimeric receptor was verified by Western Blotting (Fig. 21B). Previously, we have shown that the TM of CEACAM1 directs the receptor into membrane microdomains, whereas the CEACAM3 TM prohibits lipid raft localization (Muenzner, Bachmann et al. 2008). Involvement of lipid rafts can be revealed by methyl- β -cyclodextrin (M β CD), a cholesterol chelator, which removes cholesterol from cellular membranes, thereby disrupting lipid raft integrity and interfering with CEACAM1-mediated uptake of gonococci (Schmitter, Pils et al. 2007; Muenzner, Bachmann et al. 2008). As expected and similar to CEACAM3, bacterial endocytosis via CEACAM1/CEACAM3-TM was insensitive to methyl- β -cyclodextrin (M β CD) treatment (Fig. 21D), whereas total numbers of cell-associated bacteria were not altered (Fig. 21C). Nevertheless, bacterial internalization via CEACAM1/CEACAM3-TM was still sensitive to PI3K inhibition by LY294002 (Fig. 21F) suggesting that this lipid raft-independent uptake still involved PI(3,4,5).

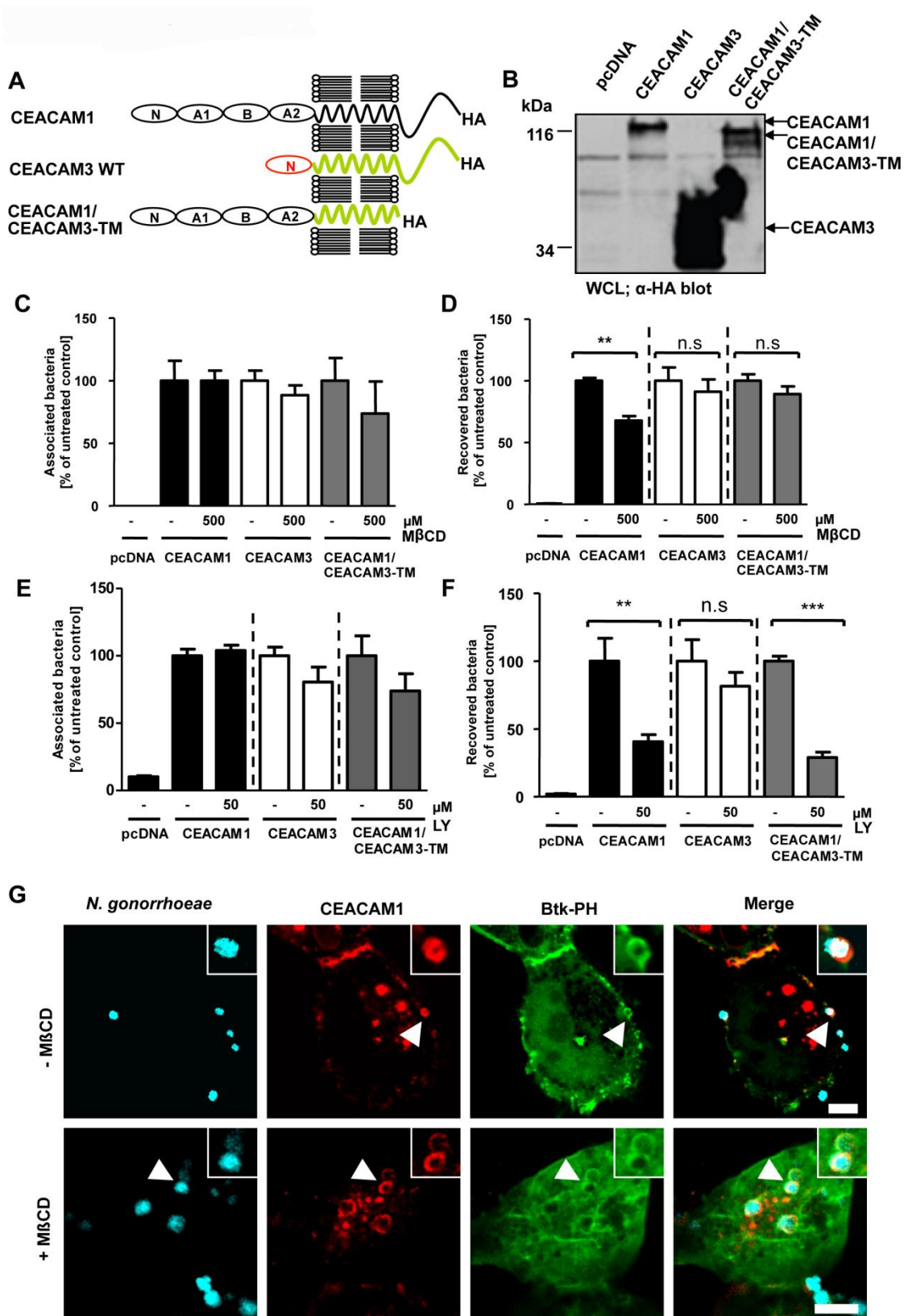


Fig. 21 Membrane microdomain localization is not required for CEACAM1-initiated PI(3,4,5)P generation. (A) Domain organization of the used CEACAM constructs. N – Ig_v-like N-terminal domain; A1, B, A2 – Ig_{C2}-like domains. (B) 293 cells were transfected with empty vector (pcDNA) or constructs encoding HA-tagged CEACAM1, CEACAM3, or a chimeric protein consisting of the extracellular domains of CEACAM1 fused to the transmembrane domain of CEACAM3 (CEACAM1/CEACAM3-TM). Expression was verified by Western blot-

ting with α -HA antibody. **(C, D)** Cells transfected as in **(B)** were pretreated for 30 min with 500 μ M methyl- β -cyclodextrin (M β CD) or left untreated. After infection for 2 h with Opa_{CEA}-expressing gonococci total cell-associated bacteria **(C)** or recovered intracellular bacteria **(D)** were quantified. Bars represent mean \pm SEM of three independent experiments done in triplicate (n = 9). Numbers are expressed relative to cells expressing the respective receptor without M β CD treatment. Significance was tested using an unpaired, two-sided Student's t-test; **, p<0.01; n.s. – not significant. **(E, F)** Cells transfected as in **(B)** were pretreated with 50 μ M of LY294002 (LY). Cells were infected as in **(C, D)** and total cell-associated bacteria **(E)** or recovered intracellular bacteria **(F)** were quantified. Bars represent mean \pm SEM of three independent experiments done in triplicate (n = 9). Numbers are expressed relative to cells expressing the respective receptor without LY treatment. Significance was tested using an unpaired, two-sided Student's t-test; ***, p<0.001; **, p<0.01; n.s. – not significant. **(G)** 293 cells were transfected with mKate-tagged CEACAM1 together with GFP-tagged Btk-PH domain and treated with M β CD before infection with Pacific Blue labelled Opa_{CEA}-expressing gonococci. After 60 min, samples were fixed and analyzed via confocal microscopy. Arrowheads highlight gonococci associated with clustered CEACAM1. Insets show enlargement of the highlighted area. Bars represent 5 μ m.

To confirm that membrane microdomain localization of CEACAM1 is not directly connected to CEACAM1-dependent PI(3,4,5)P generation, cells expressing CEACAM1-mKate together with the GFP-tagged BTK-PH domain were treated or not with M β CD. Confocal microscopy confirmed that the Btk-PH domain was recruited to the site of bacterial CEACAM1 engagement (Fig. 21G).

Interestingly, cholesterol depletion by M β CD only slightly reduced the local accumulation of PI(3,4,5)P (Fig. 21G). Therefore, the integrity of lipid rafts is not essential for PI3K activation in response to CEACAM1 stimulation. Together, these results suggest that redistribution into membrane microdomains and the receptor-initiated generation of PI(3,4,5) are two independent events needed for optimal CEACAM1-mediated endocytosis.

Bacterial uptake via several epithelial CEACAMs requires PI3K activity

Neither the cytoplasmic part of CEACAM1 nor the transmembrane domain-mediated recruitment to membrane microdomains were mechanistically linked to the PI3K-dependent endocytosis of bacteria. Therefore, we wondered, whether bacterial uptake via GPI-linked epithelial CEACAMs, which lack cytoplasmic and transmembrane do-

mains, also requires the generation of PI(3,4,5)P. Accordingly, cells were transfected with constructs encoding HA-tagged CEACAM1, CEA, CEACAM6, or the empty control vector (pcDNA). Expression levels were confirmed by Western blot with a monoclonal antibody recognizing several human CEACAMs (Fig. 22A). Next, cells were infected for 2 h with Opa_{CEA} -expressing gonococci in the presence or absence of the PI3K inhibitor wortmannin and adherence of the bacteria to the cells as well as internalization of the bacteria were quantified (Fig. 22B and C).

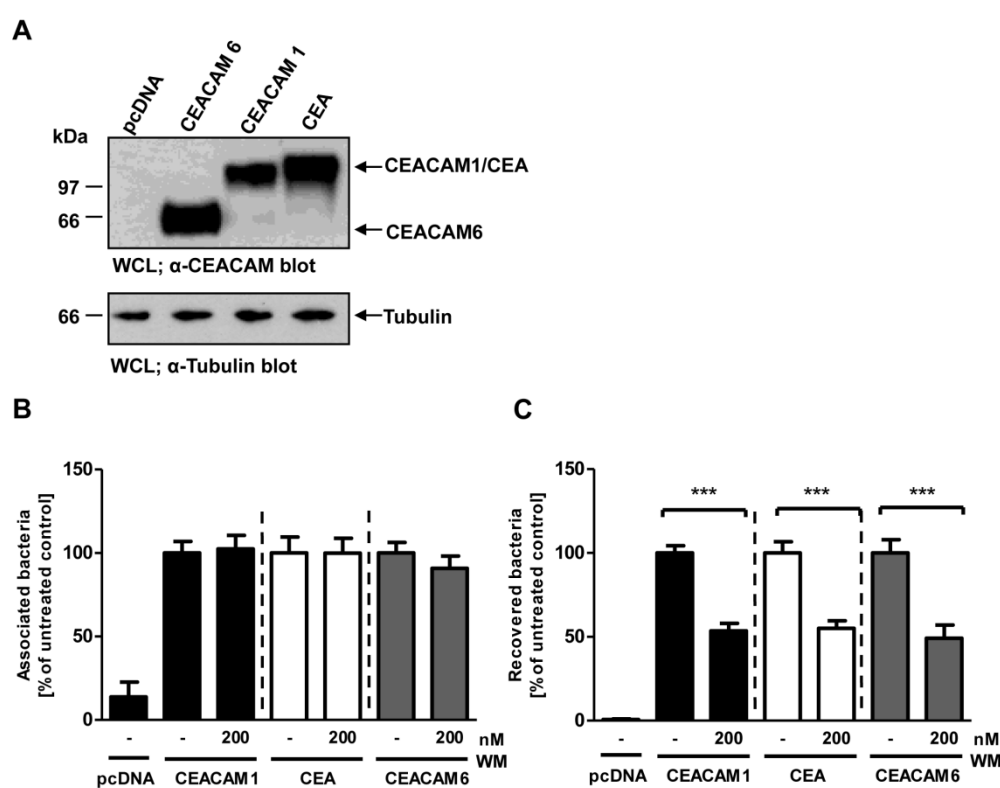


Fig. 22 Bacterial uptake via several epithelial CEACAMs requires PI3K activity. (A) 293 cells were transfected with empty vector (pcDNA) or constructs encoding CEACAM1, CEA, or CEACAM6. CEACAM expression was confirmed by Western blotting of whole cell lysates (WCLs) with α -CEACAM antibody (upper panel). Equal loading of the samples was verified by α -tubulin blot (lower panel). (B, C) Cells transfected as in (A) were pretreated for 30 min with 200 nM wortmannin (WM) or left untreated. After infection for 2 h with Opa_{CEA} -expressing gonococci, total cell-associated bacteria (B) or recovered intracellular bacteria (C) were quantified. Bars represent mean \pm SEM of three independent experiments done in triplicate ($n = 9$). Numbers are expressed relative to cells expressing the respective receptor without WM treatment. Significance was tested using an unpaired, two-sided Student's t-test; ***, $p < 0.001$.

As observed for CEACAM1, a significant reduction of bacterial internalization was seen for CEA and CEACAM6 upon addition of the PI3K inhibitor, whereas the total

amount of cell-associated bacteria was not affected (Fig. 22B and C). These results point to a general requirement for PI3K activity and PI(3,4,5)P during endocytosis of bacteria via epithelial CEACAMs.

Extracellular Ig_{C2}-like domains of CEACAM1 control the PI3K dependency during pathogen uptake

Besides the localization in membrane microdomains, CEA and CEACAM6 share with CEACAM1 the presence of additional extracellular Ig_{C2}-like domains. Interestingly, CEACAM3 does not encompass Ig_{C2}-like domains and has only the single N-terminal Ig_V-like domain, which is characteristic of CEACAM family members. As both the transmembrane and the cytoplasmic domain of CEACAM1 were not involved in the PI3K-dependent uptake of bacteria, we asked, whether the extracellular Ig_{C2}-like domains of CEACAM1 could be a critical determinant for this process. Therefore, we generated different deletion constructs of CEACAM1 lacking either one (CEACAM1-NA1B), two (CEACAM1-NA1), or all (CEACAM1-N) extracellular Ig_{C2}-like domains (Fig. 23A). Cells were transfected with the different constructs and all proteins were expressed at similar levels except for CEACAM1-N, which was expressed at slightly lower levels (Fig. 23B). The transfected cells were treated or not with PI3K inhibitor prior to infection with fluorescein-labelled Opa_{CEA}-expressing gonococci. 2 h after the infection, cells were analysed by flow cytometry for intracellular bacteria. As before, PI3K inhibition reduced internalization of bacteria via wildtype CEACAM1, but also CEACAM1-NA1B and CEACAM1-NA1 showed decreased uptake of Opa_{CEA}-expressing *N. gonorrhoeae* upon PI3K inhibition (Fig. 23C). Significantly, for CEACAM1-N, which lacks all extracellular Ig_{C2}-like domains and which supports only low level of internalization, no further reduction of bacterial uptake upon PI3K inhibition was observed compared to the uptake in the absence of the inhibitor (Fig. 23C).

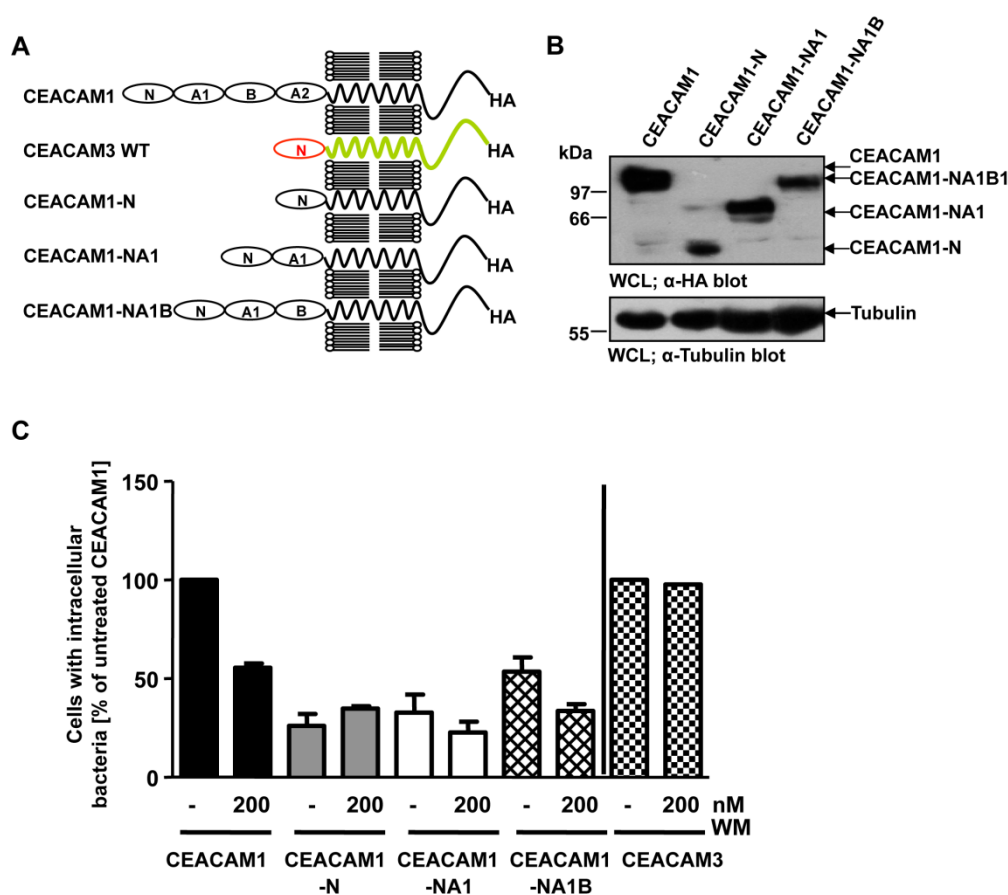


Fig. 23 Extracellular Ig_{C2}-like domains of CEACAM1 are required for the PI3K dependency during pathogen uptake. (A) Domain organization of the used CEACAM constructs. N – Ig_V-like N-terminal domain; A1, B, A2 – Ig_{C2}-like domains. (B) 293 cells were transfected with HA-tagged CEACAM1, CEACAM1-N, CEACAM1-NA1 or CEACAM1-NA1B. CEACAM1 expression was confirmed by Western blotting with α -HA antibody (upper panel). Equal loading of the samples was verified by α -tubulin blot (lower panel). (C) Cells transfected as in (B) were pretreated for 30 min with 200nM wortmannin (WM) or left untreated before infection with fluorescein-labelled Opa_{CEA}-expressing gonococci for 2 h. Infected cells were analysed by flow cytometry and the fluorescein signal from internalized bacteria was detected in the presence of trypan blue, which quenches fluorescence derived from extracellular gonococci. Bars represent mean values \pm S.E.M from three independent experiments. Numbers are expressed relative to cells expressing CEACAM1 without WM treatment.

Clearly, the absolute number of endocytosed bacteria was lower for CEACAM1 deletion mutants lacking one or more Ig_{C2}-like domains compared to wildtype CEACAM1. Together with the marginal or absent effect of wortmannin on uptake via the CEACAM1 deletion mutants, these results suggest that the Ig_{C2}-like domains are responsible for connecting CEACAM1 to a PI3K-dependent endocytosis pathway.

Overexpression of a CEACAM8/1 chimera interferes with CEACAM1-mediated uptake

If the Ig_{C2}-like domains connect epithelial CEACAMs to additional membrane protein(s), then overexpression of Ig_{C2}-like domains in the absence of a bacteria-binding Ig_V-like domain should block CEACAM1-mediated internalization. To test this idea, we took advantage of CEACAM8, a member of the CEACAM family, which is not recognized by Op_{aCEA}-expressing gonococci (Popp, Dehio et al. 1999; Schmitter, Pils et al. 2007). The CEACAM8 N-terminal Ig_V-like domain was fused to the Ig_{C2}-like domains of CEACAM1 creating a CEACAM8/1 chimera (Fig. 24A). Next, we co-transfected increasing amounts of the construct encoding the HA-tagged CEACAM8/1 chimera together with a constant amount of GFP-tagged CEACAM1 lacking the cytoplasmic domain (CEACAM1 ΔCT) and verified the expression in whole cell lysates by Western blotting (Fig. 24B). Control cells were transfected with the empty vector (pcDNA) or with the CEACAM8/1 chimera alone (Fig. 24B). Importantly, increasing amounts of CEACAM8/1 chimera on the cell surface did not alter the total amount of cell-associated bacteria (Fig. 24C). However, the increased presence of CEACAM8/1 resulted in a corresponding decrease in bacterial internalization (Fig. 24D). These results support the idea that the Ig_{C2}-like domains of CEACAM1 are involved in the uptake of bacteria, most likely via lateral interaction with other cell surface receptor(s). Together, our data point to a CEACAM co-receptor, which might be able to associate with epithelial CEACAMs via their Ig_{C2}-like domains and which appears to regulate endocytosis in a PI3K- and PI(3,4,5)P-dependent manner.

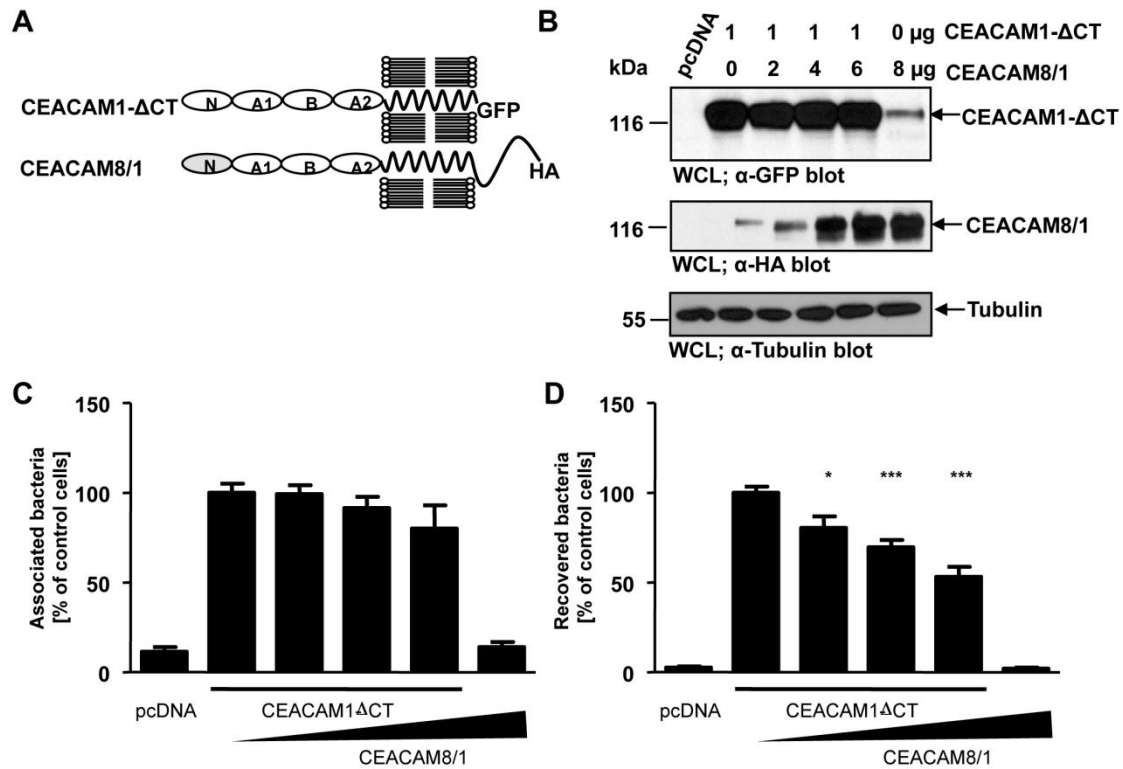


Fig. 24 Overexpression of a CEACAM8/1 chimera interferes with CEACAM1-mediated uptake. (A) Domain organization of the used CEACAM constructs. N – Ig_v-like N-terminal domain; A1, B, A2 – Ig_{C2}-like domains. (B) 293 cells were transfected with empty vector (pcDNA) or a construct encoding CEACAM1 ΔCT-GFP (1 μg) together with increasing amounts of a plasmid encoding the HA-tagged CEACAM8/1 chimera (0 to 6 μg). One sample was transfected with CEACAM8/1-HA (6 μg) only. Differential expression of CEACAM1 ΔCT-GFP and CEACAM8/1-HA was verified by Western blotting of whole cell lysates (WCLs) using α-GFP antibody (CEACAM1 ΔCT; upper panel) or α-HA antibody (CEACAM8/1; middle panel), respectively. Equal loading of samples was demonstrated by Western blotting with α-tubulin antibody (lower panel). (C, D) Cells transfected as in (B) were infected with Opa_{CEA}-expressing gonococci. After infection for 2 h, total cell-associated bacteria (C) or recovered intracellular bacteria (D) were quantified. Numbers are expressed relative to cells expressing CEACAM1 ΔCT in the absence of CEACAM8/1. Bars represent mean ± SEM of three independent experiments done in triplicate (n = 9). Significance was tested using an unpaired, two-sided Student's t-test; ***, p<0.001; *, p<0.05.

6.5 Discussion

CEACAM family members on epithelial cells are the target of several human restricted bacteria, which exploit these receptors for host cell attachment and internalization (Kuespert, Pils et al. 2006). Here we demonstrate that endocytosis of bacteria via the epithelial CEACAM family members CEACAM1, CEA, and CEACAM6 requires PI3-kinase activity. Pharmacological inhibition of PI3-kinase enzyme activity or overexpression of a PI(3,4,5)P-specific phosphatase inhibit CEACAM1-mediated uptake, whereas overexpression of class I PI3-kinase augments the internalization of bacteria via CEACAM1. PI3-kinase activity was not connected to the specific subcellular localization of CEACAM1 in cholesterol-rich membrane microdomains, but required the presence of extracellular immunoglobulin domains of CEACAM1 suggesting that CEACAM1 engages in a *cis*-interaction with other membrane proteins to trigger endocytosis.

In contrast to CEACAM1, PI3K inhibition by wortmannin does not affect CEACAM3-mediated uptake of bacteria by transfected cell lines or primary human granulocytes (Buntru, Kopp et al. 2011). Bacterial internalization via CEACAM3 has been studied in great detail and depends on actin cytoskeleton dynamics orchestrated by sequence determinants in the CEACAM3 cytoplasmic domain (Buntru, Roth et al. 2012). Indeed, CEACAM3 engagement results in massive lamellipodial protrusions on the cell surface tightly connected to bacterial engulfment (Billker, Popp et al. 2002; Schmitter, Agerer et al. 2004; Pils, Kopp et al. 2012). Therefore, the independence of CEACAM3-mediated uptake from PI3K activity is particularly intriguing, as PI(3,4,5)P has been linked to the local regulation of actin polymerization and membrane trafficking via recruitment and activation of guanine nucleotide exchange factors (GEFs) for small GTPases of the Rho and Arf families (Hawkins, Anderson et al. 2006). However, phos-

phorylated CEACAM3 can directly associate with Vav to promote GTP loading of the small G-protein Rac and this direct binding to a Rac GEF might allow CEACAM3 to bypass a requirement for PI3K activity during phagocytosis (Schmitter, Pils et al. 2007; Buntru, Roth et al. 2012). On the other hand, actin cytoskeleton dynamics play only a minor role during uptake via epithelial CEACAMs, which occur with a slower kinetic and in the absence of major surface protrusions compared to CEACAM3-mediated uptake (McCaw, Liao et al. 2004; Schmitter, Agerer et al. 2004; Schmitter, Pils et al. 2007). Therefore, PI(3,4,5)P generation in response to CEACAM1 engagement does not seem to be necessary for driving prominent actin-based changes at the cell surface. Previous studies have indicated that PI(4,5)P, which is the substrate of class I PI3K and is the major phosphoinositide synthesized at the plasma membrane, has to be removed from invaginated membranes to allow endosome formation via dynamin binding (Cremona, Di Paolo et al. 1999; Schafer, Weed et al. 2002). For example, invasion of *Yersinia* into epithelial cells can only proceed from a semi-enclosed state to a fully-enclosed state upon removal of PI(4,5)P (Sarantis, Balkin et al. 2012). Though there are multiple ways to locally reduce PI(4,5)P levels, recruitment and activation of class I PI3K, which utilizes PI(4,5)P to generate PI(3,4,5)P, is one possible route to diminish PI(4,5)P to allow completion of CEACAM1-mediated endocytosis. However, the fact that overexpression of SHIP, which degrades PI(3,4,5)P to PI(3,4)P, has a negative impact on CEACAM1-mediated internalization argues against the idea that PI3K function in this process is instrumental for reducing PI(4,5)P levels at the plasma membrane.

Another characteristic difference between epithelial CEACAMs and CEACAM3 relates to the distinct distribution in membrane microdomains (Schmitter, Pils et al. 2007; Muenzner, Bachmann et al. 2008). As a result, bacterial uptake via epithelial CEACAMs is sensitive to cholesterol chelators such as methyl- β -cyclodextrin. However, M β CD treatment did not abolish the generation of PI(3,4,5)P at sites of bacterial-

CEACAM1 contact and PI3K inhibition did not alter the distribution of CEACAM1 in membrane microdomains. These results suggest that two independent pre-requisites, membrane microdomain localization and PI3K activation, have to occur together to allow optimal CEACAM1 endocytosis. Most importantly, our results imply that these pre-requisites are coordinated by different molecular determinants of epithelial CEACAMs. On the one hand, the GPI anchor of CEA as well as CEACAM6 or the transmembrane domain of CEACAM1 direct these receptors into membrane microdomains (Muenzner, Bachmann et al. 2008). On the other hand, the Ig_{C2}-like extracellular domains, presumably by associating with a so far unknown co-receptor, are responsible for connecting CEACAM1 with PI3K activity. It is currently unknown if the Ig_{C2}-like domains of all epithelial CEACAMs connect to the same co-receptor. Sequence comparisons of Ig_{C2}-like domains of CEACAM1, CEA, and CEACAM6 reveal that these domains share 90-95% similarity between these proteins, which is comparable to the high similarity found for the N-terminal Ig_V-like domains. This degree of conservation of the Ig_{C2}-like domains could allow these proteins to interact laterally with the same kind of co-receptor. However, further investigations depend on the identification of such a co-receptor.

One hint on the identity of a potential co-receptor might come from studies of other pathogenic bacteria, which invade epithelial cells in a PI(3,4,5)P-dependent manner. For example, *Listeria monocytogenes* triggers a PI3K-dependent uptake pathway by binding to the receptor tyrosine kinase (RTK) c-Met (Iretton, Payrastre et al. 1996; Cossart 2011). RTKs such as c-Met might be perfect co-receptors for epithelial CEACAMs. Indeed, several RTKs possess, in addition to their ligand binding regions, extracellular protein-protein-interaction domains including Ig domains, fibronectin type III repeats, cadherin or discoidin domains (Lemmon and Schlessinger 2010). Previously, a functional interaction between rat CEACAM1 and the insulin receptor (IR) has been report-

ed that affects endocytosis of the IR together with its insulin ligand (Formisano, Najjar et al. 1995; Soni, Lakkis et al. 2000). At the same time, insulin triggers endocytosis of CEACAM1 in hepatocytes, presumably in a complex together with the insulin-bound IR (Choice, Howard et al. 1998). However, the IR-initiated endocytosis of CEACAM1 is regulated by amino acid residues located in the long cytoplasmic domain of CEACAM1 (Choice, Howard et al. 1998; Poy, Yang et al. 2002) making it unlikely that the IR is responsible for PI3K-dependency of endocytosis of epithelial CEACAMs.

Another example of a pathogen invading host cells in a PI3K-dependent manner is provided by *E. coli* K1, which causes meningitis in neonates. Invasion of these bacteria into brain microvascular endothelial cells is blocked by pharmacological PI3K inhibition or overexpression of dominant-negative variants of class I PI3Ks (Reddy, Prasadarao et al. 2000). Host cell receptors for the *E. coli* K1 OmpA protein on endothelial and epithelial cells have been identified as Ecgp and gp96, respectively (Prasadarao, Srivastava et al. 2003). Gp96 (also known as GRP94 or HSP90b1) is a glycoprotein of the Hsp70 chaperone family and located in the ER lumen, but small amounts are also found on the cell surface (Robert, Menoret et al. 1999). Gp96 interacts with numerous membrane proteins such as integrins and Toll-like receptors (Wu, Hong et al. 2012). How *E. coli* K1 OmpA-mediated binding to gp96 triggers PI3K-dependent internalization is currently unclear. As gp96 is linked to multiple eukaryotic surface receptors, it might be a candidate protein involved in the PI(3,4,5)P-dependent uptake of bacteria via CEACAM1 and other epithelial CEACAMs.

Streptococcus pneumoniae is a further pathogen, which invades epithelial cells in a PI3K-dependent manner (Agarwal and Hammerschmidt 2009). In this context, the *S. pneumoniae* protein PspC engages immunoglobulin-like extracellular domains of the polymeric immunoglobulin receptor (pIgR) to trigger uptake into respiratory epithelial cells (Zhang, Mostov et al. 2000). Interestingly, the physiological function of pIgR is to

regulate the transcytosis of IgA and IgM from the basolateral membrane to the apical side of mucosal epithelia. Upon arrival at the apical surface, the pIgR is proteolytically processed. Whereas the extracellular domain, as so-called secretory component, is released together with its cargo antibody, the truncated pIgR can be endocytosed from the apical membrane (Rojas and Apodaca 2002). Moreover, a minor fraction of intact pIgR seems to undergo transcytosis from the apical surface to the basolateral membrane (Rojas and Apodaca 2002). As epithelial CEACAMs have been reported to allow transcytosis of *N. gonorrhoeae* across polarized epithelia, the pIgR and its PI(3,4,5)P-dependent endocytosis appear as promising candidates for a cis-interacting membrane protein. However, pIgR is only expressed on mucosal epithelia and not found on endothelial cells and therefore, could not be responsible for the PI3K-dependent endocytosis of CEACAM1 by endothelial cells.

Together, our studies point to the existence of co-receptor(s) for epithelial CEACAMs, which confer specific signaling properties to these bacterial target molecules. Surprisingly, protein-protein interactions mediated by the Ig_{C2}-like domains of epithelial CEACAMs appear to be responsible for the PI3K-dependent endocytosis. Therefore, our novel results explain the ability of CEACAM1, CEA, and CEACAM6 to mediate bacterial uptake in the absence of cytoplasmic domains. Besides the connection to PI3K activation and the presence of extracellular protein-protein interaction domains, a putative CEACAM co-receptor should be expressed by epithelial and endothelial cells. Clearly, identification of a co-receptor for epithelial CEACAMs should be the next step to understand the role of epithelial CEACAMs as pathogen receptors and potentially obtain new insight into their physiological functions.

6.6 Acknowledgments

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6.7 Author Contributions

Conceived and designed the experiments: MV VB KK CRH. Performed the experiments: MV VB JN KK. Analyzed the data: MV CRH. Wrote the paper: MV CRH.

7 CHAPTER III

**The role of RhoG and EphA2
during CEACAM1-mediated
uptake of *N. gonorrhoeae***

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7.1 Abstract

Background. Distinct pathogenic bacteria engage members of the CEACAM family to attach and to internalize into human cells. Uptake of *Neisseria gonorrhoeae*, a gram-negative human pathogen, via epithelial CEACAMs (CEACAM1, 6, CEA) seems to require an interaction with an unknown co-receptor, mediated through the extracellular Ig_{C2}-like domains of these CEACAMs. This interaction leads to generation of phosphatidylinositol-3,4,5 trisphosphate (PI(3,4,5)P), which is necessary for efficient uptake of pathogens via epithelial CEACAMs in contrast to pathogen uptake via the granulocyte-specific CEACAM3, which does not contain any Ig_{C2}-like domains.

Principal Findings. We find that the small GTPase RhoG is recruited to site of infection via CEACAM1 as well as CEACAM3. Overexpression of an effector-binding mutant and a constitutive active form of RhoG demonstrate the involvement of RhoG in CEACAM1-mediated uptake. Knockdown of endogenous RhoG leads to decreased pathogen uptake via CEACAM1, the effect can be rescued by overexpression of PI3K, which links the both signaling pathways together. Ephexin4, a guanine nucleotide exchange factor (GEF) specific for RhoG, is recruited to site of infection via CEACAM1 and knockdown of endogenous Ephexin4 disturb the efficient invasion of pathogens. Ephexin4 is connected to the receptor tyrosine kinase EphA2, which is recruited to site of infection and knockdown of endogenous EphA2 also leads to decreased bacterial uptake via CEACAM1 but not via CEACAM3. In addition, pharmacological inhibition of EphA2 selectively affects CEACAM1-mediated uptake of *Neisseria gonorrhoeae*.

Conclusions. Our results demonstrate the new discovered role of RhoG during CEACAM1-mediated uptake of *Neisseria gonorrhoeae*, linked to the previously reported involvement of the PI3K. Moreover, we suppose EphA2 as possible co-receptor for epithelial CEACAMs, important for signal transduction into mammalian cells.

7.2 Introduction

The carcinoembryonic antigen (CEA) family is involved in various processes like tissue morphogenesis, apoptosis, insulin homeostasis, angiogenesis and regulation of T-cell activity (Kuespert, Pils et al. 2006). The members of the family (12 genes and a number of pseudogenes) belong to a group of mammalian immunoglobulin-related glycoproteins (Zebhauser, Kammerer et al. 2005; Kuespert, Pils et al. 2006). All members of the family possess one amino-terminal immunoglobulin variable-like domain (Ig_V-like), followed by up to 6 immunoglobulin constant type2-like (Ig_{C2}-like) domains (Barclay 2003; Kuespert, Pils et al. 2006). Some members of the family contain a transmembrane domain connected to a cytoplasmic domain (CEACAM1, CEACAM3) whereas other members like CEA (the product of the *CEACAM5* gene) and CEACAM6 contain a glycosylphosphatidylinositol (GPI) anchor without cytoplasmic part. The tissue specific expression of the family members is different; CEACAM1 for example is widely expressed on epithelial and endothelial cells, lymphocytes and myeloid cells, whereas CEACAM3 is only expressed on granulocytes (Gray-Owen and Blumberg 2006). Some pathogens gain members of the CEACAM family (CEACAM1, 3, CEA, CEACAM6) for binding and invasion into the host cells. This interaction is mediated by binding of specific surface proteins (Opa_{CEA}) of *Neisseria* to the non-glycosylated C'CFG-face of the immunoglobulin-domain fold of the N-terminal Ig_V-like domain (Chen and Gotschlich 1996; Virji, Watt et al. 1996; Chen, Grunert et al. 1997; Voges, Bachmann et al. 2010) (Toleman, Aho et al. 2001; Hauck, Agerer et al. 2006). Besides *N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica*, binding to the Ig_V-like domain of CEACAMs is known from *Moraxella catarrhalis*, *Haemophilus influenza*, and *Escherichia coli* via specific surface proteins (Hill, Toleman et al. 2001; Hill and Virji 2003; Berger, Billker et al. 2004). Binding of bacteria to CEACAM receptors lead to their ability for effective

colonization of mucosal epithelia in the host because they trigger increased matrix adhesion of the infected host cell and prevent of exfoliation of epithelial cells in urogenital tissue (Muenzner, Rohde et al. 2005; Muenzner, Bachmann et al. 2010). Also, binding of bacteria to CEACAMs can trigger engulfment by various cell types (Bos, Grunert et al. 1997; Chen, Grunert et al. 1997; Gray-Owen, Lorenzen et al. 1997; Hauck, Meyer et al. 1998; Muenzner, Dehio et al. 2000). Another important mechanism of binding and internalization of pathogens via CEACAMs is transcytosis through an intact cell layer in polarized epithelial cells, the regulation of this mechanism is unknown until now (Wang, Gray-Owen et al. 1998; Wang, Meyer et al. 2007). Endocytosis via epithelial CEACAMs differs entirely compared to endocytosis via CEACAM3, which is only expressed on granulocytes. For CEACAM3, the cytoplasmic domain is essential for bacterial uptake. After binding of bacteria, the immunoreceptor tyrosine-based activation motif (ITAM) is phosphorylated by Src kinases, which leads to recruitment and activation of the guanine exchange factor (GEF) Vav and recruitment of the adaptor molecule Nck, a known interaction partner of the small GTPase Rac. Activation of Rac via Vav triggers massive actin rearrangements required for CEACAM3-mediated endocytosis (Billker, Popp et al. 2002; Buntru, Roth et al. 2012). In contrast, epithelial CEACAMs (CEACAM1, CEA and CEACAM6) mediate uptake of pathogens independent of their cytoplasmic domain (CEACAM1) (Muenzner, Bachmann et al. 2008; Kuespert, Roth et al. 2011) or even do not possess any cytoplasmic domain (CEA, CEACAM6). In addition, rearrangement of the actin cytoskeleton is only partially important for efficient uptake of bacteria via epithelial CEACAMs (Billker, Popp et al. 2002; McCaw, Liao et al. 2004; Schmitter, Pils et al. 2007). Epithelial CEACAMs, but not CEACAM3, relocalize in membrane microdomains after bacterial binding, and cholesterol depletion impairs uptake of bacteria (Schmitter, Pils et al. 2007; Muenzner, Bachmann et al. 2008). Recently we could also show, that PI3K activity is very important for bacterial

uptake via epithelial CEACAMs, whereas uptake of pathogens via CEACAM3 is PI3K independent, although later on the PI3K activity is very important for the oxidative burst (Buntru, Kopp et al. 2011; Voges, Bachmann et al. 2012). Phosphoinositides are often involved in endocytotic processes because of their regulating function in actin cytoskeleton and microtubule rearrangements and the influence on the small GTPases (Vanhaesebroeck and Waterfield 1999) (Takenawa and Itoh 2001; McLaughlin, Wang et al. 2002). Surprisingly, the extracellular Ig_{C2}-like domains of the epithelial CEACAMs are responsible for the PI3K dependency during bacterial uptake, so we hypothesize an unknown co-receptor for CEACAM1-mediated signaling into mammalian cells after bacterial binding (Voges, Bachmann et al. 2012). Earlier it became clear, that the uptake mechanism of bacteria via epithelial CEACAMs is quite distinct from CEACAM3-mediated internalization of pathogens. The small GTPase Rac is connected to CEACAM3-mediated uptake of bacteria (Schmitter, Pils et al. 2007) and in general, it is known that small GTPases are important during endocytosis and vesicle trafficking, mostly by regulating the actin cytoskeleton and microtubule dynamics by activation of responsible proteins for this processes (Jaffe and Hall 2005; D'Souza-Schorey and Chavrier 2006; Prieto-Sanchez, Berenjano et al. 2006; Doherty and McMahon 2009). Here we report, that the small GTPase RhoG is not only recruited to site of infection, but also strongly involved in CEACAM1-, but not in CEACAM3-mediated endocytosis of pathogens. Also a link between RhoG and the PI3K pathway could be demonstrated, possibly by direct interaction of RhoG with the p85 subunit of the class I PI3K (Hiramoto-Yamaki, Takeuchi et al. 2010). To get more insight in upstream signaling processes, we made a shRNA-based functionally screen of all known guanine exchange factors (GEFs) which are able to catalyse the nucleotide exchange of RhoG. We could show that especially one GEF, named Ephexin4, is recruited to site of infection via CEACAM1 and is also strongly required for efficient bacterial uptake. For CEACAM3,

no recruitment and no effect on bacterial uptake was observed. The only known upstream interaction partner for Ephexin4 is the receptor tyrosine kinase EphA2 (Hiramoto-Yamaki, Takeuchi et al. 2010; Harada, Hiramoto-Yamaki et al. 2011), leading us to the investigation of the role of EphA2 during CEACAM1-mediated internalization of pathogens. We could demonstrate that EphA2 is also recruited to site of bacterial-host interaction and kinase activity of EphA2 is directly involved in CEACAM1-, but not CEACAM3-mediated uptake of gonococci, pointing to EphA2 as unknown co-receptor for epithelial CEACAMs. These data provide new insights in the signaling transduction into mammalian cells via epithelial CEACAMs and for the first time, a connection to another membrane receptor could be shown.

7.3 Material & Methods

Neisserial strains and growth conditions

Opa₅₂ protein-expressing, non-piliated *Neisseria gonorrhoeae* strain MS11 (Ngo Opa_{CEA}) and the isogenic non-opaque strain (Ngo Opa₋) were kindly provided by Thomas F. Meyer (Max-Planck-Institut für Infektionsbiologie, Berlin, Germany). *N. gonorrhoeae* was grown as described before (Kuespert, Weibel et al. 2007) on GC agar plates (Difco BRL, Paisley, UK) supplemented with vitamins at 37°C, 5% CO₂. For infection, overnight grown bacteria were taken from GC agar plates, suspended in PBS, and colony forming units (cfu) were estimated by OD₅₅₀ readings according to a standard curve.

Epithelial cell lines

Human embryonic kidney epithelial 293T cells (293 cells; ACC-635, DSMZ, Braunschweig, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. All cells were grown in the absence of antibiotics at 37°C in 5% CO₂ and subcultured every 2-3 days. Human brain microvascular endothelial cells (HBMEC) (Slanina, König et al. 2010) were grown in endothelial cell medium (PAA, Pasching, Austria) supplemented with L-glutamine. All cells were grown in the absence of antibiotics at 37°C in 5% CO₂ and subcultured every 2-3 days.

Recombinant DNA constructs

Mammalian expression plasmids encoding HA-tagged versions of human CEACAM1-4L (CEACAM1), CEACAM1 lacking the complete cytoplasmic domain (CEACAM1- Δ CT), CEACAM3, CEA and CEACAM6 were described previously (Schmitter, Agerer et al. 2004; Muenzner, Rohde et al. 2005). The mKate-tagged and mCerulean-tagged versions of CEACAM1, CEACAM1 Δ CT, and CEACAM3 were generated by amplify-

ing the HA-tagged versions of CEACAM1, CEACAM1 Δ CT, or CEACAM3, respectively, with primers CEACAM1-IF sense 5'-GAAGTTATCAGTCGATACCATGGGGCACTTCTCAGCCCC-3' and HA-CEACAM-IF antisense 5'-ATGGTCTAGAAAGCTTGCAGCGTAATCTGGAACGTCATATGG-3', or with CEACAM3-IF-sense, 5'-GAAGTTATCAGTCGATACCATGGGGCCCCCCTCAGCC-3', and HA-CEACAM-IF-antisense. The resulting PCR fragments were cloned into pDNR-Dual using the In-Fusion PCR Cloning Kit (Clontech, Mountain View, CA) and transferred by Cre-mediated recombination into pLPS-3'mKate (Muenzner, Bachmann et al. 2010) or pLPS-3'mCerulean resulting in mKate or mCerulean, respectively, fused to the carboxy-terminus of the expressed proteins. The CEACAM1 deletion mutant the Ig_{C2}-like extracellular domains (CEACAM1-N-Cyt-TM) is described previously (Kuespert, Roth et al. 2010). The cDNA of the enzymatic p110 subunit of PI3K was a gift from J. Downward (Cancer Research UK, London, UK). Full-length PI3K was amplified with primers PI3KCA-IF-sense-5'-GAAGTTATCAGTCGACCCTCCAAGAC-CATCATCAG-3' and PI3KCA-IF-anti-5'-ATGGTCTAGAAAGCTTAGGCGGCTCAGTTCAATGCATGCTG-3'. The resulting PCR fragment was cloned into pDNR-Dual using the In-Fusion PCR Cloning Kit (Clontech, Mountain View, CA) and transferred by Cre-mediated recombination into a modified pcDNA vector with loxP site 3' of the cerulean coding sequence (pcDNA Cerulean loxP). The cloning and production of CEACAM1-GFP or GFP encoding lentiviral particles and transduction of cells was performed as described previously (Muenzner, Rohde et al. 2005). The GFP-tagged RhoG, RhoGF37A, RhoA, RhoA T19N, Cdc42, Cdc42 T17N, Rac1 and Rac1 N17V12 constructs were a kind gift from T.F. Meyer (MPI Berlin). For further investigation, RhoGV12 and RhoGF37A were

subcloned in pDNRdual using the Infusion PCR cloning Kit (Clontech, Mountain View, CA) with primers RhoG_IF_forward -5'- GAAGTTATCAGTCGACATGCAGAG-CATCAAGTGC-3' and RhoG_IF_reverse -5'- ATGGTCTAGAAAGCTTTCACAA-GAGGATGCAGGAC-3', followed by Cre-mediated recombination into pLP-3xmyc with loxP site 5' of myc-coding sequence. Flag-tagged Ephexin4 and myc-tagged EphA2, EphA2 Δ SAM and EphA2 Δ KD were a kind gift of H. Katoh (Kyoto University, Japan) and EphA2 was subcloned into pLPS-mCherry vector with primers EphA2 for XhoI -5'- GACTCGAGCGGACCATGGAGCTCCAGGCAGCC-3' and EphA2 rev HindIII -5'- CTAAGCTTGATGGGGATCCCCACAGTGTTTC-3'.

shRNA construction and lentiviral production

Recombinant lentiviral particles were generated using the plasmids pLKO.1, pLKO.1-shScrambled (containing a non-targeting control shRNA-sequence), pMD2.G, and psPAX2 provided by Addgene (www.addgene.org) and maintained in E. coli STBL4 (Invitrogen, Carlsbad, CA). Using the algorithm AAGN18TT (available online at <http://jura.wi.mit.edu/bioc/siRNAext/>) we identified sequences that could silence expression of human RhoG, Ephexin4 or EphA2, respectively. According to this prediction, complementary primers were synthesized: shEphrinA2-sense1 -5'- ccggaAGTAAACAGGGTACCTCAAGctcgagCTTGAGGTACCCTGTTTACTTtttttg-3' and shEphrinA2-antisense1 -5'- aattcaaaaaAAGTAAACAGGGTACCTCAA-GctcgagCTTGAGGTACCCTGTTTACTT-3'; Ephexin4 shRNA sense -5'- ccggaGAGCGAGGAGAGCTACATGctcgagCATGTAGCTCTCCTCGCTCttttttg-3' and Ephexin4 shRNA antisense -5'- aattcaaaaaaGAGCGAGGAGAGCTACATGctcgag-CATGTAGCTCTCCTCGCTCt-3'; RhoG shRNA sense -5'- ccggaCACTGGGTATTCTCATGAGCTCGAGCTCATGAGAATACCCAG-TGttttttg-3' and RhoG shRNA antisense -5'-

aattcaaaaaaCACTGGGTATTCTCATGAGCTCGAGCTCATGAGAATACCCAG-TGTTCCGGtt-3'. The oligos were annealed and cloned into the AgeI and EcoRI site of pLKO.1. The correct insertion of the shRNA cassette was verified by sequencing. For lentiviral production, 2×10^6 293 cells were transiently transfected with 14 μg of the respective pLKO.1 vector together with 10 μg of packaging plasmid psPAX2 and 7 μg of envelope-coding plasmid pMD2.G. 48 h later, the virus-containing cell culture supernatant was collected, centrifuged at 2000 rpm at 4°C for 7 min and filtered through a 0.45 μm pore-size filter. 3 ml of the cleared viral supernatant was used to transduce 293 cells in 10 cm culture dishes. After 24 h, puromycin (0.4 $\mu\text{g}/\text{ml}$) was added and the puromycin-resistant stable cell population was used in experiments after 6 days of selection.

Antibodies and reagents

Monoclonal antibody (mAb) against CEACAMs (clone D14HD11; recognizing CEACAM1, CEACAM3, CEA and CEACAM6) were purchased from Genovac (Freiburg, Germany), polyclonal antibodies against EphA2 (sc-924), RhoG (sc-160151) and Ephexin4 (sc-1007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), mAbs against phospho-EphA2 (Tyr 594, cat#3970) was purchased from Cell Signaling Technology (Danvers, MA, USA), mAbs against the HA-tag (clone 12CA5), against tubulin (clone E7), and against transferrin (clone G1/221/12) were purified from hybridoma cell supernatants. Rabbit polyclonal α -GFP antibody was custom made at our in-house Animal Research Facility (Universität Konstanz, Germany) and affinity purified. Secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA).

Transfection of cells, cell lysis and Western blot

293 cells were transfected by calcium phosphate precipitation using 5- 8 μg of appropriate cDNA in each case. The transfection efficiency ranged between 30% and 70% as reported (Schmitter, Pils et al. 2007). Transfected 293 cells were employed in experiments 48h after transfection. Cell lysis and Western blotting were performed as described (Schmitter, Agerer et al. 2004; Buntru, Kopp et al. 2011).

Gentamicin protection assay

293 cells were seeded at 5×10^5 cells/well in 24-well plates or 2.5×10^5 cells/well in 48-well plates coated with poly-L-lysine (10 $\mu\text{g}/\text{ml}$). A multiplicity of infection (MOI) of 30 bacteria per cell was routinely used, and after 2 hour of infection, extracellular bacteria were killed by 60 min incubation in 50 $\mu\text{g}/\text{ml}$ gentamicin in DMEM. Following gentamicin treatment, cells were lysed with 1% saponin in PBS for 15 min. The samples were diluted in PBS, and the number of viable bacteria was determined by plating suitable dilutions on GC agar. For inhibition studies, cells were treated with EphA2 inhibitor (Joachim Braun) (30 or 50 μM) 30 min prior to infection.

Bacterial adherence assay

Cells were seeded and infected as described for gentamicin protection assays. After the infection, the cells were gently washed three times, before they were lysed by addition of 1% saponin in PBS for 15 min. Total cell-associated bacteria were suspended by vigorous pipetting, and colony forming units were determined by plating of serial dilutions on GC agar.

Flow cytometry-based invasion assay

Bacterial uptake by 293 cells was analysed by flow cytometry as described (Pils, Schmitter et al. 2006). Prior to infection, bacteria were surface labelled with 0.2 µg/ml 5-(6)-carboxyfluorescein-succinimidylester (fluorescein; Invitrogen-Molecular Probes, Karlsruhe, Germany) in PBS at 37°C for 30 min prior to infection. 1×10^6 cells were seeded in 6-well plates coated with poly-L-lysine (10 µg/ml) and the next day, cells were infected with labelled bacteria at an MOI of 30 for 2 h. After infection, cells were washed with PBS and the samples were analysed on a LSR II flow cytometer (BD Bioscience) by gating on the cells based on forward and sideward scatter. Cell-associated fluorescein fluorescence was measured in the presence of 2 mg/ml trypan blue to quench fluorescence derived from extracellular bacteria and to selectively detect the fluorescence derived from intracellular bacteria. The percentage of fluorescein-positive cells within the transfected cell population was multiplied by the mean fluorescence intensity of the sample to obtain an estimate of the total number of internalized bacteria (uptake index). In each sample at least 4,000 cells were counted.

Immunofluorescence staining

For microscopic analysis of 293 cells, 7×10^4 cells were seeded onto poly-L-lysine- and fibronectin-coated (10 and 4 µg/ml, respectively, in PBS) glass-coverslips in 24-well plates. Cells were infected with Pacific blue-labeled bacteria for 1h at an MOI of 40. Samples were washed once and fixed with 4% paraformaldehyde. After three washes with PBS, samples were incubated in blocking buffer (PBS, 10% fetal calf serum) for 5 min. Ephexin4 was stained with α -Flag antibody for 1 h. Following three washes, probes were incubated with secondary antibody α -mouse Cy3, following by three washes. Afterwards samples were embedded in mounting medium (Dako, Glostrup, Denmark). Samples were analysed with a Leica TCS SP5 confocal laser scanning micro-

scope (Leica Microsystems, Wetzlar, Germany). Fluorescence signals of triple-labelled specimens were serially recorded with appropriate excitation and emission filters to avoid bleed-through. Images were digitally processed with ImageJ and merged to yield pseudo-coloured pictures.

7.4 Results

RhoG is recruited to site of infection via CEACAM1 and CEACAM3

It is well known, that small GTPases are involved in trafficking and endocytosis in cells (Qualmann and Mellor 2003; Symons and Rusk 2003). Hence, we were interested in the role of small GTPases during CEACAM1-mediated uptake of pathogens. CEACAM1 and CEACAM3 are members of the same family, but it is known that entirely distinct processes occur during uptake: CEACAM3 is highly dependent on rearrangements of the actin cytoskeleton and independent of PI3K activity because of direct recruitment and activation of guanine exchange factor Vav1, leading to activation of small GTPase Rac1. For CEACAM1-mediated uptake of pathogens actin cytoskeleton rearrangements are not so important, whereas relocalization into lipid rafts and PI3K activity is required (Muenzner, Bachmann et al. 2008) (Kuespert, Pils et al. 2006). Therefore, we co-transfected 293 cells with constructs encoding wild-type, dominant negative or rather effector binding mutants of Rac1, RhoA, Cdc42 and RhoG together with either CEACAM1 or CEACAM3 in 293 cells and infected the cells with *Neisseria gonorrhoeae* expressing a CEACAM-binding Opa protein (Ngo Opa_{CEA}) for 2 h. Following the infection, cells were washed three times and total cell-associated bacteria were enumerated by plating dilutions on selective media (adherence assay; Fig. 25A, C, E, G). Parallel samples were treated with gentamicin for 1 h to kill extracellular bacteria, and the intracellular bacteria were then released by mild detergent lysis of the cells (invasion assay; Fig. 25B, D, F, H). It became clear, that Cdc42 and RhoA are not involved in CEACAM1-mediated uptake, whereas overexpression of effector binding mutant RhoG and dominant negative Rac1 led to decreased uptake of gonococci. The effect of RhoG arouses our interest in particular, because for CEACAM3-mediated uptake we could not

see any influence on internalization, whereas Rac1 seems to play an important role in bacterial uptake in both processes (Fig. 25).

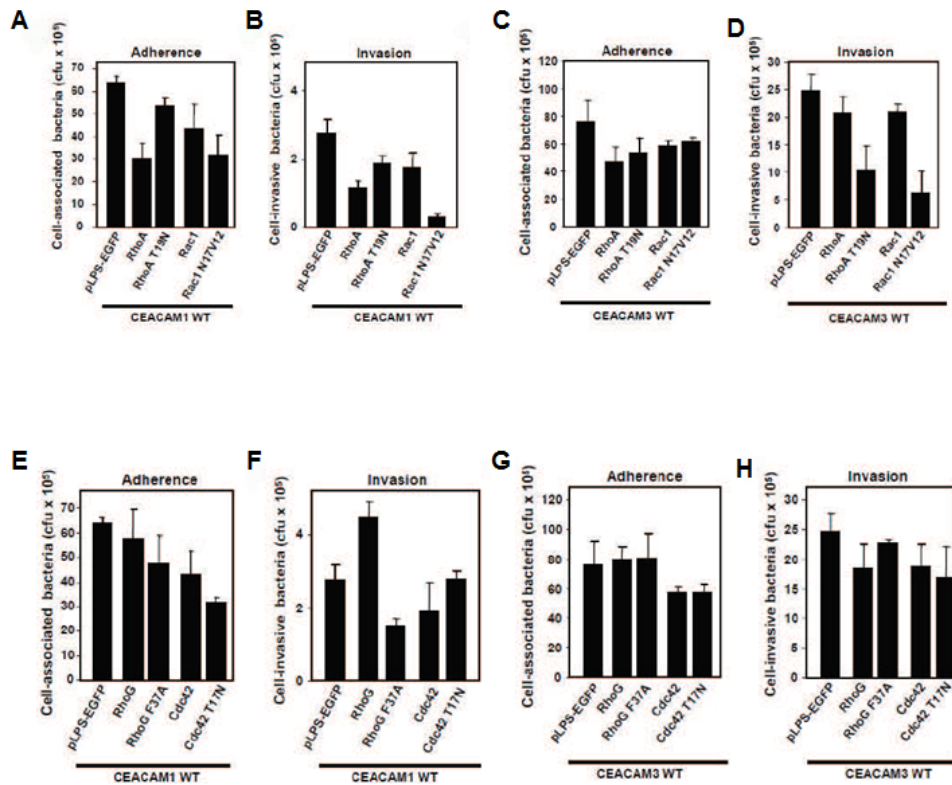


Fig. 25 RhoG and Rac1 are involved in CEACAM1-mediated uptake of *Neisseria gonorrhoeae*, in contrast to Cdc42 and RhoA. (A - D) 293 cells were co-transfected with constructs encoding HA-tagged CEACAM1-4L or CEACAM3 together with GFP-tagged RhoA/ Rac1 or dominant-negative mutant of RhoA (RhoT19N)/ Rac1(Rac1N17V12) or empty vector control (pLPS-EGFP). After infection for 2 h with Opa_{CEA}-expressing gonococci, the number of total cell-associated bacteria was determined. Bars represent mean value \pm SEM of one experiment, done in triplicate (A, C). Viable intracellular bacteria were determined in gentamicin protection assay. Bars represent mean value \pm SEM of two experiments, done in triplicate (B, D). (E-G) 293 cells were co-transfected with constructs encoding HA-tagged CEACAM1-4L or CEACAM3 together with GFP-tagged RhoG/Cdc42 or effector binding mutant of RhoG (RhoF37A)/dominant negative mutant of Cdc42 (Cdc42T17N) or empty vector control (pLPS-EGFP). After infection for 2 h with Opa_{CEA}-expressing gonococci, the number of total cell-associated bacteria was determined. Bars represent mean value \pm SEM of one experiment, done in triplicate (E, G). Viable intracellular bacteria were determined in gentamicin protection assay. Bars represent mean value \pm SEM of two experiments, done in triplicate (F, H).

Furthermore, we co-transfected 293 cells with constructs encoding GFP-tagged RhoG together with mKate-tagged CEACAM1 or CEACAM3. The transfected cells were either left uninfected or were infected for 60 min with Pacific-Blue-labelled Opa_{CEA} ex-

pressing gonococci. After infection, the cells were fixed and analyzed via confocal microscopy. In uninfected cells, RhoG was evenly distributed in the cytoplasm and the cellular membrane, but binding of CEACAM1 as well as CEACAM3 by *N. gonorrhoeae* led to a strong recruitment of RhoG to the site of bacteria-host-cell interaction (Fig. 26A and B). After infection, binding of CEACAM1 and CEACAM3 led to a massive relocation of RhoG to site of bacteria-host-cell contact (Fig. 26A and B). To summarize, these results indicate a strong recruitment of the small GTPase RhoG to site of CEACAM1- or CEACAM3-mediated bacterial uptake..

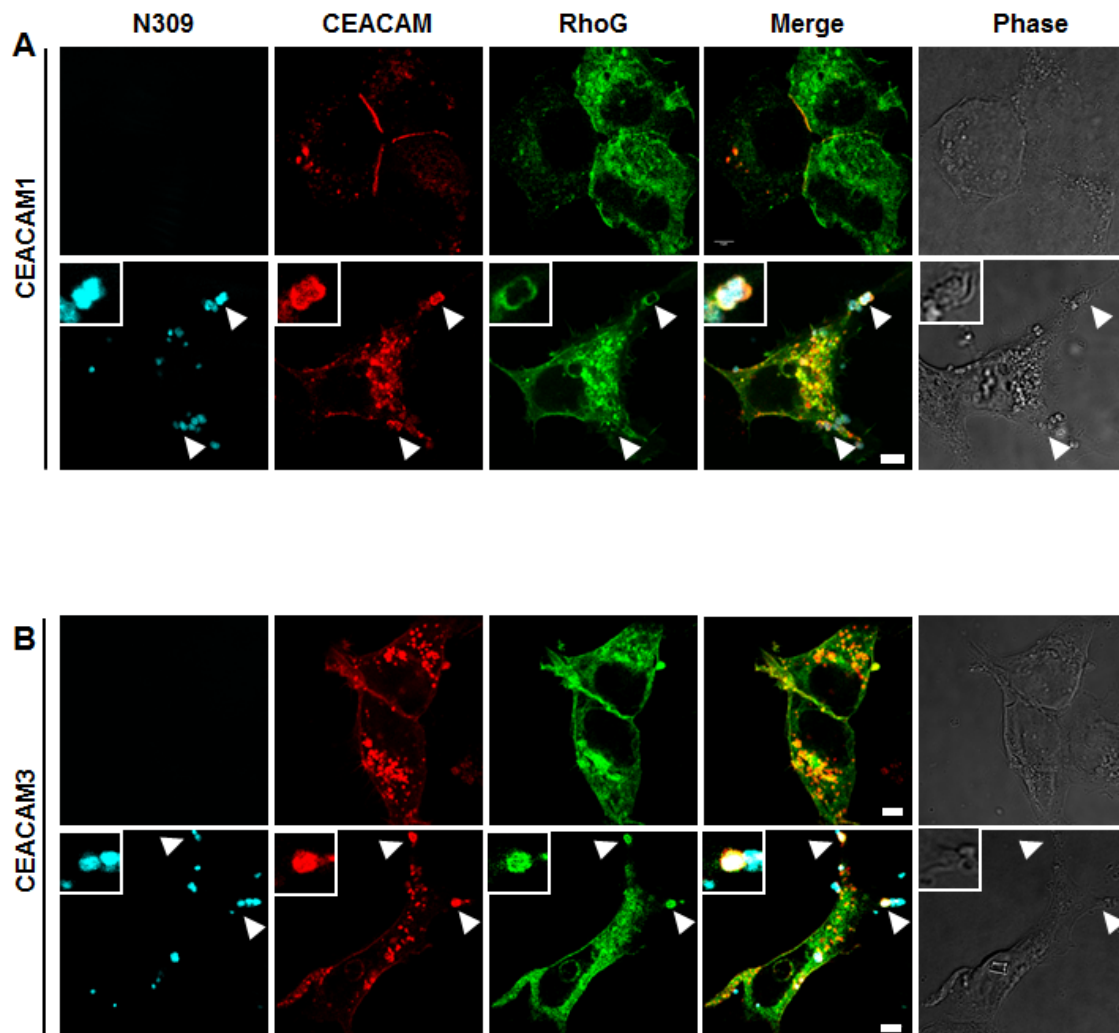


Fig. 26 RhoG is recruited to site of infection during CEACAM1- and CEACAM3-mediated uptake of *Neisseria gonorrhoeae*. (A) 293 cells were co-transfected with constructs encoding mKate-tagged CEACAM1-4L together with GFP-tagged RhoG. Cells were left uninfected or were infected with Pacific Blue labeled Opa_{CEA}-expressing *N. gonorrhoeae* for 60 min, fixed and analyzed by confocal microscopy. Arrowheads highlight bacteria associated with CEACAM1 and RhoG. Insets show enlargement of highlighted area. Bars represent 5 μ m. (B)

293 cells were co-transfected with constructs encoding mKate-tagged CEACAM3 together with GFP-tagged RhoG. Cells were left uninfected or were infected with Pacific Blue labeled Opa_{CEA}-expressing *N. gonorrhoeae* for 60 min, fixed and analyzed by confocal microscopy. Arrowheads highlight bacteria associated with CEACAM3 and RhoG. Insets show enlargement of highlighted area. Bars represent 5 μ m.

RhoG is important during CEACAM1-mediated uptake of *Neisseria gonorrhoeae*

To further investigate the role of RhoG during CEACAM1-mediated uptake we co-transfected 293 cells with constructs encoding either cerulean-tagged CEACAM1 or CEACAM3 together with empty control vector, a constitutive active form of RhoG (V12) or an effector binding mutant of RhoG (F37A). Expression of the constructs was verified via western blot with antibodies against HA-tag or RhoG (Fig. 27A). Cells were infected for 2 h with CFSE-labelled Opa_{CEA}-expressing gonococci and, after washing, the infected cells were analyzed via flow cytometry by gating on the cerulean-positive, transfected cells. Fluorescence signal from cell-associated extracellular bacteria was quenched by the addition of trypan blue and CFSE signal was recorded. Whereas overexpression of the effector binding mutant form of RhoG (F37A) in CEACAM3 transfected cells showed no effect on internalization, uptake via CEACAM1 was clearly decreased by about 30% (Fig. 27B). Overexpression of the constitutive active form of RhoG (V12) lead to more than 50% increase in uptake of gonococci via CEACAM1, while CEACAM3-mediated uptake was only slightly increased (Fig. 27B). Furthermore we established a stable knockdown of endogenous RhoG in 293 cells via lentiviral shRNA constructs or shScramble as control to verify the role of RhoG in CEACAM1-mediated uptake of gonococci. Efficiency of the knockdown was verified via western blot with antibody against RhoG (Fig. 27C). A decreased uptake of gonococci via CEACAM1 could be clearly observed, compared to cells which were only treated with scramble shRNA as positive control, whereas in CEACAM3 transfected cells no effect appeared (Fig. 27D). In previous publications we could show, that uptake via CEA-

CAM1 is strongly PI3K dependent whereas PI3K activity is not required for uptake of pathogens via CEACAM3 (Buntru, Kopp et al. 2011; Voges, Bachmann et al. 2012).

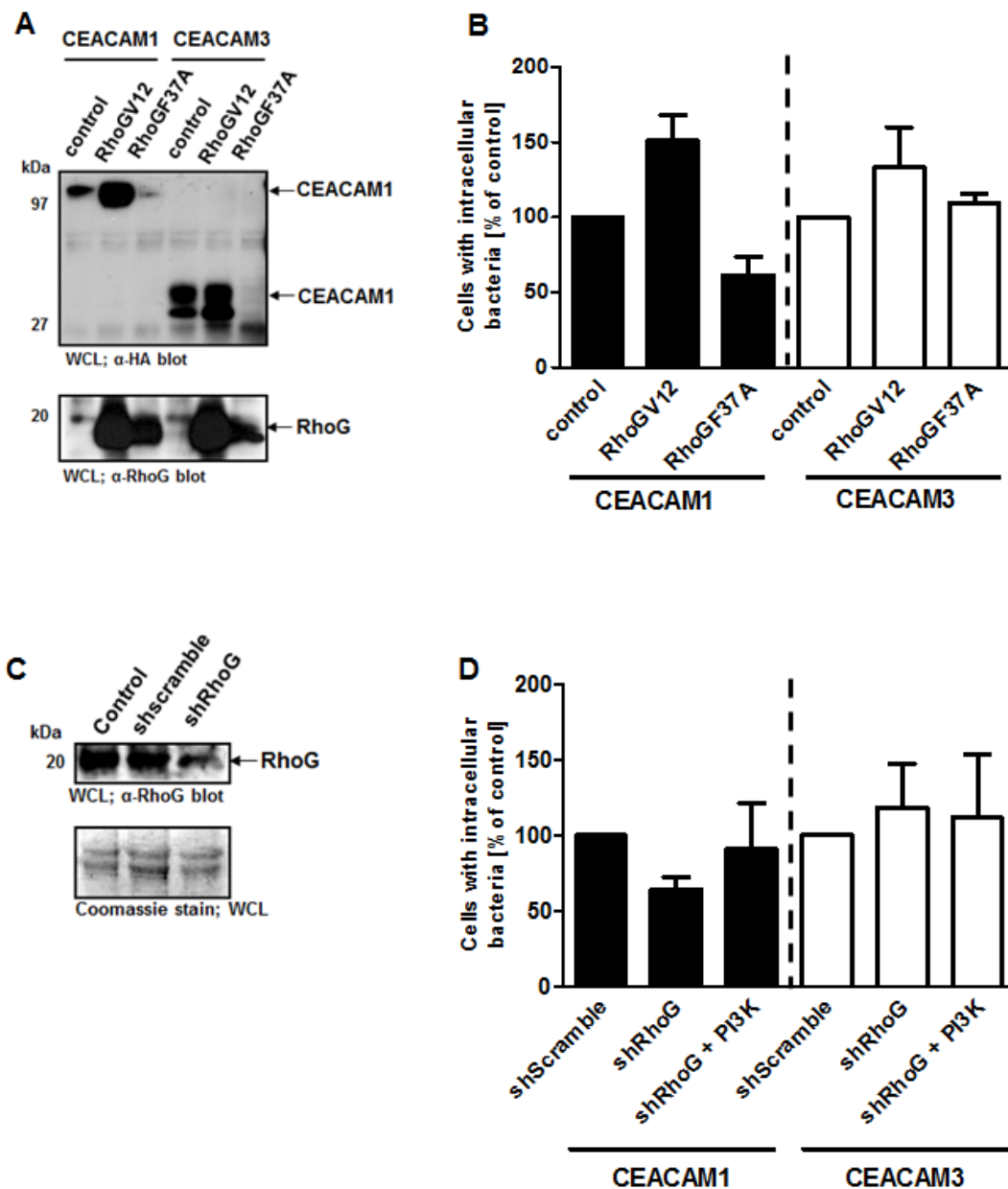


Fig. 27 RhoG is involved in CEACAM1- but not in CEACAM3-mediated uptake of *Neisseria gonorrhoeae*. (A) 293 cells were co-transfected with constructs encoding HA-tagged CEACAM1-4L or CEACAM3 together with myc-tagged constitutive active mutant of RhoG (RhoGV12), effector-binding mutant of RhoG (RhoGF37A) or empty vector control. CEACAM and RhoG expression was verified by Western blotting of whole cell lysates (WCL) using α -HA and α -RhoG antibody. (B) Cells were transfected as in (A) and infected for 2 h with fluorescein-labeled Opa_{CEA}-expressing gonococci. Samples were analyzed using a flow cytometer; the fluorescein signal was quantified in the presence of trypan blue, which quenches fluorescence derived from extracellular gonococci. Bars represent \pm SEM of three independent experiments. Control was set on 100% each with CEACAM1 or CEACAM3. (C) To generate stable cell lines, 293 cells were transduced with shScramble or shRhoG-containing virus or left without virus (control). Efficiency of the knockdown of endogenous RhoG was verified by Western

blotting of whole cell lysates (WCL) using α -RhoG antibody and equal loading was verified by Coomassie staining of the membrane after transfer. **(D)** Stable transduced 293 cell lines (shScramble and shRhoG) as described in **(C)** were co-transfected with constructs encoding HA-tagged CEACAM1-4L or CEACAM3 together with cerulean-tagged PI3K (shRhoG + PI3K) or with empty vector control and infected for 2 h with fluorescein-labeled Opa_{CEA}-expressing gonococci. Samples were analyzed using a flow cytometer; the fluorescein signal was quantified in the presence of trypan blue, which quenches fluorescence derived from extracellular gonococci. Bars represent \pm SEM of two independent experiments. Control was set on 100% each with CEACAM1 or CEACAM3.

To prove the possible linkage of PI3K dependency and the role of the small GTPase RhoG during bacterial uptake via CEACAM1, we co-transfected shRhoG treated cells with constructs encoding HA-tagged CEACAM1 or HA-tagged CEACAM3 together with cerulean-tagged PI3K. In shRhoG treated cells which express CEACAM1 and PI3K we could clearly demonstrate a rescue effect, the uptake efficiency was comparable to cells treated with shScramble as control (Fig. 27D). CEACAM3-mediated uptake of gonococci was not affected at all (Fig. 27D).

We can conclude that RhoG is very important for CEACAM1-mediated uptake of gonococci and is upstream of PI3K in the signaling pathway.

Ephexin4 is a further component of the signaling pathway in CEACAM1-mediated uptake of gonococci

Next, we were interested in guanine exchange factors (GEFs) which are involved in RhoG activation after binding of the bacteria to CEACAM1. There are several GEFs known which are linked to RhoG activation, like SGEF, Ephexin4, Kalirin, Dbs and PLEKGH6 (Ellerbroek, Wennerberg et al. 2004; Hiramoto-Yamaki, Takeuchi et al. 2010; Samson, Welch et al. 2010). Via lentiviral shRNA constructs against the different GEFs we generated several stable knockdown cell lines, which we transfected either with cerulean-tagged CEACAM1 or cerulean-tagged CEACAM3 to prove effect of GEF knockdown in bacterial uptake (data not shown). Cells were infected for 2 h with fluorescein-labelled Opa_{CEA}-expressing gonococci and, after washing, samples were

analyzed by gating on cerulean-positive cells during flow cytometry. Internalization of fluorescein-labeled bacteria was measured in the presence of trypan blue, which quenches fluorescein fluorescence derived from extracellular bacteria, and in the most cases no effect of bacterial uptake via CEACAMs in the different shRNA treated cells could be observed (data not shown).

However, downregulation of endogenous Ephexin4 led to a decreased uptake only via CEACAM1 by around 50% whereas CEACAM3-mediated internalization was not affected (Fig. 28B). Decreased level of endogenous Ephexin4 in 293 cells was verified via western blotting with antibodies against Ephexin4 and tubulin as loading control (Fig. 28A). Next, we wanted to examine, if Ephexin4 is also recruited to site of infection. We co-transfected 293 cells with constructs encoding GFP-tagged CEACAM1 or GFP-tagged CEACAM3 together with myc-tagged Ephexin4. After infection of the transfected cells with Pacific-Blue labeled Opa_{CEA}-expressing gonococci for 1 h, cells were fixed and permeabilized, followed by antibody-staining of Ephexin4. Samples were analyzed via confocal microscope and we could demonstrate clear recruitment of Ephexin4 to site of infection via CEACAM1 (Fig. 28C). Furthermore, no co-localization for CEACAM3 and Ephexin4 at site of bacteria-host interaction could be shown (Fig. 28C). These results suggest that Ephexin4 plays a role in CEACAM1- but not in CEACAM3-mediated uptake of gonococci and is recruited to site of infection in CEACAM1-transfected cells.

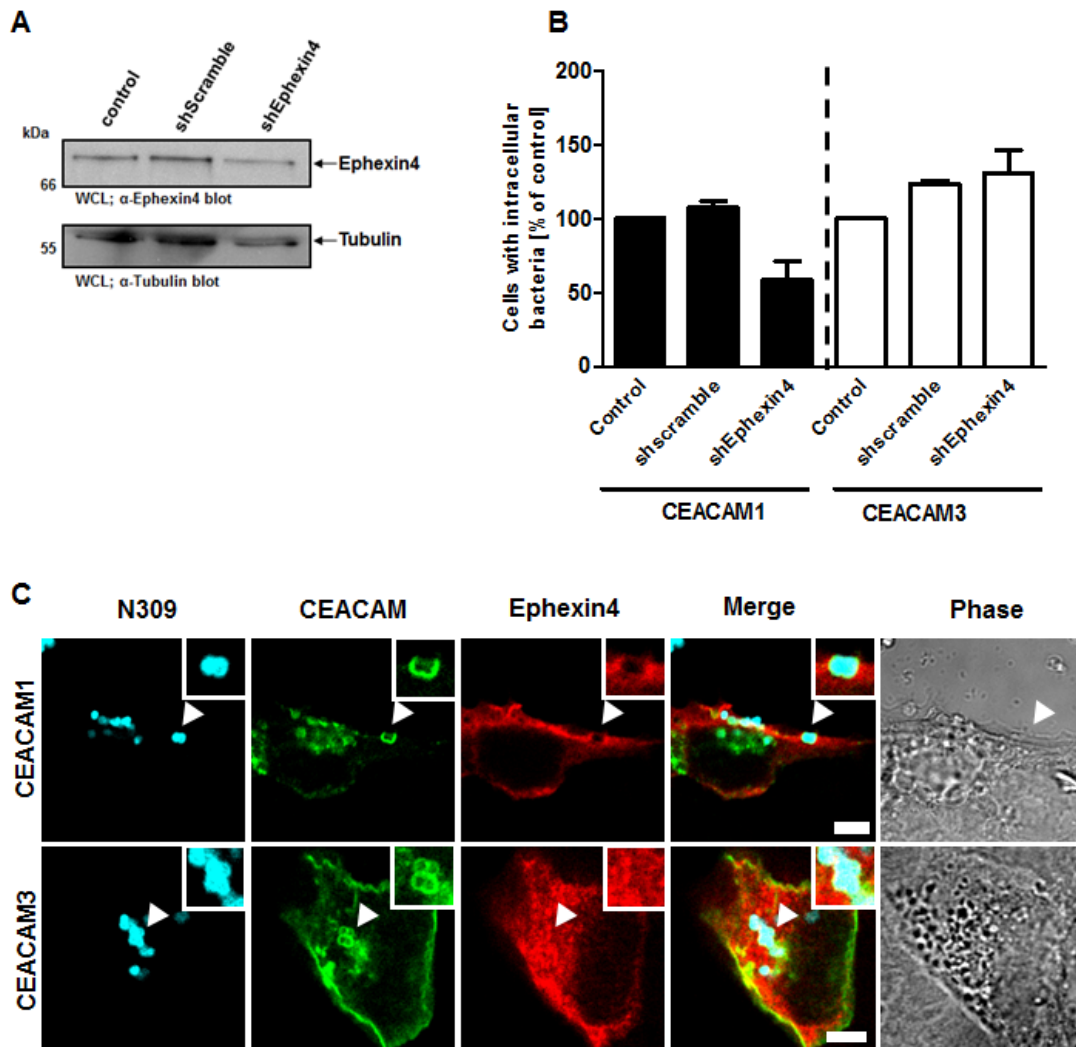


Fig. 28 RhoG guanine exchange factor Ephexin4 is involved in CEACAM1-mediated uptake of *Neisseria gonorrhoeae* and is recruited to site of infection. (A) To generate stable cell lines, 293 cells were transduced with shScramble or shEphexin4-containing virus or left without virus (control). Efficiency of the knockdown of endogenous Ephexin4 was verified by Western blotting of whole cell lysates (WCL) using antibodies against Ephexin4 and tubulin as loading control. (B) Stable transduced 293 cell lines (shScramble and shEphexin4) or cells left without virus (control) as described in (A) were transfected with constructs encoding cerulean-tagged CEACAM1-4L or CEACAM3 and infected for 2 h with fluorescein-labeled Opa_{CEA}-expressing gonococci. Samples were analyzed using a flow cytometer by gating on cerulean-positive cells; the fluorescein signal derived from cerulean-positive cells was quantified in the presence of trypan blue, which quenches fluorescence derived from extracellular gonococci. Bars represent \pm SEM of two independent experiments. Control was set on 100% each with CEACAM1 or CEACAM3. (C) 293 cells were co-transfected with constructs encoding GFP-tagged CEACAM1-4L or CEACAM3 together with myc-tagged Ephexin4. Cells were infected with Pacific Blue labeled Opa_{CEA}-expressing *N. gonorrhoeae* for 60 min, fixed and permeabilized, followed by antibody-staining of Ephexin4. Samples were analyzed by confocal microscopy and arrowheads highlight bacteria associated with CEACAM and Ephexin4. Insets show enlargement of highlighted area. Bars represent 5 μ m.

EphA2 co-localize with CEACAM1, 3 and CEACAM1 lacking extracellular Ig_{C2}-like domains during infection with *Neisseria gonorrhoeae*

Our previous and current findings show, that CEACAM1-mediated uptake of gonococci is PI3K- as well as RhoG-dependent. It is known, that RhoG can activate class I PI3K by direct interaction with the p85 subunit (Murga, Zohar et al. 2002). The involved guanosin exchange factor seems to be mainly Ephexin4, which is connected to PI3K and RhoG pathway during anoikis, regulated by the activation of the receptor tyrosine kinase EphA2 (Harada, Hiramoto-Yamaki et al. 2011). Activation of EphA2 leads to autophosphorylation of the cytoplasmic tyrosine motif, which in turn leads to recruitment and activation of Ephexin4, followed by activation of RhoG. A few years ago, another group could demonstrate, that expression of the EphA2 receptor is upregulated in epithelial cells after infection with Opa_{CEA}-expressing gonococci (Plant, Asp et al. 2004), so we were interested if this interaction of EphA2 with Ephexin4, RhoG and PI3K is also important for uptake of gonococci via CEACAM1. First of all, we investigated a possible co-localization of EphA2 receptor and CEACAM receptors during infection. We co-transfected 293 cells with constructs encoding GFP-tagged CEACAM1 or GFP-tagged CEACAM3 together with a construct encoding mCherry-tagged EphA2 receptor and infected the cells for 60 min with Pacific Blue- labeled Opa_{CEA}-expressing *Neisseria gonorrhoeae*. After fixation of the cells, the samples were analyzed by confocal microscopy (Fig. 29A). A strong co-localization of the CEACAM receptors together with EphA2 receptor to site of bacterial-host contact could be observed, in CEACAM1- as well as in CEACAM3-transfected cells (Fig. 29A). But the co-localization was also present at regions of the cells, where no bacteria were associated, so it could be also a general co-localization between EphA2 and CEACAM receptors (Fig. 29A). In a previous publication we could show, that the extracellular Ig_{C2}-like domains of CEACAM1 are responsible for the PI3K dependency during CEACAM1, CEA and CEACAM6-

mediated uptake of gonococci (Voges, Bachmann et al. 2012). We were speculating of an unknown surface receptor interacting with the extracellular Ig_{C2}-like domains, responsible for signal transduction into the cells, because for CEACAM1 the cytoplasmic domain is not necessary for internalization and CEACAM6 and CEA are only anchored in the membrane via GPI (Muenzner, Bachmann et al. 2008) (Gray-Owen and Blumberg 2006). So we generated stable transduced MCF7 cells expressing either only lentiviral GFP construct as control, a lentiviral vector expressing GFP-tagged CEACAM1-4L or GFP-tagged CEACAM1 mutant lacking the immunoglobulin constant domains (CEACAM1-N-TM). Expression of the CEACAMs was verified via western blotting with anti-CEACAM antibody (Fig. 31A). Furthermore, these cells were additionally transfected with a construct expressing mCherry-tagged EphA2 receptor, followed by infection with Pacific Blue-labeled Opa_{CEA}-expressing *Neisseria gonorrhoeae* for 60 min. After fixation of the cells, samples were analyzed by confocal microscopy (Fig. 29B). Whereas the GFP control was evenly distributed all over the cytoplasm in the cell, a strong recruitment of CEACAM1 or CEACAM1-N-TM together with EphA2 receptor to site of infection could be observed (Fig. 29B). Altogether, the expression of GFP-tagged CEACAM1 or CEACAM1-N-TM was very weak in these cells and could be observed only high concentrated after strong recruitment at site of infection via confocal microscopy, admitting no assumption about co-localization independent of infection in these cells (Fig. 29B).

Together, EphA2 is generally recruited to site of infection, independent of extracellular Ig_{C2}-like extracellular domains and tentatively the co-localization is independent of infection but is present in general in cellular context.

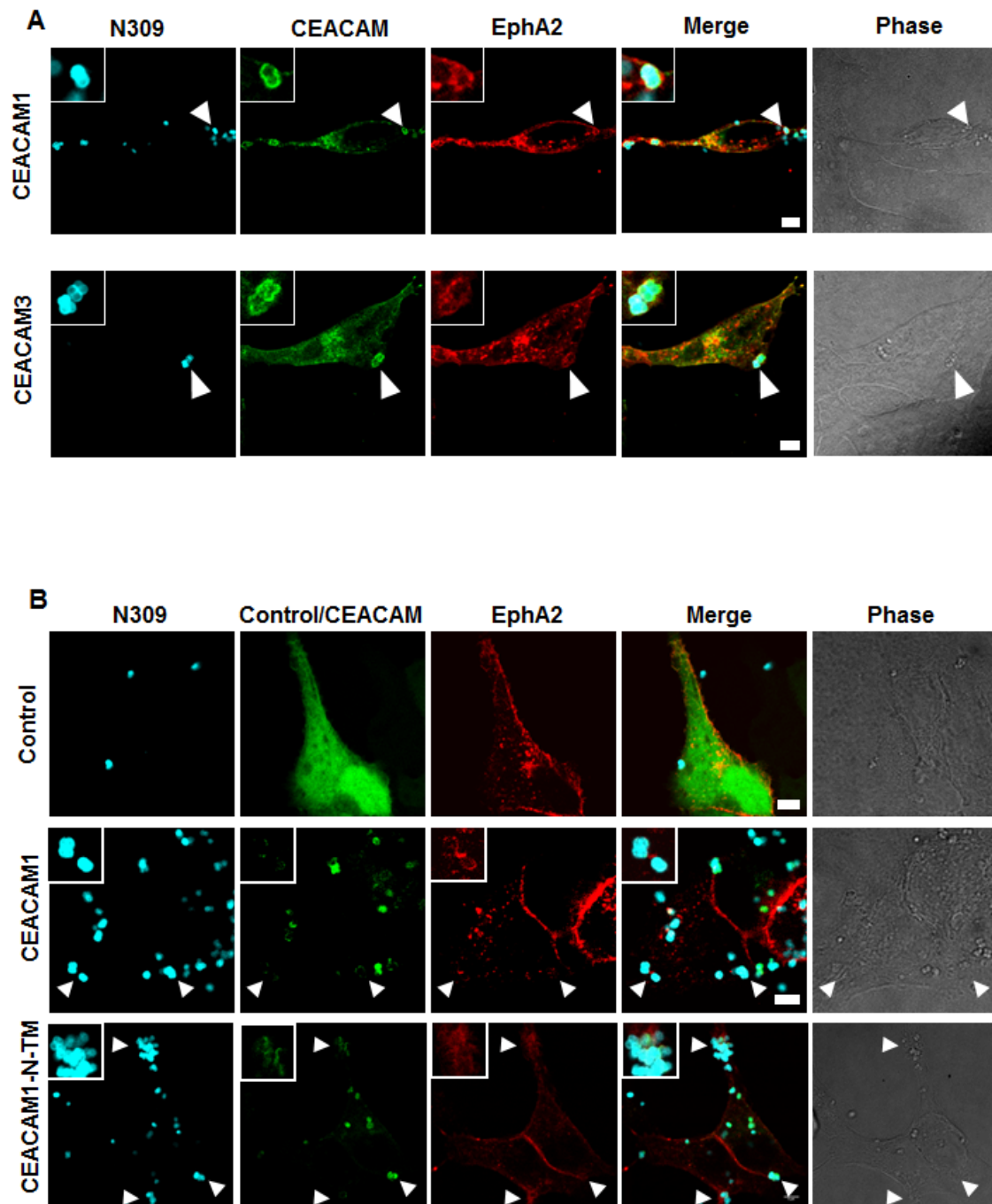


Fig. 29 EphA2 receptor tyrosine kinase co-localize with CEACAM receptors in the membrane and is recruited during uptake of *Neisseria gonorrhoeae* to site of infection. **(A)** 293 cells were co-transfected with constructs encoding GFP-tagged CEACAM1-4L or CEACAM3 together with mCherry-tagged EphA2. Cells were infected with Pacific Blue labeled Opa_{CEA}-expressing *N. gonorrhoeae* for 60 min, fixed and analyzed by confocal microscopy. Arrowheads highlight bacteria associated with CEACAM1 and EphA2. Insets show enlargement of highlighted area. Bars represent 5 μ m. **(B)** To generate stable cell lines, MCF-7 cells were transfected with GFP-CEACAM1-4L, GFP-CEACAM1 lacking the Ig_{C2}-like domains (N-TM) or only GFP (control)-containing virus. Stable transfected cells were infected with Pacific Blue labeled Opa_{CEA}-expressing *N. gonorrhoeae* for 60 min, fixed and analyzed by confocal microscopy. Arrowheads highlight bacteria associated with CEACAM and EphA2. Insets show enlargement of highlighted area. Bars represent 5 μ m.

Knockdown of EphA2 leads to decreased internalization via CEACAM1 and can be rescued by overexpression of constitutive active RhoG

For further clarification of the involvement of EphA2 during CEACAM1-mediated uptake of gonococci we generated 293 cells with stable knockdown of endogenous EphA2 via lentiviral shRNA construct. Efficiency of the downregulation of endogenous EphA2 was verified via western blotting with antibodies against EphA2 and tubulin as loading control (Fig. 30A). Additionally, we co-transfected these cells with constructs encoding HA-tagged CEACAM1 or CEACAM3 followed by infection with Opa_{CEA}-expressing *N. gonorrhoeae* for 2 hours. Afterwards, cells were washed and total cell associated bacteria were enumerated by plating dilutions on selective media (adherence assay). Parallel samples were treated with gentamicin for 1 hour to kill extracellular bacteria, and the intracellular bacteria were released by mild detergent lysis of the eukaryotic cells (invasion assay). The results were calculated, implicating the amount of all cell-associated bacteria to consider differences caused by unequal transfection efficiency (Fig. 30B). Uptake of gonococci via CEACAM1 was decreased by around 50% in shEphA2 treated cells compared to cells which were only treated with positive control shScramble, whereas uptake of bacteria in CEACAM3-expressing shEphA2 treated cells was not affected compared to shScramble control (Fig. 30B). To determine our hypothesis, that EphA2 receptor activates RhoG, we co-transfected the shEphA2 treated cells with constructs encoding HA-tagged CEACAM1 or HA-tagged CEACAM3 together with a construct encoding myc-tagged constitutive active mutant of RhoG (RhoGV12). Expression was verified via western blotting with antibodies against CEACAM and myc-Tag (Fig. 30C). Cells were infected with Opa_{CEA}-expressing gonococci for 2h, and adherence and invasion assay followed by calculation of the results was performed as described before. Again, uptake of pathogens via CEACAM1 was decreased by around 50% in shEphA2 treated cells compared to shScramble control, but

co-expression of the constitutive active mutant of RhoG (RhoGV12) led to entire rescue of the observed effect (Fig. 30D). Like before, internalization via CEACAM3 in shEphA2 treated cells was not affected, and co-expression of the constitutive active RhoG led even to reduced uptake of gonococci (Fig. 30D). These results led us suggest that EphA2 is important for efficient internalization of *Neisseria gonorrhoeae* via CEACAM1 and seems to be upstream connected to RhoG during the process of bacterial uptake.

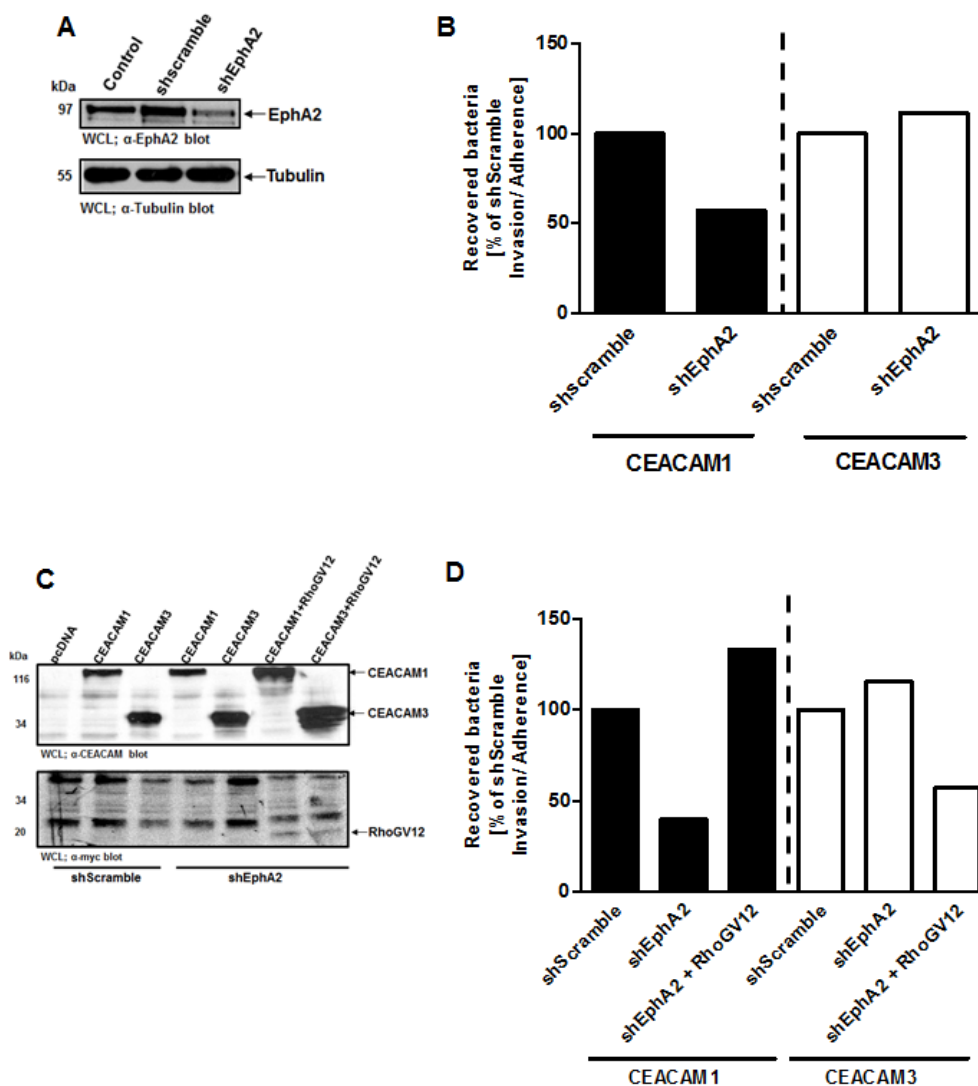


Fig. 30 EphA2 receptor tyrosine kinase is involved in CEACAM1-mediated uptake of *Neisseria gonorrhoeae* and functions upstream of RhoG. (A) To generate stable cell lines, 293 cells were transduced with shScramble or shEphA2-containing virus or left without virus (control). Efficiency of the knockdown of endogenous EphA2 was verified by Western blotting of whole cell lysates (WCL) using antibodies against EphA2 and tubulin as loading control. (B) 293 cells transduced with shScramble or shEphA2-containing virus were transfected with con-

structs encoding HA-tagged CEACAM1-4L or CEACAM3 and infected for 2 h with Opa_{CEA}-expressing *N. gonorrhoeae*. The number of total cell-associated bacteria and viable intracellular bacteria (gentamicin protection assay) were determined and results were calculated by implicating both values to consider differences caused by unequal transfection efficiency. Bars represent \pm SEM of one representative experiment. shScramble treated sample was set on 100% each with CEACAM1 or CEACAM3. **(C)** 293 cells transduced with shScramble or shEphA2-containing virus were co-transfected with constructs encoding HA-tagged CEACAM1-4L or CEACAM3 together with myc-tagged constitutive active mutant of RhoG (RhoGV12) or empty control vector. CEACAM and RhoG expression was verified by Western blotting of whole cell lysates (WCL) using α -CEACAM and α -myc antibody. **(D)** Stable transduced cells were transfected as in **(C)** and infected for 2 h with Opa_{CEA}-expressing *N. gonorrhoeae*. The number of total cell-associated bacteria and viable intracellular bacteria (gentamicin protection assay) were determined and results were calculated by implicating both values to consider differences caused by unequal transfection efficiency. Bars represent \pm SEM of one representative experiment. shScramble treated sample was set on 100% each with CEACAM1 or CEACAM3.

Chemically inhibition of EphA2 receptor leads to decreased bacterial uptake via CEACAM1

In 2010 a group from Moscow published five different novel fragment-like inhibitors (Stroylov, Rakitina et al. 2010) of EphA2 kinase activity. We resynthesized the compound 2 (5.8 Synthesis of 4-(3,4-dihydroxyphenyl)thiazol-2-aminium bromide) and tested the effect of this compound on bacterial uptake via CEACAM receptors. First we used the previous described stable transduced MCF7 cells expressing GFP construct as control, CEACAM1-GFP or CEACAM1 lacking the Ig_{C2}-like domains (N-TM)-GFP construct (Fig. 31A) and pretreated the cells with two different concentrations of the EphA2 kinase inhibitor 30 min before infection with Opa_{CEA}-expressing *Neisseria gonorrhoeae*. After 2 h, cells were washed and total cell adhesion was determined by plating dilutions on selective media (adherence assay, Fig. 31B). Parallel samples were treated with gentamicin for 1 h to kill extracellular bacteria, followed by release of the bacteria from cells by mild detergents (invasion assay; Fig. 31C). For CEACAM1, a slight increase in associated bacteria could be observed after treatment with 30 μ M of the inhibitor, although the uptake of gonococci was significantly decreased in a concentration-dependent manner. This result matches very well to the observation, that knock-down of EphA2 in cells leads to an increased amount of CEACAMs on the surface (Fig.

32). In this approach we transfected shScramble- or shEphA2 treated 293 cells with constructs encoding cerulean as control, cerulean-tagged CEACAM1 or CEACAM3. After 48 h, extracellular CEACAM was stained with antibody against CEACAM and fluorescence was measured at flow cytometer, gating on cerulean positive cells to get only the transfected cells. For CEACAM1-N-TM no effect of the inhibitor could be observed on total cell association and on uptake (Fig. 31B and C). But compared to MCF7 cells without CEACAM on the surface (control), almost no difference of bacterial uptake could be shown, so most of the bacterial uptake in CEACAM1-N-TM cells seems to be CEACAM1-independent. Because of the high amount of CEACAM-independent bacterial uptake we used again 293 cells, which we transfected with constructs expressing CEACAM1, CEACAM1 lacking cytoplasmic domain (Δ CT) and CEACAM3. After treatment with 50 μ M of inhibitor for 30 min, cells were infected with Opa_{CEA}-expressing gonococci for 2 h and the same procedure as described before was performed (adherence assay & invasion assay). Expression of CEACAM1, CEACAM1 Δ CT and CEACAM3 was proved via western blot (Fig. 31D). Bacterial uptake via CEACAM1 and CEACAM1 Δ CT was strongly decreased after treatment with inhibitor, whereas for CEACAM3 no effect of the inhibitor on bacterial uptake occurred (Fig. 31F). Total cell association was not influenced by EphA2 inhibitor treatment of the cells (Fig. 31E).

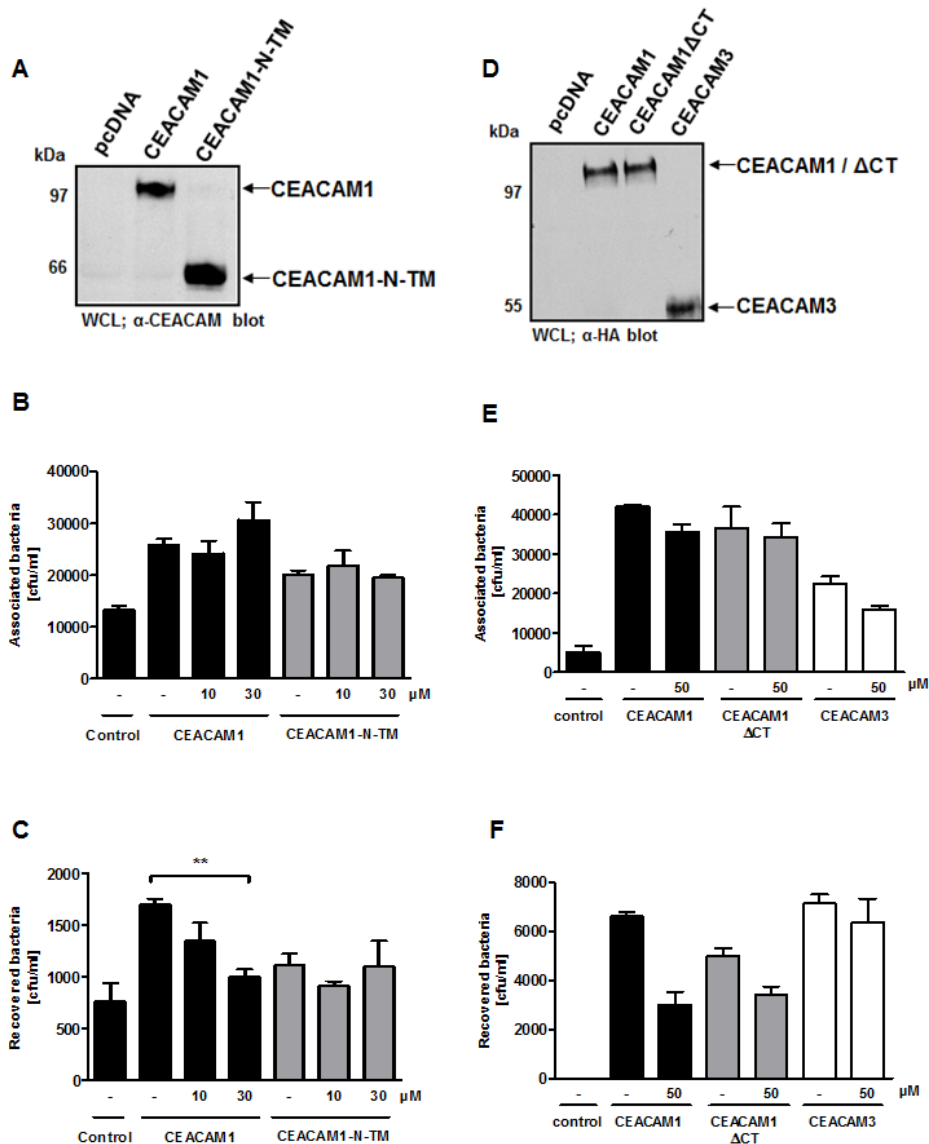


Fig. 31 Extracellular Ig_{C2}-like domains and not the cytoplasmic domain are responsible for EphA2 involvement during pathogen uptake via CEACAM1. (A) MCF-7 cells were stable transduced with GFP-CEACAM1-4L, GFP-CEACAM1 lacking the Ig_{C2}-like domains (N-TM) or only GFP (control)-containing virus. Expression of CEACAM1-4L and CEACAM1-N-TM was verified by Western blotting using antibody against CEACAM. (B) Stable transduced MCF-7 cells described in (A) were pretreated for 30 min with two different concentrations of EphA2 kinase activity inhibitor or with DMSO as control. After infection for 2 h with Opa_{CEA}-expressing gonococci, the number of total cell-associated bacteria was determined. Bars represent mean value \pm SEM of two experiments, done in triplicate. (C) Cells were infected and pretreated as in (B). Viable intracellular bacteria were determined in gentamicin protection assay. Bars represent mean value \pm SEM of two experiments, done in triplicate. Significance was tested using an unpaired, two-sided Student's t-test, **, $p < 0.01$. (D) 293 cells were transfected with constructs expressing HA-tagged CEACAM1-4L, CEACAM1 missing the cytoplasmic domain (Δ CT), CEACAM3 or pcDNA as empty vector control. Expression of CEACAMs was verified by Western blotting with antibody against HA. (E) Cells were transfected as in (D) and were pretreated for 30 min with 50 μ M of EphA2 kinase activity inhibitor or with DMSO as control. After infection for 2 h with Opa_{CEA}-expressing gonococci, the number of total cell-associated bacteria was determined. Bars represent mean value \pm SEM of two experiments, done in triplicate. (F) Cells were transfected, infected and pretreated as in (E). Viable intracellular bacteria

were determined in gentamicin protection assay. Bars represent mean value \pm SEM of one experiments, done in triplicate.

These results support the idea that uptake of gonococci via CEACAM1 is regulated via activation of EphA2 as co-receptor, which activates Ephexin4, RhoG and the PI3K, finally leading to endocytosis of the whole EphA2 – CEACAM – pathogen complex, whereas for CEACAM3 the activation of EphA2 is not required for efficient uptake of pathogens.

A

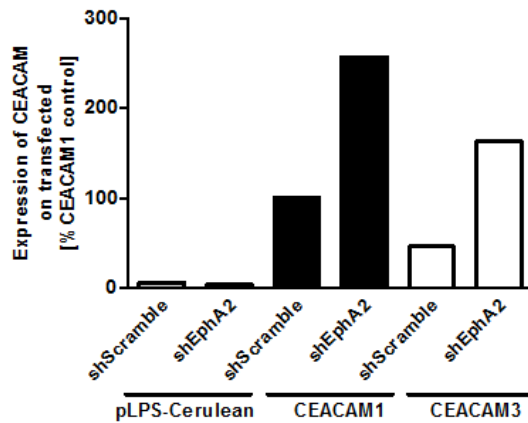


Fig. 32 Downregulation of endogenous EphA2 leads to increased surface levels of CEACAM1 and CEACAM3. (A) Stable transduced 293 cells with shScramble or shEphA2-containing virus were transfected with constructs expressing cerulean-tagged CEACAM1-4L, CEACAM3 or empty vector control (pLPS-Cerulean). Samples were analyzed using flow cytometry by gating on cerulean positive cells. By staining with CEACAM antibody, CEACAM receptors localized extracellular on the surface of cells were detected. The staining signal was quantified only in cerulean positive cells. Bars represent mean value of one experiment after analysis of 10,000 cells.

7.5 Discussion

Several human restricted bacteria are using CEACAM receptors on epithelial cells for internalization and host cell attachment (Kuespert, Pils et al. 2006; Muenzner, Bachmann et al. 2010; Voges, Bachmann et al. 2010). The signal transmission inside cells after bacterial binding via epithelial CEACAMs, namely CEACAM1, CEA and CEACAM6, was unclear until now, because of the further observation that the cytoplasmic domain of CEACAM1 is not required for bacterial uptake (Muenzner, Bachmann et al. 2008) and besides, CEA and CEACAM6 are only linked via GPI anchor into the membrane. Recently, we made the surprising observation that the extracellular Ig_{C2}-like domains of epithelial CEACAMs are responsible for PI3K dependency during pathogen uptake (Voges, Bachmann et al. 2012). Here, we demonstrate that the small GTPase RhoG is recruited to site of infection, and overexpression of an effector-binding mutant of RhoG or downregulation of endogenous RhoG via shRNA results in decreased bacterial uptake via CEACAM1, but not via CEACAM3. Overexpression of PI3K in the shRhoG treated cells could rescue the effect on bacterial uptake via CEACAM1. The shRNA-based screen of different RhoG-GEFs pointed out the role of Ephexin4, which is also localized at site of infection. Finally, we could demonstrate the involvement of the receptor tyrosine kinase (RTK) EphA2 in uptake of bacteria via CEACAM1, most likely by its functions as co-receptor for bacterial uptake via epithelial CEACAMs through activation of RhoG guanine exchange factor Ephexin4, which catalyze GTP exchange of the small GTPase RhoG followed by activation of the PI3K in a direct way. RTKs are promising candidates to act as co-receptor, because they contain a lot of protein-protein interaction domains in their extracellular part, for example immunoglobulin domains, fibronectin type III domains or cadherins (Lemmon and Schlessinger 2010). It is also known, that RTK c-Met function as receptor for uptake of

Listeria monocytogenes, which triggers a PI3K-dependent uptake pathway (Ireton, Payrastra et al. 1996; Cossart 2001). In general, small GTPases are linked to vesicular transport, actin cytoskeleton and microtubule remodeling, nuclear transport, gene expression and survival (for reviews (Zerial and Stenmark 1993; Sazer and Dasso 2000; Stenmark and Olkkonen 2001; Takai, Sasaki et al. 2001; Karnoub and Weinberg 2008)(Aspenstrom, Fransson et al. 2004; D'Souza-Schorey and Chavrier 2006). Especially the Rac subfamily of the Ras superfamily is linked to endocytotic processes by its influence on actin filaments and microtubules, and RhoG contains to the Rho family inside the Rac subfamily (Etienne-Manneville and Hall 2002). The cellular functions of RhoG are not well understood until now, but most of the RhoG dependent processes are linked to Rac1 activation via ELMO2-DOCK complex (Katoh and Negishi 2003; Ho and Dagnino 2011). The small GTPase Rac1 was shown to be very important for CEACAM3-mediated uptake of gonococci (Billker, Popp et al. 2002; Schmitter, Pils et al. 2007; Pils, Kopp et al. 2012), based on its ability to promote actin cytoskeleton rearrangements leading to phagocytosis and its role in oxidative burst by activation of the NADPH oxidase (Diekmann, Abo et al. 1994; Dinauer 2003; Niedergang and Chavrier 2005). In fact, CEACAM3 activation via bacterial binding leads to massive lamellipodial protrusions on the cell surface in a PI3K independent manner (Billker, Popp et al. 2002; Schmitter, Agerer et al. 2004; Buntru, Kopp et al. 2011; Pils, Kopp et al. 2012) because of the direct association with GEF Vav to promote GTP loading of the small GTPase Rac1, bypassing requirement for PI3K activity during phagocytosis (Schmitter, Pils et al. 2007; Buntru, Kopp et al. 2011). Although actin cytoskeleton remodeling plays only a minor role during bacterial uptake via epithelial CEACAMs (Muenzner, Bachmann et al. 2008) we observed involvement of Rac1 in CEACAM1-mediated uptake. Rac1 is not only linked to actin cytoskeleton rearrangements, it has also an important role in regulating microtubule destabilization by modulating of p21-activated

kinases (PAK), followed by activation of Op18/stathmin, which destabilize microtubules by sequestering tubule dimers (Wittmann, Bokoch et al. 2004). It could be shown, that microtubule are very important for uptake of Afa/Dr expressing *Escherichia coli* via epithelial CEACAMs (Guignot, Hudault et al. 2009) and we also observed requirement of microtubule dynamics during internalization of *Neisseria gonorrhoeae* via epithelial CEACAMs (unpublished data) in contrast to CEACAM3-mediated uptake of pathogens, which is completely independent of microtubules. Anyway, Rac1 activation seems not to be the only explanation for RhoG influence on bacterial uptake via epithelial CEACAMs, because overexpression of an effector-binding mutant of RhoG or knockdown of endogenous RhoG did not influence CEACAM3-mediated uptake. Recently, a Rac1-independent pathway for RhoG was discovered: Activation of the receptor tyrosine kinase EphA2 stimulates the guanine exchange factor Ephexin4 (also known as ARHGEF16). A known substrate for Ephexin4 is RhoG, catalyzing the nucleotide exchange from GDP to GTP (Hiramoto-Yamaki, Takeuchi et al. 2010). This leads to conformational changes in RhoG following of effector binding and activation of downstream signaling pathways. On the one hand, activation of ELMO2-Dock complex is described, which leads to Rac1 activation, but on the other hand, it could be shown that RhoG can directly bind to the p85 subunit of class I PI3K leading to activation (Katoh and Negishi 2003; Hiramoto-Yamaki, Takeuchi et al. 2010; Harada, Hiramoto-Yamaki et al. 2011). This matches to our further findings of PI3K dependency during uptake of pathogens via epithelial CEACAMs and so we were interested in the role of Ephexin4 and EphA2 in bacterial uptake. As mentioned before, we performed a small screen of known GEFs for RhoG via shRNA downregulation, and Ephexin4 was one of the most promising candidates, because of its influence on bacterial uptake only via CEACAM1 and not via CEACAM3. So the next step was to prove the role of the upstream regulator of Ephexin4, the receptor tyrosine kinase EphA2, dur-

ing pathogen uptake via epithelial CEACAMs. Knockdown of EphA2 receptor via shRNA and chemical inhibition leads to reduced bacterial uptake via CEACAM1 and CEACAM1 missing the cytoplasmic domain (Δ CT), whereas for CEACAM3 and CEACAM1 missing the extracellular domains (N-TM) no effect was observed. The cytoplasmic domain of CEACAM1 is not involved in bacterial uptake (Muenzner, Bachmann et al. 2008) and so we could demonstrate, that it is also not involved in activation of the possible co-receptor EphA2, but the extracellular immunoglobulin-like domains of CEACAM1 seems to be required for effective uptake connected with EphA2 receptor. Overexpression of constitutive active mutant of RhoG in shEphA2 treated cells could rescue the decreased bacterial uptake, which facilitate our model of upstream regulation of RhoG via EphA2 receptor and Ephexin4. EphA2 is a promising candidate as co-receptor, because of its expression in epithelial cells and localization in lipid rafts after activation (like CEACAM1) (Wykosky and Debinski 2008; Bocharov, Mayzel et al. 2010). The extracellular domains of Eph receptors contain a ligand binding domain, a cysteine rich region and two fibronectin (FN) type III domains (Pitulescu and Adams 2010). FN type III domains are known to associated with immunoglobulin domains (Barclay 2003), leading us to the speculation that activation of EphA2 by interaction with extracellular immunoglobulin constant type2-like (Ig_{C2}) domains of epithelial CEACAMs is responsible for the further observed PI3K dependency during pathogen uptake. Another hint of EphA2 involvement in bacterial uptake comes from a study from 2004, where expression of different genes in epithelial cells after infection with *Neisseria* was investigated, and EphA2 appeared as one of the upregulated genes (Plant, Asp et al. 2004). Eph receptors are in general very important for tissue assembly and in maintaining tissue homeostasis, especially for embryonic development including axon guidance, cell migration and segmentation (reviewed in (Pitulescu and Adams 2010)). Activation of Eph receptors by binding of ephrin ligands (and possibly by *cis-*

interaction with Ig_{C2}-like domains of CEACAMs) leads to clustering of Eph receptors and transphosphorylation of the cytoplasmic domains together with often associated Src-family kinases or GEFs like Vav and Ephexin4 (Hunter, Zhuang et al. 2006; Harada, Hiramoto-Yamaki et al. 2011). EphA2 is an exception within its family, because kinase activity is not crucial controlled via activation by extracellular stimuli (Wykosky and Debinski 2008). To terminate signaling, one of the possible mechanism is endocytosis of the intact ligand-EphA2 complex. This internalization complex contains also cytoplasmic proteins together with surrounding plasma membrane (Pitulescu and Adams 2010) and it could be possible, that the interaction between EphA2 and epithelial CEACAMs leads to co-internalization of both surface proteins. The higher CEACAM surface levels in the shEphA2 treated cells promote this theory. Furthermore, the general co-internalization without stimulus seems not to be regulated via the extracellular Ig_{C2}-like domains of CEACAMs, because of the higher CEACAM3 levels on surface of the shEphA2 treated cells, which do not contain any Ig_{C2}-like domains. In this case the signal for internalization could be regulated by interaction between EphA2 and its ligand EphrinA1, which leads also to internalization of EphA2 together with surrounding membrane. We are speculating that there is in general a stable co-localization of CEACAM receptors and EphA2, but increased activation and internalization of the complex after pathogen binding can only occur with epithelial CEACAMs because of their Ig_{C2}-like domains. The internalization of EphrinB1 is connected to classical clathrin-dependent endocytosis, regulated by Rac1 activation (Marston, Dickinson et al. 2003), which is not the endocytotic pathway responsible for epithelial CEACAM internalization, but it could be shown that EphB1 and EphA2 contain a caveolin-1 binding motif and both of them could be localized in caveolae (Vihanto, Vindis et al. 2006). Endocytosis of Eph receptors (especially EphA2) is PI3K dependent, shown by recruitment of SHIP-2, a 5' phosphatase of PI(3,4,5)P via the SAM domain of EphA2

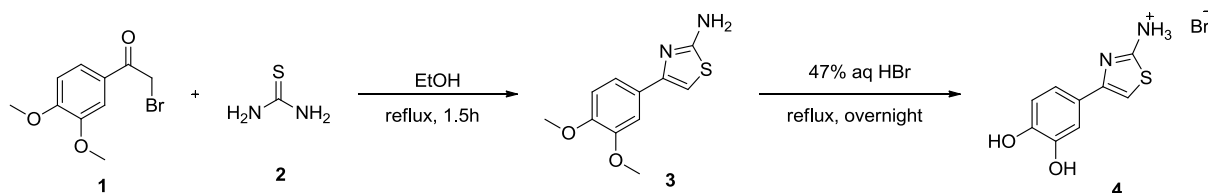
(Zhuang, Hunter et al. 2007). To sum up, there is more than one way of internalization of Eph receptors and further investigation has to be done to get more insight in these processes. There is also a possibility of an interaction between EphA2 and CEACAM1 in a more cellular context independent of bacterial internalization, because both receptors are involved in angiogenesis (Wykosky and Debinski 2008; Herbert, Huisken et al. 2009; Pitulescu and Adams 2010) (Ergun, Kilik et al. 2000; Wagener and Ergun 2000) (Kuespert, Pils et al. 2006). Furthermore, other groups linked EphA2 to uptake of pathogens, like *Kaposi's sarcoma associated herpesvirus* (KSHV), which activates EphA2 receptor during invasion via integrins to amplify KSHV-induced Src- and PI3K signals in lipid rafts (Chakraborty, Veettil et al. 2012). For *Hepatitis C* virus it is known, that EphA2 regulates the CD81-claudin-1 co-receptor association and viral-glycoprotein-dependent membrane fusion during clathrin-dependent internalization of the virus (Lupberger, Zeisel et al. 2011). The paramyxoviridae *Nipah* and *Hendra* viruses bind directly to EphrinB2 (Bonaparte, Dimitrov et al. 2005; Negrete, Levroney et al. 2005), and for *Mycobacterium tuberculosis* regulation of the T-cell response by binding and activation of EphA2 via unknown surface proteins was observed (Khounlotham, Subbian et al. 2009).

Together, in our studies we could demonstrate that the receptor tyrosine kinase EphA2 is involved in CEACAM1-mediated uptake of bacteria and is regulated via the extracellular Ig_{C2}-like domains, which matches perfectly to our previous findings (Voges, Bachmann et al. 2012). We are speculating that activation of epithelial CEACAMs via pathogens (or other factors) leads to a *cis*-interaction with EphA2 receptors in close proximity, followed by activation of EphA2 kinase activity. Phosphorylation of the cytoplasmic domain of EphA2 leads to recruitment and activation of the guanine exchange factor Ephexin4, which catalyze the nucleotide exchange of the small GTPase RhoG. Afterwards RhoG-GTP binds and activates PI3K in a direct way, leading to generation

of PI(3,4,5)P and internalization of the whole EphA2-CEACAM-pathogen complex in a microtubule-dependent endocytotic process.

Certainly, the signaling pathways during pathogen uptake via epithelial CEACAMs interacting with EphA2 should be clarified in more detail, and it would be also very interesting to investigate the role of EphA2 and CEACAM1 interaction in cellular context, for example in lumen formation of breast epithelial cells or angiogenesis to get more insight into the physiological function of CEACAM1-EphA2 interaction.

7.6 Synthesis of 4-(3,4-dihydroxyphenyl)thiazol-2-aminium bromide



General

^1H and ^{13}C NMR spectra were recorded on Avance 400 NMR spectrometer from Bruker at room temperature and the chemical shifts are reported relative to the residual solvent peak. The ESI mass spectrum was obtained with the Esquire 3000 plus from Bruker Daltronics and for the HRMS spectrum the microTOF-QII ESI-Qq-TOF from Bruker Daltronics was used. The reported yield refers to the analytically pure substance and is not optimized.

Synthesis

The preparation of 4-(3,4-dihydroxyphenyl)thiazol-2-aminium bromide 4 is related to methods described in literature (Suter and Johnson 1930; Wang, Chai et al. 2008).

2-Bromo-1-(3,4-dimethoxyphenyl)ethanone **1** (603 mg, 2.3 mmol) and thiourea **2** (212 mg, 2.8 mmol) were suspended in 15 ml ethanol and subsequently refluxed for 1.5h. After cooling to room temperature the precipitated 4-(3,4-dimethoxyphenyl)thiazol-2-amine **3** was isolated by filtration (540 mg, 2.3 mmol, quant., off-white solid).

¹H NMR (400 MHz, DMSO): δ [ppm] = 8.77 (s, broad, NH₂), 7.31 (d, J = 2.1 Hz, 1H), 7.27 (dd, J = 8.3, 2.1 Hz, 1H), 7.15 (s, 1H), 7.06 (d, J = 8.4 Hz, 1H), 3.83 (s, 3H), 3.80 (s, 3H)

4-(3,4-dimethoxyphenyl)thiazol-2-amine **3** (100 mg, 0.42 mmol) was suspended in 10 ml 47% *aq* HBr and heated to reflux overnight. The precipitate was separated, washed with 47% *aq* HBr and dried over P₂O₅ to yield 4-(3,4-dihydroxyphenyl)thiazol-2-aminium bromide **4** (70 mg, 0.24 mmol, 58%) as an off-white solid.

¹H NMR (400 MHz, MeOD) δ [ppm] = 7.09 (d, J = 1.8 Hz, 1H), 7.01 (dd, J = 8.3, 1.7 Hz, 1H), 6.90 (d, J = 8.2 Hz, 1H), 6.82 (s, 1H).

¹³C NMR (101 MHz, MeOD) δ [ppm] = 172.51 (s), 148.77 (s), 147.24 (s), 141.09 (d, J = 11.1 Hz), 121.20 (s), 119.06 (s), 116.77 (s), 114.19 (s), 100.58 (s).

MS (ESI, positive mode): [M-Br⁻]⁺

m/z calc. for C₉H₉N₂O₂S: 209.04

m/z found: 209.1

HRMS: [M-Br⁻]⁺

m/z calc. for C₉H₉N₂O₂S: 209.0379, 210.0412, 211.0337

m/z found: 209.0384, 210.0412, 211.0362

7.7 Acknowledgments

We thank T.F. Meyer (MPI for Infection Biology, Berlin, Germany) and A. Unkmair (Universität Würzburg, Germany) for the bacterial strains used in this study. We are grateful to T. Balla (NIH, Bethesda, MD), J. Downward (Cancer Research UK, London, UK), G. Krystal (British Columbia Cancer Agency, Vancouver, Canada), B.G. Neel (University of Toronto, Toronto, Canada) and H. Katoh (University of Kyoto, Japan) for supplying different cDNA constructs used in this study, Eva Martin and Konstantin Bode for working under supervision on this project, and R. Hohenberger-Bregger, S. Feindler-Boeckh and Petra Zoll-Kiewitz for expert technical assistance. This study was supported by funds from the Deutsche Forschungsgemeinschaft (Ha2856/6-1) to C.R.H.

7.8 Author Contributions

Conceived and designed the experiments: MV VB CRH. Performed the experiments: MV VB JB. Analyzed the data: MV CRH. Wrote the paper: MV CRH.

8 GENERAL DISCUSSION

Some members of the human CEACAM family serve as host receptors for several human-restricted, gram-negative, mucosa associated pathogens like *Neisseria gonorrhoeae*, *N. meningitidis*, *N. lactamica*, *N. subflavia*, *Moraxella catarrhalis*, *Haemophilus influenzae* and *Escherichia coli* (Leusch, Drzeniek et al. 1991; Virji, Makepeace et al. 1996; Chen, Grunert et al. 1997; Hill, Toleman et al. 2001; Toleman, Aho et al. 2001; Hauck and Meyer 2003; Hill and Virji 2003; Gray-Owen and Blumberg 2006). These pathogens express distinct afimbrial adhesins to associate with the CC'-FG interface of the N-terminal immunoglobulin variable-like domains of human CEACAM1, CEACAM3, CEACAM6, and CEA leading to internalization into mammalian cells. Although all CEACAMs belong to the same family, it became clear that bacterial uptake via CEACAM3, exclusively expressed on granulocytes, differs mechanistically from the pathogen uptake by epithelial CEACAMs (CEACAM1, 6 and CEA) (Schmitter, Agerer et al. 2004; Buntru, Roth et al. 2012). In the last years, the mechanism and the involved proteins in CEACAM3-mediated uptake were mainly revealed, whereas little is known for the epithelial CEACAMs. CEACAM3-mediated uptake of pathogens is very fast and strictly dependent on massive actin rearrangements to engulf the bacteria for phagocytosis. In contrast, actin cytoskeleton rearrangements play only a minor role for bacterial uptake via epithelial CEACAMs (CEACAM1, 6, CEA). Also the cytoplasmic part of CEACAM1 is not involved in pathogen internalization and activation of CEACAM1 leads to re-localization of the receptor into lipid rafts (Muenzner, Bachmann et al. 2008).

8.1 Coevolution of pathogens with receptors

Some pathogens have evolved distinct CEACAM-binding adhesins to promote colonization of the mucosa. The most widely investigated adhesins are human-restricted pathogens like *Neisseria gonorrhoeae*, *N.meningitidis*, *Moraxella catarrhalis*, *Haemophilus influenza* and some more (Leusch, Drzeniek et al. 1991; Virji, Makepeace et al. 1996; Chen, Grunert et al. 1997; Hill, Toleman et al. 2001; Toleman, Aho et al. 2001; Hauck and Meyer 2003; Hill and Virji 2003; Gray-Owen and Blumberg 2006). They have been shown to bind to human CEACAMs only and not to engage orthologous of other mammalian species (Voges, Bachmann et al. 2010), possibly explaining the strong restriction of the pathogens to the human host. Moreover, the mouse hepatitis virus strain A59 (MHV-A59) is known to bind only to one of the two murine CEACAM1 alleles. Although both of the alleles code for CEACAM1, the divergence in the pathogen binding interface is so high, that the virus only recognize the N-terminal domain of CEACAM1a (Dveksler, Dieffenbach et al. 1993; Dveksler, Pensiero et al. 1993; Zelus, Wessner et al. 1998). This example demonstrates that the engagement of CEACAM receptors by pathogens is highly specific, and the relatively high amino acid divergence between different species, especially in the CC'C'' FG-face of the N-terminal domain, is the reason for the strong species restriction for the pathogens mentioned before. The higher amino acid divergence in the CC'C''FG face compared to the rest of the CEACAM1 molecule might be explained by the evolutionary pressure from CEACAM utilization by pathogens. This selective pressure likely also account for the rapid diversification of CEACAMs in different mammalian lineages. However, pathogens also continuously adapted these modifications during evolution, resulting in an everlasting co-adaption between hosts and pathogens. Based on the cellular functions of CEACAM1, binding has considerable advantages for the pathogen, due to the ability to exploit mammalian host cell

functions like signaling transduction pathways, cytoskeletal rearrangements, or vesicular trafficking (Finlay and Cossart 1997). Also, receptor binding promotes the establishment of infection because increased attachment prevents the excretion of the pathogens. Furthermore, it could be shown that binding of *N. gonorrhoeae* to CEACAM1 results in increased extracellular matrix adhesion by activation of integrins through triggering of CD105 expression in epithelial cells (Muenzner, Rohde et al. 2005; Muenzner, Bachmann et al. 2010). Another advantage of CEACAM1 binding by pathogens is the modulation of the host immune response by inhibiting T-cell responses (Watt, Teixeira et al. 2001; Lee, Ostrowski et al. 2008) or increasing expression of proinflammatory cytokines like TNF- α or CEACAM1 receptor itself (Gray-Owen, Lorenzen et al. 1997; Muenzner, Dehio et al. 2000; Muenzner, Naumann et al. 2001). One member of the CEACAM family, CEACAM3, has been shown to be a chimera with the immunoglobulin variable like domains derived from bacteria-recognizing CEACAMs like CEACAM1, 6 and CEA and the transmembrane and cytoplasmic domains of CEACAM4, a member of the CEACAM family which is also exclusively expressed on granulocytes. Bacterial uptake via CEACAM3 is an opsonin-independent, very effective phagocytotic process, leading to fast intracellular killing of the pathogens (Pils, Gerrard et al. 2008; Buntru, Roth et al. 2012). This could again be the result of a co-evolutionary process, to handle the highly adapted bacteria. To sum up, the species restriction of CEACAM-binding pathogens is mediated by the sequence divergence of the pathogen binding domain of CEACAMs between the different mammalian lineages and possibly this divergence originated from selective pressure of CEACAM utilization by pathogens.

8.2 The role of the PI3K during CEACAM1-mediated uptake and the participation of the extracellular Ig_{C2}-like domains of CEACAM1

Phosphoinositides are involved in almost all cellular signaling processes in a regulatory way, depending on cell localization and interaction partners. During endocytosis, they often play important roles in actin cytoskeleton rearrangements by interaction with actin-severing proteins, facilitation of actin polymerization or promoting actin uncapping (Toker 1998; Logan and Mandato 2006). Phosphoinositides are also involved in microtubule organization (McLaughlin, Wang et al. 2002), vesicle fusion and trafficking (Toker 2002), and activation of small GTPases (Hawkins, Anderson et al. 2006). Although phagocytosis via Fc γ receptors strongly requires the activity of PI3K, CEACAM3-mediated uptake of *Neisseria gonorrhoeae* is PI3K-independent (Buntru, Kopp et al. 2011). This could be explained by generating of a shortcut through direct association of the ITAM-like domain in the cytoplasmic part of CEACAM3 with an upstream and downstream effector of the small GTPase Rac1. After bacterial engagement, the tyrosines in the ITAM-like motif get phosphorylated by kinases of the Src family, leading to recruitment and activation of the guanine nucleotide exchange factor Vav. Activation of Vav results in recruitment and activation of the small GTPase Rac1 and the adaptor protein Nck, both responsible for massive lamellipodia formation and phagocytosis via the WAVE complex (McCaw, Schneider et al. 2003; McCaw, Liao et al. 2004; Schmitter, Agerer et al. 2004; Schmitter, Pils et al. 2007; Pils, Gerrard et al. 2008; Buntru, Kopp et al. 2011; Buntru, Roth et al. 2012). For CEACAM1, 6 and CEA the activity of the PI3K is essential for efficient pathogen uptake, although the actin cytoskeleton plays only a minor role during uptake process. In this case, generation of PI(3,4,5)P must have other advantages for bacterial uptake, for example the role in vesi-

cle trafficking or activation of small GTPases. It will be interesting to examine the exact role of generation of PI(3,4,5)P during CEACAM1- (and CEA-/CEACAM6-) mediated uptake in the future. Furthermore, it became clear that the extracellular Ig_{C2}-like domains are responsible for PI3K requirement during bacterial uptake via the epithelial CEACAMs. All of them contain two to six Ig_{C2}-like domains besides the Ig_V-like domain, which is present in all family members. Amino acid comparisons of the Ig_{C2}-like domains showed a high similarity between these proteins (90 – 95%), allowing laterally interaction with unknown co-receptor(s). In contrast, CEACAM3 does not contain any Ig_{C2}-like domains, but in this case, signal transduction after activation is mediated directly by the cytoplasmic part of CEACAM3 (Schmitter, Agerer et al. 2004; Schmitter, Pils et al. 2007; Buntru, Roth et al. 2012; Pils, Kopp et al. 2012). As mentioned before, CEACAM1-mediated uptake does not require the cytoplasmic domain and in addition, the other epithelial CEACAMs (CEA and CEACAM6) are linked via GPI anchor into the membrane and do not express any cytoplasmic part (Kuespert, Pils et al. 2006; Muenzner, Bachmann et al. 2008). Currently, it is entirely unclear how epithelial CEACAMs can transmit signals into mammalian cells. Lateral interaction with an unknown co-receptor, which then mediates the signal transduction, could be an explanation for this unsolved question. In summary, pathogen uptake via epithelial CEACAMs requires the activity of class I PI3K, and activation of PI3K seems to be regulated via the extracellular Ig_{C2}-like domains, possibly leading to lateral interaction with an unknown co-receptor responsible for signal transduction into the cells.

8.3 The role of RhoG and EphA2 in the uptake process of pathogens via CEACAM1

Small GTPases are often involved in endocytotic processes, mainly linked to vesicular transport, actin cytoskeleton and microtubule remodeling (Takai, Sasaki et al. 2001; Aspenstrom, Fransson et al. 2004; D'Souza-Schorey and Chavrier 2006). Besides the important role of PI3K, also the small GTPase RhoG is strongly involved in CEACAM1-mediated uptake of pathogens. It is known, that most of the RhoG dependent processes are directly connected to activation of Rac1 via the ELMO-DOCK complex (Kato and Negishi 2003; Ho and Dagnino 2011). Rac1 is an important regulator of the actin cytoskeleton, which explains the requirement during CEACAM3-mediated uptake of pathogens (Niedergang and Chavrier 2005; Schmitter, Pils et al. 2007). However, for CEACAM1 the actin cytoskeleton plays only a minor role. Rac1 could also be linked to regulation of microtubule destabilization (Wittmann, Bokoch et al. 2004), which is more important for CEACAM1-mediated uptake of pathogens (unpublished data). In addition to the role in activation of Rac1, RhoG can also directly activate class I PI3K by binding to the p85 subunit (Hiramoto-Yamaki, Takeuchi et al. 2010; Harada, Hiramoto-Yamaki et al. 2011). This direct activation might link the previous observations of PI3K dependency during CEACAM1-mediated uptake to the observed role of RhoG. Indeed, we could demonstrate a connection between these two signaling pathways with RhoG as upstream effector of PI3K. Furthermore, Ephexin4 could be identified as the involved guanine nucleotide exchange factor, which is specific for RhoG and is regulated upstream via the receptor tyrosine kinase EphA2 (Hiramoto-Yamaki, Takeuchi et al. 2010; Harada, Hiramoto-Yamaki et al. 2011). Receptor tyrosine kinases might be the perfect co-receptors for epithelial CEACAMs, because of their ability to transmit signals into mammalian cells in a direct way. There are other examples of

pathogens using receptor tyrosine kinases as receptor, like *Listeria monocytogenes*, where binding to c-Met leads to bacterial uptake in a PI3K dependent manner (Ireton, Payraastre et al. 1996; Cossart 2001). EphA2 contains fibronectin type III domains in the extracellular part, like many other receptor tyrosine kinases. These domains could be the possible interaction domain with the Ig_{C2}-like domains of epithelial CEACAMs since interaction between fibronectin type III and immunoglobulin domains has been reported before (Barclay 2003; Pitulescu and Adams 2010). In fact, a direct role in CEACAM1-mediated uptake of pathogens of EphA2 could be demonstrated, suggesting EphA2 as co-receptor for signal transduction into mammalian cells after CEACAM activation. Another hint of a connection between epithelial CEACAMs and EphA2 gives a study from 2004, where a group observed upregulation of gene expression of EphA2 after infection of epithelial cells with *Neisseria* (Plant, Asp et al. 2004). In conclusion, we identified several signaling molecules involved in pathogen uptake via epithelial CEACAMs such as the receptor tyrosine kinase EphA2, the guanine nucleotide exchange factor Ephexin4, the small GTPase RhoG and the PI3K.

8.4 Proposed model of *N. gonorrhoeae* uptake via CEACAM1

In the present study we could identify several new signaling molecules involved in bacterial uptake via CEACAM1 and possibly also via CEA and CEACAM6. The results are shown schematically in Fig. 33.

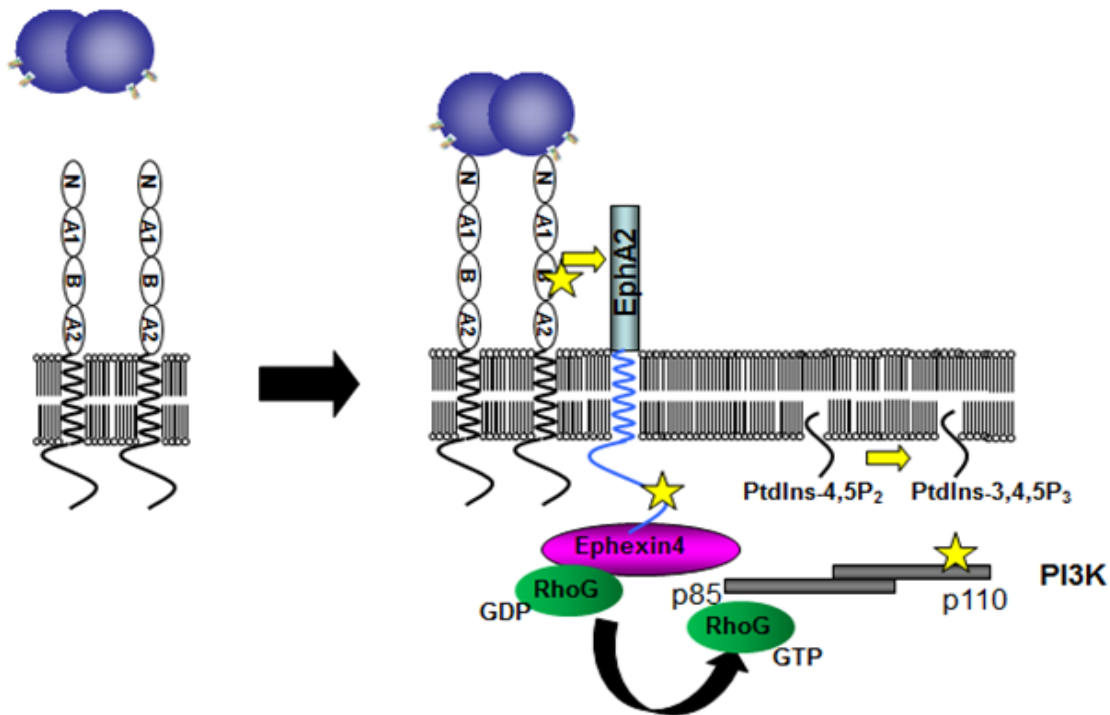


Fig. 33 The schematic working model for CEACAM1-mediated internalization of *Neisseria gonorrhoeae*. Binding of pathogens to CEACAM1 induces activation, clustering and *cis*-phosphorylation of EphA2 via extracellular Ig_{C2}-like domains of CEACAM1. Phosphorylated EphA2 recruits and activates RhoG GEF Ephexin4, which catalyze nucleotide exchange of RhoG from GDP to GTP. GTP-bound RhoG binds to p85 subunit of class I PI3K for activation, leading to generation of PI(3,4,5)P in close proximity to bacteria-host-interaction and endocytosis of the pathogen-CEACAM-EphA2 complex.

Binding of the bacteria to CEACAM1 leads to clustering of several CEACAMs, relocalization into lipid rafts and possibly to an activation signal in the Ig_{C2}-like domains. Another possibility is that only the accomplishing of CEACAM and EphA2 leads to an interaction between the Ig_{C2}-like domains of CEACAMs and the Fibronectin type III domains of EphA2. The *cis*-interaction between these molecules trigger signal transduction from EphA2 receptor into the cell. Activated EphA2 transphosphorylate the cytoplasmic domains of EphA2 receptors in close proximity and also phosphorylate and activates associated guanine nucleotide exchange factors like Vav and Ephexin4 (Hunter, Zhuang et al. 2006) (Harada, Hiramoto-Yamaki et al. 2011). Furthermore, activated Ephexin4 catalyze the nucleotide exchange of the small GTPase RhoG, which can bind to the p85 subunit of class I PI3K, leading to activation and generation of

PI(3,4,5)P at the site of infection. Accordingly, uptake of the whole EphA2-CEACAM-pathogen complex occurs, but the internalization mechanism remains unclear. It is known, that Eph receptors get internalized after activation via their ephrin ligands together with the surrounding plasma membrane (Pitulescu and Adams 2010). Therefore, the CEACAM interaction with the EphA2 receptor might on the one hand be necessary for transmitting signals into mammalian cells, but on the other hand also be very important for internalization by general invagination and endocytosis of the surrounding membrane together with the CEACAM-pathogen complex. In conclusion, we could identify new players in CEACAM1- (and possibly also CEA- and CEACAM6-) mediated uptake of pathogens, leading to the perception that another membrane protein is responsible for signal transduction into the cell. This could be an explanation for the open question, how GPI-anchored CEACAMs (like CEA and CEACAM6) can transmit signals and why the cytoplasmic domain of CEACAM1 is not involved in the uptake process of the bacteria.

DECLARATION OF AUTHOR'S CONTRIBUTIONS

Maike Voges, Verena Bachmann, Robert Kammerer, Uri Gophna and Christof R. Hauck:

CEACAM1 recognition by bacterial pathogens is species-specific.

The experiments were designed by Maike Voges and Christof R. Hauck. Maike Voges and Verena Bachmann performed the experiments. Christof R. Hauck, Maike Voges, Uri Gophna and Robert Kammerer conceived of the study. Maike Voges and Christof R. Hauck wrote the paper. All authors read and approved the final manuscript.

Maike Voges, Verena Bachmann, Jan Naujoks, Kathrin Kopp and Christof R. Hauck:

Extracellular Ig_{C2}-like domains of CEACAMs mediate PI3K sensitivity during uptake of pathogens.

The experiments were conceived and designed by Maike Voges, Verena Bachmann, Kathrin Kopp and Christof R. Hauck. Maike Voges, Verena Bachmann, Jan Naujoks and Kathrin Kopp performed the experiments. The data were analyzed by Maike Voges and Christof R. Hauck. Maike Voges and Christof R. Hauck wrote the paper.

Maike Voges, Verena Bachmann, Joachim Braun, Laura Reckzeh, Ulrich Groth, Christof Hauck: *The role of RhoG and EphA2 during CEACAM1-mediated uptake of N. gonorrhoeae.*

The experiments were conceived and designed by Maike Voges, Verena Bachmann and Christof R. Hauck. Maike Voges, Verena Bachmann, and Laura Reckzeh performed the experiments. The data were analyzed by Maike Voges and Christof R. Hauck. Maike Voges and Christof R. Hauck wrote the paper.

9 MATERIAL

9.1 Bacteria

Opa₅₂-expressing (Opa_{CEA}), non-piliated *N. gonorrhoeae* MS11-B2.1 (strain N309), and non-piliated, non-opaque gonococci MS11-B2.1 (strain N302) were kindly provided by T.F. Meyer (Max-Planck Institut für Infektionsbiologie, Berlin, Germany) and were cultured as described previously (Schmitter, Agerer et al. 2004). Opa-expressing, non-encapsulated *N. meningitidis* (SiaD mutant of strain MC58) was obtained from Matthias Frosch (Institut für Hygiene und Mikrobiologie, Universität Würzburg, Germany). *M. catarrhalis* strain ATCC 25238 was obtained from DSMZ (Braunschweig, Germany). Both *Moraxella* and *Neisseriae* were grown on GC agar plates (Difco BRL, Paisley, UK) supplemented with vitamins at 37°C, 5% CO₂ and subcultured daily. For infection, over-night grown bacteria were taken from GC agar plates, suspended in PBS, and colony forming units (cfu) were estimated by OD₅₅₀ readings according to a standard curve.

9.2 Cells

The human embryonic kidney cell line 293T (293 cells) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum at 37°C in 5% CO₂ and subcultured every second to third day. 293T cells were transfected by calcium-phosphate coprecipitation using 5 - 8 µg of plasmid DNA for each 10 cm culture dish.

Human brain microvascular endothelial cells (HBMEC) (Slanina, König et al. 2010) were grown in endothelial cell medium (PAA, Pasching, Austria) supplemented with L-glutamine.

Michigan Cancer Foundation – 7 cells (MCF-7) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. MCF-7 cells were transfected with FuGENE®HD (Promega) using 5 - 8 µg of plasmid DNA for each 10 cm culture dish.

All cells were grown at 37°C in 5% CO₂ and subcultured every 2-3 days.

9.3 Medium

9.3.1 Medium for agar plates and bacteria

LB medium	1% w/v Tryptone; 0.5% w/v yeast extract; 0.5% w/v NaCl; pH 7.0 with NaOH (autoclaved)
LB agar plates	1% w/v Tryptone; 0.5% w/v yeast extract; 0.5% w/v NaCl; 10 mM MgCl ₂ ; 1.2% w/v Agar-Agar; pH 7.0 with NaOH (autoclaved)
GC agar plates	3.375% w/v GC-Agar (autoclaved); 1% v/v Vitamin solution
BHI agar plates	5.25% w/v BHI-Agar; 20 µg/ml Hemin; 20 µg/ml NAD
Vitamin solution	10% w/v Dextrose; 1% w/v L-Glutamin; 2.6% w/v L-Cystein Cl; 0.01% w/v Cocarboxylase; 0.002% w/v Fe(NO) ₃ ; 0.0003%w/v Thiamin-HCl; 0.025% w/v NAD; 0.001% w/v Vitamin B12;0.015% w/v L-Arginin; 0.0013% w/v)-aminobenzoe acid; 0.11% w/v L-Cystein; 0.1% w/v Adenin; 0.05% w/v Uracil; 0,003% w/v Guanin; 3% v/v 3N NaOH; (pH3.5)
Refreezing medium	50% v/v LB medium; 50% v/v Glycerol (50%)

9.3.2 Antibiotics

Ampicillin (amp)	100 mg/ml
Chloramphenicol (cam)	30 mg/ml
Erythromycin	7 µg/ml
Gentamicin	50 µg/ml
Kanamycin	50 mg/ml
Penicillin	10 mg/ml
Streptomycin	10 mg/ml
Tetracyclin	10 µg/ml

9.3.3 Medium for cell culture

Medium for 293T cells	DMEM (PAA) + 10% v/v CS (calf serum)
Medium for MCF-7 cells	DMEM (PAA) + 10% v/v FCS (fetal bovine serum) + 1% v/v Pyruvat (PAA)
Medium for HBMEC	Endothelial Medium (PAA)
Refreezing medium for cells	70% v/v DMEM, 20% v/v CS, 10% v/v DMSO

9.4 Antibodies and Enzymes

9.4.1 Antibodies

9.4.1.1 Primary Antibodies

Antigen	Name	Isoform	Species	Company
CEACAM1/3	Clone 18/20		Mouse	AG Hauck
CEACAM-pan	D14HD11		Mouse	
Tubulin	Clone E7		Mouse	AG Hauck
GFP	JL-8	IgG2a	Mouse	Clontech
HA	Clone12CA5		Mouse	AG Hauck
Flag	M2	IgG1	Mouse	Sigma
Flotillin2/ESA	Clone 29	IgG1	Mouse	BD Bioscience
Opa	Clone B11C12		Mouse	Prof. Achtmann

Transferrin receptor	Clone G1/221/12	IgG	Mouse	AG Hauck
EphA2			Rabbit	Santa Cruz
Ephexin4			Goat	Santa Cruz
RhoG			Rabbit	Santa Cruz
Myc	Clone 9E10	IgG	Mouse	AG Hauck
DsRed			Rabbit	BD Bioscience
pTyr	PY-72		Mouse	AG Hauck
GST			Rabbit	AG Hauck

9.4.1.2 Secondary Antibodies

Name	Company
Cy-3 conjugated AffiniPure goat anti-mouse IgG	Jackson ImmunoResearch Laboratories
Cy-5 conjugated AffiniPure goat anti-mouse IgG	Jackson ImmunoResearch Laboratories
Cy-2 conjugated AffiniPure goat anti-mouse IgG	Jackson ImmunoResearch Laboratories
Cy-3 conjugated AffiniPure goat anti-rabbit IgG	Jackson ImmunoResearch Laboratories
Cy-5 conjugated AffiniPure goat anti-rabbit IgG	Jackson ImmunoResearch Laboratories
Cy-2 conjugated AffiniPure goat anti-rabbit IgG	Jackson ImmunoResearch Laboratories
HRP conjugated AffiniPure goat anti-mouse IgG	Jackson ImmunoResearch Laboratories
HRP conjugated AffiniPure goat anti-rabbit IgG	Jackson ImmunoResearch Laboratories
HRP conjugated AffiniPure donkey anti-goat IgG	Jackson ImmunoResearch Laboratories

9.4.1.3 Antibody- independent staining reagents

Name	Company
Cholera toxin subunit B- FITC	Sigma
Cholera toxin subunit B- AlexaFluor555	Sigma
FITC-Streptavidin	ICN
Rhodamine-Streptavidin	Jackson ImmunoResearch Laboratories
CFSE (5-(6)-carboxyfluorescein-succinylester)	Molecular Probes
Pacific Blue	Molecular Probes
AlexaFluor647	Molecular Probes
Rhodamine (5-(6)-carboxytetramethylrhodamine-succinimidylester)	Molecular Probes

9.4.2 Enzymes

Name	Company
Taq-Polymerase	AG Hauck
Pfu-Polymerase	AG Hauck
T4 Ligase	AG Hauck

Cre Recombinase	AG Hauck
InFusion	Clontech
Antarctic Phosphatase	NEB
Trypsin	PAA Laboratories
Restriction Enzymes	NEB or Fermentas

9.4.3 Proteins/ Additional

Name	Company
Fibronectin	AG Hauck
Sulfo-NHS-LC Biotin	Pierce
Poly-L-Lysin	Sigma

9.5 Inhibitors

Name	Target	Company
Wortmannin	PI3K	Calbiochem
LY294002	PI3K	Calbiochem
Methyl- β -Cyclodextrin	Cholesterol	Sigma
Aprotinin	Serin Proteases	Sigma
Pepstatin A	Aspartatic Proteases	Sigma
Leupeptin	Serin Proteases	Sigma
EphA2-Inhibitor	EphA2 Receptor	AG Groth/Hauck

9.6 Oligonucleotides

9.6.1 Cloning oligonucleotides

Name	Sequence	Plasmid
mCEA-CAM1-sense	5'-GAAGTTATCAG-TCGACATGGAGCTGGCCTCAGCAC-3'	pDNR dual mCEACAM1 Gesamt (Short)
mCEA-CAM1-anti	5'-ATGGTCTAGAAAGCTTCCGCCAGACTTCCTGG-3'	pDNR dual mCEACAM1 Gesamt (Short)
mCEA-CAM1-N-anti	5'-ATGGTCTAGAAAGCTTGGGTG-TACATGAAATCGC-3'	pDNR dual mCEACAM1 N-Terminus (Short)
bovine CEA-	5'-GAAGTTATCAGTCGACATGGG-	pDNRdual CEA1-

CAM1abN for	GACCCCCTCAG-3'	3NaNbta/nNbta
bovine CEA-CAM1aN rev	5'-ATGGGTCTAGAAAGCTTGGGAGTATGTG-GAGGTGTCCAG-3'	pDNRdual CEA1-3NaNbta
bovine CEA-CAM1bN rev	5'-ATGGTCTAGAAAGCTTTGGAGTACGTG-GAGGTGTCC-3'	pDNRdual CEA1-3NbNbta
canine CEA-CAM1N for	5'-GAAGTTATCAGTCGACATGGAGCCCCCCTCG-3'	pDNR dual CEA1-3Ncfa
canine CEA-CAM1N rev	5'-ATGGTCTAGAAAGCTTGGGAATACTT-GGAGCTGTCC-3'	pDNR dual CEA1-3Ncfa
sense-Chime-ra3cd-HA	5'-ATAATGGCCATAGTGGCGCTGGTGGCCGCAC-TGGTGTGTTTCCTGCTCCTTCATTTCTGGGAAA-TATCCCTATGACG-3'	pcDNA Chim3abcd
rev-Chime-ra3ab	5'-ATAATGGCCACTCCGACCAG-GACCCCGGTCACGATCCCAGGTGAGAGGCC-3'	pcDNA Chim3abcd
PI3KCA-IF-sense-5'	GAAGTTATCAGTCGACCCTCCAAGAC-CATCATCAG-3'	pcDNA Cerulean PI3KcA
PI3KCA-IF-anti	5'-ATGGTCTAGAAAGCTTAGGGCGGCTCAG-TTCAATGCATGCTG-3'	pcDNA Cerulean PI3KcA
SHIP-PD-IF sense	5' GAAGTTATCAGTCGAC-GAGCCAGAGCCTGAC-3'	pLPS-5'YFP-FKBP SHIP PD / PD D675G
SHIP-PD-IF anti-sense	-5' ATGGTCTAGAAA-GCTTAAGGGACCCTGCCAGAAGG-3'	pLPS-5'YFP-FKBP SHIP PD / PD D675G
CEA-CAM1-Nhe_sense	5'-GAACTGCTAGCACCATGGGGCACCTCTCAG-3'	pLL3.7 CEA-CAM1-4L
CEA-CAM1-AgeI_reve rse	5'-GCTAGACCGGTATGTCATAGGGATACTGC -3'	pLL3.7 CEA-CAM1-4L
EphA2 for XhoI	5'-GACTCGAGCGGAC-CATGGAGCTCCAGGCAGCC-3'	pLPS-3'2xmyc EphA2WT/Eph A2ΔKD
EphA2 rev HindIII	5'-CTAAGCTTGATGGGGATCCCCACAGTGTTCC-3'	pLPS-3'2xmyc EphA2WT/Eph A2ΔKD

mCherry For AgeI	5'- GAACCGGTAAGGGTGAGCAAGGGCGAGGAG- GATAAC-3'	pLPS-3'mCherry loxP
mCherry rev NotI	5'- TAGCGGCCGCAATTAATT- GTACAGCTCGTCCATGC-3'	pLPS-3'mCherry loxP
CEA1-N AgeI rev	5'- CCAACCGGTGTGTCATAGGGA- TACTGCTTTTTACTTCTG-3'	pLL3.7 CEA- CAM1-N

9.6.2 shRNA

Name	Sequence
shEphA2-sense1	ccggaaGTAAACAGGGTACCTCAAGctcgagCTT- GAGGTACCCTGTTACTTtttttg
shEphA2- antisense1	aattcaaaaaAAGTAAACAGGGTACCTCAAGctcgagCTT- GAGGTACCCTGTTACTt
shRhoG-sense	ccggaaCACTGGGTATTCTCATGAGctcgagCTCATGAGAA- TACCCAGTGTTtttttg
shRhoG- antisense	aattcaaaaaAACACTGGGTATTCTCATGAGctcgagCTCATGA- GAATACCCAGTGtt
shEphexin4- sense	ccggaaGAGCGAGGAGAGCTACATGctcgag- CATGTAGCTCTCCTCGCTCttttttg
shEphexin4- antisense	aattcaaaaaGAGCGAGGAGAGCTACATGctcgag- CATGTAGCTCTCCTCGCTct

9.7 Plasmids

Vector	Insert	Cloning strategy or source
pLPS-3'EGFP	mCEACAM1 Gesamt (Short) /N-Terminus; soluble mCEACAM1-N, cfaCEACAM1-N, btaCEACAM1a/b-N, hCEACAM1-N; CEACAM1-4L, CEACAM1 Δ CT, CEACAM1-4S, CEACAM1-N-TM-Cyt, CEACAM3; PLC- δ PH, BTK-PH; RhoGcA, RhoG, RhoGdN	Cre-Recombination between the loxP sites
pLPS-3'Cerulean	CEACAM1-4L, CEACAM3, PI3KcA	Cre-Recombination between the loxP sites
pLPS-3'mKate	CEACAM1-4L, CEACAM1 Δ CT, CEACAM3	Cre-Recombination between the loxP sites
pcDNA 3.1 Hygro	CEACAM1-4L, CEACAM3, CEACAM1 Δ CT, CEA, CEACAM6, Chimera3abcd, CEACAM8/1	Restriction digest with HindIII / NotI and ligation
pLPS-3'2xHA	CEACAM1-N, CEACAM1NA1,	Cre-Recombination between

	CEACAM1NA1B	the loxP sites
pGEX4 TiLoxP	SHP2-C-SH2, PI3K3R-N-SH2	Cre-Recombination between the loxP sites
pLPS-3'2xmyc	EphA2wt, EphA2ΔKD; mCEACAM1 Gesamt (Short) /N-Terminus; RhoGcA, RhoG, RhoGdN	Restriction digest with XhoI / HindIII and ligation or Cre-Recombination between the loxP sites
pLPS-3'mCherry	EphA2 wt	Restriction digest with XhoI / HindIII and ligation
pLPS-5'YFP-FKBP	SHIP1-PD, SHIP1 D675G	Cre-Recombination between the loxP sites
pEXN2-Flag	hEphexin4-WT, hEphexin4ΔDH	Katoh et al
pLL3.7	CEACAM1-4L, CEACAM3, CEACAM1-N-TM-Cyt	Restriction digest with AgeI/NheI and ligation
pLKO.1	shRhoG, shEphexin4, shEphA2, shscramble	Restriction digest with AgeI / EcoRI and ligation of annealed oligos

9.8 Media and Buffer

Name	Recipe
Ammonium Persulfate (APS)	10% w/v APS
Annealing buffer	100 mM K-acetate, 30 mM HEPES-KOH pH7.4, 2 mM Mg-acetate
Anode buffer (5x)	125 mM Tris-Base, 200 mM 6-aminohexanacid pH 9.4
Birnboim-Doly P1	50 mM Tris/HCl; 10 mM EDTA; 100 mg/ml RNaseA
Birnboim-Doly P2	0.2 mM NaOH; 20% w/v SDS
Birnboim-Doly P3	3 M potassic acetate; pH 5.5 with acetic acid
Blocking buffer	10% v/v FCS in PBS
Blotto	2% w/v BSA; 0.05 %w/v Sodium azide; in 1x TBS-T
Borate buffer	0.2 M borate buffer prepare by adding 1 M NaOH to boric acid until pH 8.5
Carbodiimide	2% carbodiimide in 0.1 M MES buffer
Carbonate buffer [0.1 M]	adding 0.1 M Na ₂ CO ₃ to 0.1M NaCO ₃ until pH 9.6
Cathode buffer (5x)	125mM Tris-Base, pH 10.4
Coomassie Stain (PVDF Membrane)	25% v/v Isopropanol; 10% v/v Pure acetic acid; 3% w/v Coomassie
Cre-Buffer	33 mM NaCl; 50 mM Tris/HCl pH7.5; 10 mM MgCl ₂
Cristal violet	50mg/ml in 96% Ethanol
Destain (PVDF Membrane)	45.5% v/v Methanol; 9.1% v/v Pure acetic acid
DNA-Ladder (1 kb)	1 kb DNA-Ladder in 6x loading dye solution

Material

Marker)	
dNTPs [20 mM]	20 mM dGTP; 20 mM dATP; 20 mM dCTP; 20 mM dTTP in ddH ₂ O
ECL	0.225 mM ρ -coumaric acid, 1.25 mM luminol, 0.1 M Tris-Base, pH 8.5
Ethanolamine [0.25 M]	20 μ l ethanolamine in 1.3ml borate buffer
Ethidium bromide	10 mg/ml EtBr
FACS buffer	5% heat inactivated FCS; 0.1% Azide; in 1x PBS
CFSE	4 mg CFSE in 1 ml DMSO
GEBS	20% w/v Glycerol; 0.5% w/v Sarkosyl; 50 mM EDTA; 0.05% bromphenol blue
H ₂ O ₂ solution	30% v/v H ₂ O ₂
HBS (2x)	50 mM Hepes; 280 mM NaCl; 1.5 mM Na ₂ HPO ₄ ; pH 7.1-7.05 with NaOH
Homogenization Buffer	50mM Tris pH7.4; 2mM MgCl ₂ ; 8% w/v Sucrose
Lysis buffer	50 mM Tris Base; 10 mM EDTA; 150 mM NaCl;
	10% Glycerol; 0.025% Triton X-100; 2.5 mM DTT;
	10 μ M Benzamidin; 5 μ g/ μ L Leupeptin; 10 μ g/ μ L
	Aprotenin; 10 μ g/ μ L Pefablock
MES buffer	0.1M MES buffer, pH 5.2-6
PBS (10x)	1.37 M NaCl; 26.8 mM KCl; 14.7 mM KH ₂ PO ₄ ; 78.1 mM Na ₂ PO ₄
PBS++	0.9 mM Ca ⁺⁺ (CaCl ₂); 0.5 mM Mg ⁺⁺ (MgCl ₂); in 1x PBS
PFA	4% w/v PFA, in Aq.bidest pH 7.3
Pfu-buffer	10 mM Tris/HCl pH 7.5 ; 50 mM KCl, 1.5 mM MgCl ₂
Permeabilization buffer	10% v/v FCS, 0.2% Saponin; in PBS
RIPA-buffer	1% Triton X-100; 50 mM Hepes; 150 mM NaCl; 10% Glycerol; 1.5 mM MgCl ₂ ; 1 mM EGTA;
	10mM Sodium pyrophosphat; 100 mM NaF; 1 mM Natrium Orthovanadat;
	5 μ g/ml Leupeptin; 10 μ g/ml Aprotinin; 10 μ g/ml Pefablock; 5 μ g/ml Pepstatin;
	10 μ M Benzamidin; 0,1% w/v SDS; 1% v/v Deoxycholin acid
Running-buffer (10x)	3% w/v Tris Base; 14.4% w/v Glyzin; 1% w/v SDS
Sample buffer 2x/4x	4% w/v SDS; 20% w/v Glycerol; 125 mM Tris HCL; 10/20% v/v -2-Mercaptoethanol;
	1% w/v Bromphenol blue; pH6.8
Saponin (gentamicin-assay)	1% in 1x PBS
Saponin (immunofluorescence)	0.1% in 1x PBS
SDS-PAGE-Marker (HMW)	0.5 mg/ml Horseradish-peroxidase (40 kDa); 0.5 mg/ml Bovine Serum Albumin (66 kDa);
	0.5 mg/ml Lipoxidase (96 kDa); 0.5 mg/ml -Galactosidase (116 kDa);
	0.5 mg/ml Myosin rabbit muscle (205 kDa) in Triton-buffer
SDS-PAGE-Marker (LMW)	0.5 mg/ml Lysozym (14.4 kDa); 0.5 mg/ml Soybean Trypsin Inhibitor (22 kDa);

	0.5 mg/ml Horseradish-peroxidase (40 kDa); 0.5 mg/ml Bovine Serum Albumin (66 kDa);
	0.5 mg/ml Lipoxidase (96 kDa) in Triton-buffer
Sephadex G10-solution	10% w/v Sephadex G10; in Triton-buffer
Sodium dodecyl sulfat (SDS)	20% w/v SDS
Stacking gel buffer	0.5 M Tris Base; pH 6.8
Staining solution (crystal violet)	0.1 M Borat buffer; 1% v/v crystal violet
Storage buffer	0.01 M phosphate buffer (pH 7.4), 1% BSA, 0.1% sodium acid, 5% glycerol
Stripping buffer	1% w/v SDS; 62.5 mM Tris HCl, 0.8% v/v mercaptoethanol
TAE-buffer (50x)	500 mM TrisBase; 50 mM EDTA; 5.7% w/v acetic acid
TBS (10x)	500 mM Tris Base; 1.5 M NaCl; pH 7.5
TBS-T	0.05% Tween in 1x TBS
TE-buffer	10 mM TrisBase; 1 mM EDTA; pH 8.5 (autoclaved)
T4 Ligase buffer	50 mM Tris/HCl pH 7.5; 10 mM MgCl ₂ ; 10 mM Dithiothreitol; 1 mM ATP
TNE-buffer	20 mM Tris/HCl pH8 ; 130 mM NaCl ; 5 mM EDTA ; 10µg/ml Aprotinin; 10µg/ml Leupeptin;
	1µg/ml Pefablock; 1mM PMSF; 0.2% v/v Triton X-100
Triton-buffer	1% v/v Triton X-100; 50 mM Hepes; 150 mM NaCl; 10% Glycerol; 1.5 mM MgCl ₂ ;
	1 mM EGTA; 10 mM Sodium pyrophosphate; 100 mM NaF; 1 mM Sodium Orthovanadat;
	5 µg/ml Leupeptin; 10 µg/ml Aprotinin; 10 µg/ml Pefablock; 5 µg/ml Pepstatin; 10 µM Benzamidin
Tween-solution	10% v/v Tween 20
Western transfer buffer	0.3% w/v Tris Base; 1.44% w/v Glycin; 21.5% v/v Methanol; 10% w/v SDS; 30 min devolatilization

9.9 KITS

Application	Name	Manufacturer
Gel Extraction	QIAquick Gel Extraction Kit	Qiagen
InFusion	InFusion PCR Clonig System	Clontech
Mini-preparation	QIAprep Spin Miniprep Kit	Qiagen
Midi-preparation	Nucleobond AX Pc	Macherey-Nagel
Transfection	Lipofectamine Plus Reagent	Invitrogen
	FuGene®HD Transfection Reagent	Promega

9.10 Laboratory Equipment and Consumption Items

Instrument	Notation	Manufacturer
Autoclave	Autoclave	Sauter, Sulgen Switzerland
Centrifuges	5415R / 5417R	Eppendorf
	Multifuge 1s-R	Hereaus Instruments
	Centrifuge H-401	Hermle
	Laboratory centrifuges	Sigma
	E Centrifuge CLP3401	Pathtech
	SORVALL RC6+	Thermo
Cryostat	Vacutom HM500	Microm
Ultracentrifuge	Optima L-80 XP	Beckman Coulter
Cell counter	Casy Model TT	Schärfe System
Blotter	Semidry Blotter	PeqLab
Developer	Curix 60	AGFA
Electrophoresis System (agarose)	Perfect Blue Gelsystem Mini	PeqLab
Electrophoresis System (SDS)	Mini Protean: 3Cell/Tetra cell	BioRad
Flow cytometry device	LSRII	BD Biosciences
Gel Documentation	GelDoc XR System	BioRad
Incubator – bacteria	Celsius 2005	Memmert
Incubator –cells	Innova Co 170 CO2	New Brunswick Scientific
Microscopes	ECLIPSE TC100	Nikon
	AF 6000LX	Leica
	TCS SP5	Leica
Microwave	646223M	Privilege
Mini UV Table	60 ECX 20M	PeqLab
NanoDrop		PeqLab
Neubauer Chamber		Roth
PCR Cycler	Primus 25 advanced	PeqLab
	Master Cycler Gradient	Eppendorf
pH Meter	Seven Easy	Mettler Toledo
Photometer	Biophotometer 6131	Eppendorf
	Libra S4	Biochrom
Power Supply Units	EV202 / Power Pac Basics	Consort / BioRad
Rotator	STR 4	Roth
Spectrophotometer	NanoDrop ND-1000	PeqLab
Spectrofluorometer	Varioskan Flash	Thermo Scientific
Sterilisation Sterilisation	966-HS-1ELD	Belimed / Sauter

Product	Supplier
Plastic Ware	Greiner Bio-one
Cover Slips	WTC Binder
Eppendorf tubes	Eppendorf
Filter Paper	Whatman
Glass Ware	Schott

Glutathion Sepharose	Amersham Biosciences
Object Slides	Menzel Gläser
Pasteur Pipettes (glass)	Roth
Pipette Tips	Sarstedt
Plastic Pipettes	Nalge Nunc
PVDF-Membranes	Millipore
Sepharose (Sephadex G 10)	Sigma
Sterile Filter (Poresize: 0.2 μm & 0.45 μm)	Schleicher & Schuell
X-Ray Films	Typon Imaging AG

9.11 Software

Adobe Photoshop 6.0, Clone Manager Professional 9, FACSDiva software, GraphPad Prism5, ImageJ, LAS software, MS Office, Quantity One, Cyflogic, LSM 5 Image Browser, EndNote.

10 METHODS

10.1 Cell culture

10.1.1 Culturing

Human embryonic kidney (HEK) 293T cells, human brain microvascular endothelial cells (HBMEC) and Michigan Cancer Foundation – 7 (MCF-7) cells were cultured in 10 cm dishes with 293T medium (DMEM + 10% CS), HBMEC medium (endothelial medium, PAA) or MCF-7 medium (DMEM + 10% FCS + 1% Pyruvat in a water saturated 95% air / 5% CO₂ atmosphere at 37°C. 293t cells and MCF-7 cells were splitted 1:5 or 1:10 when reaching 60-80% confluence, HBMECs were splitted 1:4 or 1:8. For splitting, cells were washed once with PBS (37°C) and trypsinized with 1 ml trypsin/EDTA at 37°C. respective medium was added and cells were centrifuged for 3 min at 800 rpm. Afterwards medium supernatant was aspired and cells were resuspended and seeded.

10.1.2 Cell counting

For different experiments distinct cell numbers were necessary. Therefore cells were counted before seeded into well-plates. After trypsinizing the cells, medium was added and 25 µl of cells suspension was mixed with 5 ml CasyTon. Subsequently cells were counted by Casy Model TT with the adequate program for respective cells.

10.1.3 Cell Transfection

10.1.3.1 Calcium- Phospate Co-Precipitation

For each transfection sample, a clean mini or midi preparation of the plasmid construct was needed. The transfection samples were mixed in a 15 ml tube by adding 500 µL

sterile ddH₂O, 5 µg DNA and 500 µL 2X HBS. To this solution, 50 µL 2.5 M CaCl₂ were added drop wise during vortexing, followed by an incubation time of 15 minutes. During that time, 10 µL 25 mM chloroquin, which inhibits the acidification of the lysosomes and therefore counteracts the degradation of the inserted DNA, were added to the cells. Subsequently, transfection sample was dropwise added to the cells. Transfected cells were cultured in a water saturated 95% air / 5% CO₂ atmosphere at 37°C for 5-8 h. Afterwards medium was changed and cells were cultured for further 12 – 36 h. To determine the transfection efficiency cells were analyzed either by fluorescence microscopy, flow cytometry or Western Blot analyzes.

10.1.3.2 FuGene®HD

For each transfection sample, a clean mini or midi preparation of the plasmid construct was needed. The transfection samples were mixed in an eppendorf tube by adding 100 µL sterile OptiMEM with 5 µg DNA and 7 µl FuGene®HD. After an incubation time of 20 minutes transfection sample was dropwise added to the cells. Transfected cells were cultured in a water saturated 95% air / 5% CO₂ atmosphere at 37°C for 12 – 36 h. To determine the transfection efficiency cells were analyzed either by fluorescence microscopy, flow cytometry or Western Blot analyzes.

10.1.4 Unfreezing and Refreezing of Cells

When unfreeze cells it is very important to unfreeze the cryo-tube quick in a 37°C water-bath. Afterwards cell suspension was transferred to a 15 ml tube and centrifuged for 3 min at 800 rpm. Subsequently, medium was removed and cells resuspended in 10 ml fresh medium and transferred to a prepared culture dish. Fresh seeded cells were splitted for the first time one or two days later. For refreezing semiconfluent or confluent cell cultures were chosen. After trypsinizing, centrifugation and washing cells were resuspended in 1 ml refreezing medium and transferred to cryo-tubes. Tubes were cooled

overnight in an isopropanol box at -80°C . The next day tubes were transferred for permanent storage to liquid nitrogen.

10.2 Cloning procedures

10.2.1 Polymerase-Chain-Reaction (PCR)

DNA fragments were generated by PCR using appropriate oligonucleotides (primers) and Pfu- or Taq-polymerase. For cloning strategies the following protocol was used:

Reaction Mix:

4 μl 10x Pfu-Buffer
 1 μl Forward Primer (10pmol)
 1 μl Reverse Primer (10pmol)
 1 μl dNTPs (20mM)
 1 μl Template DNA (100ng)
 Ad 40 μl ddH₂O

Enzyme Mix:

1 μl 10x Pfu Buffer
 0,5 μl Pfu-Polymerase (10U/ μl)
 0,5 μl Taq-Polymerase (10U/ μl)
 ad 10 μl ddH₂O

For amplification in the thermocycler variations of the following standard program were used:

hot start	94°C		
	80°C	pause	
denaturation	94°C	20sec	} 30 cycles
hybridization	57-65°C	20sec	
elongation	72°C	60sec/1000bp	
final elongation	72°C	5-10min	
stop	4°C	forever	

Afterwards PCR-product was purified for the further cloning strategy and examined by restriction digest or colony PCR. The colony PCR provides a quick test for positive clones. The protocol equates to the standard protocol, except for using one bacterial colony instead of purified plasmid DNA for template. Also appropriate oligonucleotides were used.

10.2.2 Agarose-Gel-Electrophoresis

By agarose-gel-electrophoresis DNA fragments can be separated and identified. The separation of the fragments in the gel matrix is dependent on their size, whereas the velocity is proportional to the logarithm of their size. For an optimal separation, the concentration of agarose in the gel was chosen dependent on the size of the fragments. The suitable amount of agarose was solved in 1x TAE buffer by heating and poured into the gel chamber after cooling down to about 50°C. Slots were formed by a comb. The DNA samples were mixed with GEBS, applied to the slots and then separated in 1x TAE at 70-90 V. A DNA standard (Gene Ruler DNA Ladder, Fermentas) that was also applied in one slot facilitated a direct comparison of the fragment sizes. DNA bands were visualized by staining the gel in an ethidium-bromide (EtBr)/ GelRed bath (10 min), as after intercalation into the DNA, EtBr/GelRed fluoresces under UV light ($\lambda = 366 \text{ nm}$).

10.2.3 Purification of PCR products

After PCR the obtained products were purified by QIAquick Gel Extraction KitTM (QIAGEN), following the provided manufacturers protocol. The correct size was determined by agarose gel electrophoresis.

10.2.4 Gel extraction

DNA fragments analyzed by agarose-gel-electrophoresis were cut out under UV light. Afterwards the fragment was purified by QIAquick Gel Extraction KitTM (QIAGEN),

following the provided manufacturers protocol. The concentration of the DNA in elution buffer was determined with NanoDrop

10.2.5 InFusion Cloning

A method to insert a PCR product into a cloning vector is the In-Fusion™ Dry-Down PCR Cloning Kit from Clontech. As a precondition, the oligonucleotides must contain a specific InFusion sequence, which consists of a 15 basepair long overlap that is homologous to the ends of the linearized vector (pDNRdual). The InFusion enzyme is able to insert the PCR fragment via homologous recombination (without restriction and ligation) in the linearized vector, leading to a cyclization. One Dry-Down sample was completed by adding 200 ng linearized pDNRdual, 12 µl 10x InFusion buffer and ddH₂O to a total volume of 120 µl. When used, the molar ratio Insert:Vector should be 2:1. The concentration of the PCR product was measured photometrically with NanoDrop, and assuming that 1 mol DNA fragment of 1 kbp weighs 660 kg, the molar concentration of each PCR product could be calculated and therefore added to the IF sample correctly, in a maximum volume of 2 µl. Once the volume was filled up to 10 µl, if necessary by ddH₂O, the whole preparation was incubated at room temperature for 30 minutes and then stored on ice until being transformed into *E. coli* NovaBlue.

10.2.6 Ligation

The method of ligation was used to integrate an insert of interest into a plasmid vector.

The following reaction mix was prepared:

x µl	plasmid vector (100 ng)
x µl	insert (300 ng)
1 µl	T4-ligase (1U)
1 µl	10x T4-ligase buffer

ad 10 μ l ddH₂O

The reaction mix was incubated for 1 h at 20°C or at 4°C over night. Subsequently, the sample was directly transformed into competent *E. coli* NovaBlue.

10.2.7 Chemically competent Bacteria

For an effective uptake of DNA, the bacterial cell membrane has to be permeabilised. This is warranted through a MnCl₂ treatment. Therefore *E. coli* Nova Blue were cultivated overnight on an agar plate at 37°C or appropriate 30°C. Three of these colonies were cultivated overnight in 2ml LB medium at 37°C or 30°C under shaking. After that 250 ml SOB medium is inoculated with the grown bacteria in LB medium and incubated overnight at 19°C and 200 rpm, till an optical density (OD) of 0.5. The bacterial culture is incubated on ice for 10 min and centrifuged for 10 min at 4°C and 4000 rpm. The pellet is resuspended in 80 ml ice cold and incubated for 10 min on ice. Again bacteria are centrifuged for 10 min at 4°C and 4000 rpm. The bacterial pellet is resuspended in 20 ml ice cold TB and 1.4 ml DMSO and 200 μ l aliquots are frozen in liquid nitrogen and stored at -80°C.

10.2.8 Transformation of Bacteria

First competent bacteria were thawed on ice. Second competent bacteria were mixed with 0.5 μ l DNA or 5 μ l InFusion sample and incubated for 30 min on ice. Third heat shock at 42°C for 75 sec followed. Finally the sample was cooled on ice for 5 min and 1ml LB-medium was added. The sample was incubated for 1 h at 37°C on a shaker. After centrifugation at 5000 rpm for 5 min, supernatant was discarded and the pellet resuspended in the residual medium (approximately 100 μ l), which was plated on adequate selective medium plates and incubated overnight at 37°C. The next day, from

grown colonies, clones were selected, plated on new selective medium plates and incubated under the same conditions.

10.2.9 DNA-Preparation

From the grown bacteria, the plasmids were isolated following the protocol of Birnboim and Doly (Pils, Schmitter et al. 2006) one inoculating loop full of bacteria of one clone in 300 µl cold buffer P1. After adding 300 µl buffer P2 and inverting the tubes 6 times, the bacteria were lysed and the proteins were precipitated. Within the following 5 min, 300 µl cold buffer P3 were added and the tubes inverted to precipitate chromosomal DNA. By centrifugation for 10 min at 13000 rpm, the precipitated proteins and the genomic DNA formed a pellet. The supernatant, containing the plasmids, was transferred into a fresh tube. After adding 0.7x volume of isopropanol, the tube was inverted three times to precipitate the plasmid DNA and centrifuged for 15 min at 13000 rpm. The supernatant was discarded. The pellet was washed by adding 0.5 ml 70% EtOH, followed by another centrifugation step for 5 minutes at 13000 rpm. To remove all EtOH, pellet was dried at 37°C for at least 10 minutes before resuspending in 40 µl TE buffer. The plasmid concentration was determined photometrically via NanoDrop. The plasmid quality and size was determined by agarose-gel electrophoreses.

For high quality plasmids that were used for further processing, e.g. transfection or sequencing, were prepared with a commercial kit (QIAprep Spin Miniprep Kit, Qiagen) following the provided manufacturer's protocol. For high quantity of plasmids a commercial kit (QIAprep Spin Midiprep Kit, Quiagen) was used following the provided manufacturer's protocol.

10.2.10 Restrictional Digestion

The cloning products generated before were restricted by enzymatic digestion by an adequate restriction enzyme for analytical purpose. For digestion reaction mix following protocol was used:

2-4 μ l	plasmid DNA (depending on concentration)
2 μ l	10x restriction buffer
2 μ l	10x BSA (optionally)
0.1 μ l	restriction enzyme (2 U)
ad 20 μ l	ddH ₂ O

The reaction mix was incubated for 3 h at 37°C or overnight at 37°C. Afterwards the digestion mix was analyzed via agarose-gel-electrophoreses. The optimal temperatures and buffers for the digestion could be extracted from the enzyme lists provided by the manufacturers (NEB and Fermentas). For double digestions, the same protocol was used, however, a compatible buffer had to be found.

10.2.11 Cre/lox Recombination

For Cre/lox recombination the BD Creator DNA Cloning Kit was used. As donor vector for Cre/lox recombination the pDNRDual vector with adequate PCR-fragment was used. The prokaryotic expression vectors pLPS3'EGFP, pLPS3'mKate, pLPS3'mCherry, pLPS3'2xmyc or pLPS3'Cerulean were used as acceptor vector. The recombination between donor and acceptor vector was mediated by the enzyme cre-recombinase. It recognizes the specific recombination sequences (loxP) on both vectors. The optimal ratio between donor and acceptor vector was 1:2.

1 μ l	10x cre-buffer
1 μ l	10x BSA
0.5 μ l	cre-recombinase

100 ng donor vector

200ng acceptor vector

The reaction mix was incubated for 15 min at RT. Heat inactivation of the enzyme followed by 70°C for 5min. Subsequently, 5 µl of the reaction was used for transformation in *E. coli* and plated on LB-plates with chloramphenicol and 7% sucrose. With chloramphenicol and sucrose it is possible to select desired recombinant colonies that contain a directionally correct gene insert. Clones containing the remaining donor vector, without the gene insert, will express SacB, and therefore, cannot be grown on media containing sucrose. Putative positive clones were verified by restrictional digestion and sequencing.

10.3 Work with proteins

10.3.1 SDS-PAGE (SDS-Poly-Acrylamide-Gel-Electrophoresis)

For protein gels gel systems and gel chambers from BioRad were used. Separation gels had a polyacrylamide concentration between 8 and 15%, according to protein size. After addition of TEMED and APS to the separation gel it was cast between two glass plates and covered with a layer of ddH₂O. After polymerization the water was removed and the stacking gel was cast on the separation gel. Before polymerization a comb was put between the glass plates. The completely polymerized gel was put in a gel chamber after removal of the comb and covered with running buffer. Protein samples were mixed with 2x SDS sample buffer and boiled for 10 min at 90°C. Subsequently they were loaded on the SDS gel. Protein separation carried out at 120 V for 1 – 2 h.

Separation Gel			Stacking Gel	
	10%	12.5%		5%
Polyacrylamid 40%	2.5 ml	3.1 ml	Polyacrylamid 40%	1.25 ml
ddH ₂ O	5.0 ml	4.3 ml	ddH ₂ O	6.15 ml
1.5M Tris pH 8.8	2.5 ml	2.5 ml	0.5M Tris pH 6.8	2.5 ml

SDS (20%)	50 μ l	50 μ l	SDS (20%)	50 μ l
APS (10%)	30 μ l	30 μ l	APS (10%)	30 μ l
TEMED	15 μ l	15 μ l	TEMED	15 μ l

For Coomassie staining the gel was fixed and stained for 2 h in Coomassie staining solution. For destaining, gels were shaken in methanol-pure acetic acid destainer until all bands were detectable.

10.3.2 Western-Blot

10.3.2.1 Wet Blot

For protein detection via antibodies firstly a separation with respect to the protein size is done by SDS-PAGE. Afterwards the SDS-gel is packed between sponges, Whatman Paper and an methanol-activated PVDF membrane. The blot chamber was filled with western transfer buffer and blotted overnight at 30 V or for 2 h at 150 mA. Through capillary forces the proteins were transferred onto the PVDF membrane. Through Coomassie staining the protein bands and marker could be detected. Afterwards the membrane was destained via the appropriate solution. Unspecific binding was blocked by incubation overnight or 1h room temperature with blotto. After incubation the appropriate first antibody was added overnight at 4°C or 2 h at room temperature. The membrane was washed three times for 10 min with TBS-T and incubated for 30 min at room temperature with the secondary HRP-conjugated antibody. After washing three times for 10 min with TBS-T the membrane was incubated with 10 ml ECL solution and 3 μ l H₂O₂ for 1 min and developed via an X-ray film and the developer machine.

10.3.2.2 Semi Dry Western Blot

For semi-dry Western Blotting the Whatman papers and PVDF membrane were saturated with anode buffer. The SDS gel was applied to the PVDF membrane and covered with cathode buffer saturated Whatman papers and blotted for 1 – 2 h at

70 mA/gel. When the blot was finished, the membrane was stained in a diluted Coomassie staining solution and destained to visualize the protein bands and to mark the protein marker. Afterwards the membrane was blocked with BSA containing blocking solution for at least 2 h. Subsequently the membrane was incubated with the primary antibody for at least 2 h on a shaker. Afterwards the membrane was washed three times 10 min with TBST and incubated with the secondary antibody (HRP-conjugated protein G for monoclonal primary antibodies; HRP-conjugated protein A for polyclonal primary antibodies) for 1 h. Membrane was washed again three times 5 min with TBST and developed via an ECL-system (10 ml ECL-solution + 3 μ l H₂O₂ (30%)) for 2 min. In some cases the membrane was used again for another primary antibody. Therefore the membrane was treated with stripping buffer for 10 min at 65°C. After blocking the membrane with blotto the membrane was ready to be incubated with the primary antibody. Hence, the detection of different proteins within one membrane was possible.

10.3.3 Whole-Cell-Lysates

For determination of cellular protein-levels a distinct number of cells was treated with RIPAbuffer and lysed via a cell scraper. Chromosomal DNA and cell debris were pelleted by addition of 100 μ l sepharose beads (5 min on the shaker) and a centrifugation step (13000 rpm, 20 min). Supernatant was mixed with 2x SDS sample buffer and boiled up for 10 min at 90°C. After SDS-PAGE proteins were transferred on a PVDF membrane and incubated with adequate antibody.

10.3.4 Flotation Assay

To analyse the association of proteins with detergent-resistant membrane fractions the flotation assay as described by (Umlauf, Mairhofer et al. 2006) was modified. Briefly,

293T cells were transfected with HA-tagged recombinant constructs of CEACAM1-4L or CEACAM3 and flotillin2. Transfected cells were left untreated or incubated with $5 \mu\text{g ml}^{-1}$ monoclonal anti-CEACAM antibody (clone 18/20, recognizing CEACAM1, 3, 6 and CEA) for 1 h at 37°C , washed with PBS and further incubated for 30 min in serum-free DMEM with a polyclonal goat anti-mouse antibody. Half of cells were pre-treated with 200nM of PI3K inhibitor wortmannin, the other half of cells not. After washing with ice-cold PBS, cells were homogenized in ice-cold buffer containing 50 mM Tris, pH 7.4, 2 mM MgCl_2 and 8% sucrose and centrifuged for 15 min at 5700 g. The pellet of unbroken cells and organelles was discarded and the supernatant was adjusted to 4 mM EDTA, before centrifugation at 48 000 rpm. using an SW 60 rotor (Beckman) for 2 h. The resulting pellet containing cellular membranes was subsequently solubilized at 4°C for 15 min in 280 μl of ice-cold TNE-T buffer (20 mM Tris-Cl, pH 8.0, 130 mM NaCl, 5 mM EDTA, 0.7% Triton X-100 and protease inhibitors ($10 \mu\text{g ml}^{-1}$ aprotinin and leupeptin, $1 \mu\text{g ml}^{-1}$ pepstatin A and 1 mM phenylmethylsulfonyl fluoride). The lysate was adjusted to 50% sucrose by the addition of 490 μl of 80% sucrose in TNE-T and overlaid with 3 ml of 35% sucrose followed by 1.2 ml of 5% sucrose in the same buffer. After ultracentrifugation at 48 000 rpm in a SW 60 rotor (Beckman) for 18 h, eight fractions were collected from the top of the gradient and analysed by Western blotting. DRM components were recovered in the low-density fractions 2–4.

10.3.5 Test Expression of Recombinant Proteins

The finished expression vector was transformed into the E. coli expression strain BL21 Rosetta. After incubation overnight, grown clones were picked, plated on LB agar with the same antibiotic and on the same time used for inoculation of 5 ml LBcultures. LB-cultures were incubated for 3 h at 37°C on a shaker. Afterwards, 1 ml

was taken off, centrifuged and the pellet mixed with 100 μ l 2X SDS buffer as the pre induction sample. The remaining 4 ml were inducted with 0.1 mM IPTG and incubated overnight at 30°C and 220 rpm for protein expression. The following day, 0.5 ml of the culture was again centrifuged and mixed with 100 μ l SDS buffer, this time as the post induction sample. Both the pre and post induction sample could now be tested via SDS-PAGE and Coomassie staining for successful over-expression.

10.3.6 Over-Expression of Recombinant Proteins

When test expression was positive, the clone was used for inoculating 30 ml LBmedium with adequate antibiotic, incubated at 30°C and 220 rpm over night. Three 350 ml LB-main-cultures were inoculated with the pre culture at an OD600 of 0.2 the next day and incubated at the same conditions. The optical density at 600 nm of all three cultures was observed by measuring every 30 min. When an OD600 between 0.6 and 0.8 was reached, the induction with 0.5 mM IPTG followed after a pre induction sample was taken as described above. The expression was performed at 37°C and 220 rpm for 3 h, followed by removal of the post induction sample. Now the three cultures could be pelleted at 5000 rpm for 15 min, resuspended in a total volume of 25 ml cold T-buffer and stored at -80°C until further treatment.

10.3.7 Purification of GST Fusion Proteins via FPLC

For purification the bacterial pellet, produced while over-expression process, was lysed. The protein-containing supernatant was transferred to an affinity chromatography column and eluted. After this purification, the pure eluates were dialyzed to remove rests of the buffers. All steps of the purification were conducted at 4°C. Firstly, the frozen pellet from the over expression was thawed in a total of 5 ml T-buffer, mixed with 1 ml lysozym (25 mg/ml) and incubated for 30 min on ice.

Secondly, 20 ml lysis buffer were added and the sample was treated with ultrasonic (50% intensity, 5 cycles of 20 sec each) for completely lysis all bacteria. For removal of all DNA molecules from the sample 300 μ l sepharose solution was added and an incubation step of 10min followed. Finally the lysates were centrifuged for 30min at 11000xg to pellet the debris and sepharose. The supernatant was filtered to remove all traces of debris and transferred to a GStrap column, which previously had been equilibrated with 10 column volumes of T-buffer. After the sample was applied, the column was washed with 10 volumes T-buffer, followed by elution of the protein by adding 10 volumes elution buffer that contained 10 mM glutathione. All steps were conducted at a flow rate of 0.5 ml per minute. The elution passage was sampled in fractions of 2 ml, and measured photometrically. Fractions containing protein could be roughly identified. The column was then recalibrated by washing with 5 volumes elution buffer, followed by washing in turns with 5 volumes regeneration buffers A and B. All proteins should be eluted by the low pH from the glutathione sepharose. Afterwards the column was washed with 3 volumes ddH₂O and conserved in 20% EtOH at 4°C. For definite identification of protein containing fractions, 2 μ l of each fraction was applied on a Whatman filter paper and stained coomassie solution. After destaining, protein positive fractions became visible as a stained dot. Furthermore, 20 μ l of each sampled fraction was run on a SDS-gel and stained for examination. The two fractions containing the highest concentration of protein were then pooled and dialyzed. The dialysis hose was deposited in ddH₂O for 30 min before the concerned fractions were transferred. Dialyzed was 2 times against 2 L PBS for 24 h, then against 1 L dialysis buffer, which contained glycerol, overnight. Aliquots of 1 ml were transferred to eppendorf tubes and stored at -80°C. The exact protein concentration of the dialysis product was conducted via SDS-PAGE or BCA protein Assay Kit. A BSA standard was applied as well as two different volumes of the

protein. After staining with coomassie and destaining, the concentration could be estimated.

10.3.8 Immunoprecipitation

An aliquot of whole cell lysate was thawed on ice and appropriate amount of lysate was diluted in triton buffer to a total volume of 800 μ l where applicable. 3 μ g of antibody was added and incubated with the lysate for 4h at 4°C on a rotary mixer prior to the addition of 25 μ l protein-A/G sepharose suspension (Santa Cruz) for another hour of rotation incubation. The sepharose beads were pelleted by centrifugation and washed three times with 500 μ l of triton or RIPA buffer by repeated spin-down and resuspension. The supernatant remaining after the last wash (~40 μ l) was supplemented with 15 μ l 4x SDS sample buffer, and the complete sample loaded onto a SDS polyacrylamide gel after heating to 95 °C for 10 min.

10.4 Infection

10.4.1 Bacterial Culture

Competent bacteria of *E. coli* were grown on LB-plates with adequate antibiotics and cultured at 37°C. Pathogenic *Neisseria* were grown on GC-plates with erythromycin and chloramphenicol and cultured at 37 C in a humidified atmosphere with 5 % CO₂. On the next day pathogens were phenotypically selected and streaked out on GC-plates without antibiotics. On the next day they were used for experiments.

Haemophilus and *Moraxella* were grown on BHI-plates and cultured at 37°C in a humidified atmosphere with 5 % CO₂.

10.4.2 Cell Infection

Cells were seeded in adequate well plates with distinct cell numbers. Infection of the cells with pathogenic bacteria followed 2 days after transfection of the cells. Bacteria were cultured on GC-plates with erythromycin and chloramphenicol over night and selected via the phenotype at the second day on GC-plates without antibiotics. The OD₅₅₀ was measured photometrically. The number of bacteria could be estimated using a standard curve. Bacteria were stained, if necessary, with an adequate fluorescence dye 30 min at 37°C. Afterward three washing steps with PBS followed. Subsequently cells were infected with MOI 30 (gentamicin-protection-assay) or MOI 40 (immunofluorescence experiments) and incubated for two hours or the indicated time points at 37°C and 5% CO₂. For intra/extra staining, the bacteria were incubated with 0.3 mg Biotin per ml 1x PBS before the first marker was added. After 2 h of infection, extracellular bacteria were then bound by a streptavidin coupled marker with a different color.

10.4.3 Gentamicin-Protection-Assay

293T cells were seeded in Poly-L-Lysine coated 24-well-plates (5×10^5 cells/well). In some cases cells were treated with inhibitors 30 min before infection in the denoted concentrations. Afterwards cells were infected with an MOI 30 and incubated for 2 h at 37°C and 5% CO₂. After infection time medium was carefully removed and replaced by fresh medium containing 50 µg/ml gentamicin. Cells were incubated for 1 h at 37°C and 5 % CO₂. After all extracellular bacteria were killed medium was removed and cells were washed once with PBS. Subsequently the cells were lysed with 1 ml 1% saponin for 10 min at 37°C. Afterwards samples were diluted (10^1 , 10^2 , 10^3) and transferred to GC-plates. GC-plates were incubated at 37°C and 5% CO₂ overnight.

10.5 Microscopy

10.5.1 Staining of Bacteria and Immunofluorescence of Proteins

Cells were seeded on gelatine coated cover slips in 24-well-plates (293T cells 7×10^4 cells/well) and incubated overnight. The next day bacteria were centrifuged and stained with either rhodamine, CFSE or incubated with biotin. Afterwards cells were infected for 2 h. Subsequently cells were washed once with PBS⁺⁺ and fixed with 4% PFA for 20 min at RT. For only blocking unspecific binding sites fixed cells were incubated with blocking solution for 10 min at RT. For simultaneously blocking and permabilization cells were incubated with permabilization solution containing BSA for 10 min at RT. Primary antibody or antibody independent staining reagent incubation followed for 1 h at RT (in a dark chamber). Afterwards cells were washed three times with PBS⁺⁺ and secondary antibody was added for 45 min at RT (in a dark chamber). Again cells were washed three times with PBS⁺⁺ and embedded with mounting medium. The next day coverslips were sealed with nail polish and stored at RT in the dark.

10.5.2 Intra/Extra-Staining of Bacteria

Bacteria were cultivated as mentioned above. Bacteria were washed with 1x PBS. The washed bacterial pellet was incubated with either CFSE or rhodamine for 15min at 37°C and 600 rpm. Afterwards bacteria were incubated with biotin (0.3 mg/ml) for 15min at 37°C and intensively washed. Cells were infected for 2 h or the indicated time points. After infection samples were washed, fixed with 4% PFA and blocked with blocking buffer. For detection of extracellular bacteria samples were incubated with Alexa-Fluor-647-streptavidine for 45 min. With this method all bacteria were either CFSE or rhodamine labelled, whereas intracellular bacteria were exclusively CFSE or rhodamine labelled and extracellular bacteria CFSE or rhodamine and Alexa-Fluor-647-streptavidine

labelled. Subsequently there was the possibility to detect intracellular, eukaryotic bacteria via blocking, permabilization and staining with primary and secondary antibody.

10.5.3 Live-Cell Imaging

Cells were seeded in poly-L-lysine and fibronectin coated 3.5cm dishes (with integrated coverslip), (1×10^5 cells/dish). The next day, exchange of DMEM medium with colorless DMEM and preparation of the CLSM (heating of incubation chamber to 37°C and connection of CO² supply) followed. If bacterial infection should be investigated, cells were directly infected under the microscope and analyzed. Firstly appropriate cells were searched. Secondly settings were adjusted and finally movie was started. Images and movies were analyzed by LAS software and ImageJ.

10.6 Flow Cytometry

10.6.1 Determination of Transfection Efficiency/ Amount of receptor on surface

Cells were seeded in 6 cm dishes or 6-well-plates, respectively (1×10^6 cells/dish or well). The next day cells were trypsinized and mixed with FACS buffer. If needed, cells were incubated with a primary antibody (in FACS buffer) for 30 h at RT. After washing the samples secondary antibody was added for 15 min at RT in the dark. If no antibody staining was necessary cells were directly analyzed by flow cytometry (LSRII, FACSDiva software).

10.6.2 Invasion Assay

Cells were transfected with the indicated constructs 2 days before the assay. One day after cells were seeded in 6 cm dishes, respectively (1×10^6 cells/dish). On the next day bacteria were marked with CFSE for 15 min at 37°C under shaking in the dark and

washed afterwards three times with 1xPBS. After determination of bacterial density via photometer cells were infected with CFSE-marked bacteria with an MOI of 30 for 2 h. Denoted inhibitor treatment was 30 min prior to infection. Afterwards cells were washed one time with 1xPBS and trypsinized with 1 ml trypsin/EDTA at 37°C. DMEM + 10% CS was added and cells were centrifuged for 3 min at 800 rpm. Cells were washed with FACS buffer and pellets were resuspended in 1 ml of FACS buffer and divided into two reaction tubes prior to analyzing by flow cytometry (LSRII, FACSDiva software). One half of the cells were analyzed after the addition of trypan blue prior to measuring quenching the fluorescence signal of the extracellular bacteria. The amount of intracellular bacteria could be obtained by multiplying the mean fluorescence intensity with the percentage of CFSE positive cells determining the uptake index (u.i.) (Naldini, Blomer et al. 1996).

10.7 Lentiviral Methods

10.7.1 Cloning Strategies

Recombinant lentiviral particles were generated using the systems developed by the groups of D. Trono (Eades-Perner, van der Putten et al. 1994) and Luc van Parijs (Schmitz, Reali et al. 2002). The used plasmids pLL3.7/ pLKO.1, pMD2.G and psPAX2 were maintained in *E. coli* *STBL4*. For shRNA production the algorithm AAGN₁₈TT (available online at <http://jura.wi.mit.edu/biocs/siRNAext/>) was used identifying sequences that could silence expression of the target gene. According to this prediction, two complementary primers were synthesized. The primers were annealed with following protocol:

1µl	sense oligo (60 pmol)
1µl	reverse oligo (60 pmol)

48µl Annealing buffer

Program:

95°C 4min

70°C 10min

decrease temperature slowly to 4°C (0.1°C/min)

4°C 10min

After that annealed primers were cloned into the XhoI and HpaI site of pLL3.7 generating pLL3.7-sh or AgeI and EcoRI site of pLKO.1 generating pLKO.1sh. The correct insertion of the shRNA cassette was verified by sequencing.

For the expression of proteins with the lentiviral system, fragments were amplified from their appropriate template. The fragments were ligated into pLL3.7 vector via AgeI and EcoRI restriction sites replacing the GFP cDNA or via AgeI and NheI restriction sites to the 5' end of GFP cDNA. The correct insertion of the fragment was verified by sequencing.

10.7.2 Production

HEK 293T cells were transfected with 14 µg of pLL3.7 containing the gene of interest, 10 µg of packaging plasmid psPAX2 and 7 µg of envelope-coding plasmid pMD2.G using calcium phosphate DNA precipitation method. After 8 h the medium was replaced with 5 ml of fresh DMEM containing 10 % CS. 48 – 72 h later the virus-containing medium was collected, centrifuged at 2000 rpm at 4°C for 7 min and filtered through a 0.45 µm pore-size filter. For concentration 4 ml of 20 % sucrose were overlaid with 25 ml of the virus-containing supernatant in an Beckman tube. Afterwards the supernatant was centrifuged at 20.000 rpm for 2 h in an ultracentrifuge to pellet the lentiviral parti-

cles. After that the viral pellet was taken up in PBS/1% BSA, flash-frozen in liquid nitrogen, and stored at -80°C for at least one day prior to usage.

10.7.3 Transduction

For titration of recombinant lentiviral particles, 1×10^6 HEK293T cells or according cell lines were transduced with serial dilutions of the lentiviral particle preparation. 48h after transduction, cells were analyzed by flow cytometry for GFP-expression to determine the infectious units. The titre of the preparations was between $3 - 7 \times 10^9$ infectious units (IFU) per ml. For in vitro application 1×10^6 cells were seeded in 6-well plates one day before transduction. Cells were infected with 1×10^8 IFU in PBS/1% BSA in 2ml of culture medium. After three days experiments were accomplished. Transduction efficiencies were among 20% and 30%.

10.8 Amino acid sequence alignment

For the amino acid sequence alignment of the N-terminal domains of CEACAM1 following sequences were used: human CEACAM1 (hCEA1, NM_001712), murine CEACAM1a (mCEA1, BC016891), canine CEACAM1 (cCEA1, NM_001097557.1), bovine CEACAM1 (bCEA1, AY345129), bovine CEACAM1 isoform b (bCEA1b, AY487418). The alignment was performed using CLUSTALW.

11 LIST OF PUBLICATIONS

11.1 Parts of this thesis are published or going to be submitted for publication

Voges M, Bachmann V, Braun J, Reckzeh L, Groth U, Hauck CR (2012). “The role of RhoG and EphA2 during CEACAM1-mediated uptake of *Neisseria gonorrhoeae*.” In preparation

Voges M, Bachmann V, Naujoks J, Roth A, Kopp K, Hauck CR (2012). “Extracellular Ig_{C2}-like domains of CEACAMs mediate PI3K sensitivity during uptake of pathogens.” Accepted, PLoS One

Voges M, Bachmann V, Kammerer R, Gophna U, Hauck CR (2010). ”CEACAM1 recognition by bacterial pathogens is species-specific.” BMC Microbiol 10:117

11.2 Publications not part of this thesis

Reiser J, Hurst J, **Voges M**, Krauss P, Münch P, Iftner T, Stubenrauch F (2011). “High-risk human papillomaviruses repress constitutive kappa interferon transcription via E6 to prevent pathogen recognition receptor and antiviral-gene expression.” J Virol 85(21):11372-80

Buntru A, Kopp K, **Voges M**, Frank R, Bachmann V, Hauck CR (2011). ”Phosphatidylinositol-3'-Kinase activity is critical for initiating the oxidative burst and bacterial destruction during CEACAM3-mediated phagocytosis”. J Chem Biol 286(11):9555-66

Voges M (2011). “Papillomviren und ihre Taktik das Immunsystem des Wirtes zu umgehen: Eine Studie zur Unterdrückung der Expression interferonstimulierter Gene durch Papillomviren.“ VDM Verlag Dr. Müller

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