Molecular Microbiology (2004) **52**(4), 963–983 doi:10.1111/j.1365-2958.2004.04033.x

# Differential recognition of members of the carcinoembryonic antigen family by Afa/Dr adhesins of diffusely adhering *Escherichia coli* (Afa/Dr DAEC)

**Cedric N. Berger,[1] Oliver Billker,[2,3] Thomas F. Meyer,[2] Alain L. Servin[1]* and Imad Kansau[1]**

[1]*Institut National de la Santé et de la Recherche Médicale (INSERM), Unité 510, Faculté de Pharmacie Paris XI, F-92296 Châtenay-Malabry, France.*

[2]*Max-Planck-Institut fur Infektionsbiologie, Abteilung Molekulare Biologie, 10117 Berlin, Germany.*

[3]*Department of Biological Sciences, Imperial College London, London SW7 2AZ, UK.*

## Summary

**Little is known about the molecular bases underlying the virulence of diffusely adhering *Escherichia coli* (DAEC) harbouring the Afa/Dr family of adhesins. These adhesins recognize as receptors the GPI-anchored proteins CD55 (decay-accelerating factor, DAF) and CD66e (carcinoembryonic antigen, CEA). CD66e is a member of the CEA-related cell adhesion molecules (CEACAM) family, comprising seven members. We analysed the interactions of Afa/Dr DAEC with the CEACAMs using CEACAM-expressing CHO and HeLa cells. The results demonstrate that only *E. coli* expressing a subfamily of Afa/Dr adhesins, named here Afa/Dr-I, including Dr, F1845 and AfaE-III adhesins, bound onto CHO cells expressing CEACAM1, CEA or CEACAM6. Whereas all the Afa/Dr adhesins elicit recruitment of CD55 around adhering bacteria, only the Afa/Dr-I subfamily elicits the recruitment of CEACAM1, CEA and CEACAM6. In addition, although CEACAM3 is not recognized as a receptor by the subfamily of Afa/Dr adhesins, it is recruited around bacteria in HeLa cells. The recruited CEACAM1, CEA and CEACAM6 around adhering bacteria resist totally or in part a detergent extraction, whereas the recruited CEACAM3 does not. Finally, the results show that recognition of CEA and CEACAM6, but not CEACAM1, is accompanied by tight attachment to bacteria of cell surface microvilli-like extensions, which are elongated. Moreover, recognition of CEA is accompanied by an activation of the Rho GTPase Cdc42 and by a phosphorylation of ERM, which in turn elicit the observed cell surface microvilli-like extensions.**

Accepted 13 January, 2004. *For correspondence. E-mail alain.servin@cep.u-psud.fr; Tel. (+33) 1 46 83 56 61; Fax (+33) 1 46 83 58 44.



## Introduction

Interest in the molecular and cellular pathogenicity of *Escherichia coli* has grown considerably in the past few years. However, little is yet known about the molecular bases underlying the virulence of diffusely adhering *E. coli* (DAEC), the sixth class of enterovirulent *E. coli*. Two subclasses of DAEC strains have recently been identified. The first is known as diffusely adhering enteropathogenic *E. coli* (DA-EPEC) and harbours a pathogenicity island of EPEC strains, the locus of enterocyte effacement (LEE) island (Beinke *et al*., 1998). The second subclass includes DAECs that express adhesins of the Afa/Dr family (Nowicki *et al*., 2001), including the afimbrial adhesins AfaE-I (Labigne-Roussel *et al*., 1984; 1985; Labigne-Roussel and Falkow, 1988), AfaE-III (Le Bouguenec *et al*., 1993; Garcia *et al*., 1994; 2000) and Dr-II (Pham *et al*., 1997), as well as the fimbrial Dr (Nowicki *et al*., 1987; Swanson *et al*., 1991) and F1845 (Bilge *et al*., 1989; 1993) adhesins. Afa/Dr adhesins have similar genetic organization, consisting of operons involving at least five genes. Genes A to C encode accessory proteins and are highly conserved between family members, whereas gene E, which encodes the adhesin molecule itself, is more divergent (Garcia *et al*., 1996; 2000; Jouve *et al*., 1997; Zalewska *et al*., 2001).

Diarrhoeagenic and uropathogenic Afa/Dr DAEC, which attach to the brush border of polarized epithelial cells (Kerneis *et al*., 1991; 1994), recognize the decay accelerating factor (DAF-CD55) as a receptor (Bernet-Camard *et al*., 1996; Goluszko *et al*., 1999; Guignot *et al*., 2000; Nowicki *et al*., 1988; 1990; 1993). Structure–function studies mapping the adhesin binding site on the CD55 molecule show that the third short consensus repeat (SCR-3) domain of CD55 plays a pivotal role (Nowicki *et al*., 1993). The interaction between the bacterial adhesins and the GPI (glycosyl-phosphatidylinositol)-anchored CD55 has been analysed further. Mutations of CD55 segments involved in its biological activity along with the use of

bacteria with mutant adhesins have suggested the involvement of the SCR1 domain of CD55 and amino acid 54 of the Dr adhesin in the mobilization of CD55 (Nowicki *et al*., 1993; Guignot *et al*., 2000; Van Loy *et al*., 2002). It has been reported recently that members of the Afa/Dr family of adhesins not only recognize the CD55 molecule, but also recognize another membrane-associated GPI-anchored protein, the carcinoembryonic antigen (CEA) (Guignot *et al*., 2000). CEA belongs to a family of molecules recently renamed the CEACAMs (carcinoembryonic antigen-related cell adhesion molecules) (Thompson *et al*., 1991; Öbrink, 1997; Beauchemin *et al*., 1999). The CEACAM family belongs to the immunoglobulin (Ig) superfamily of adhesion molecules (Öbrink, 1997), comprising seven members including CEACAM1 (biliary glycoprotein, BGP, CD66a), CEACAM3 (CEA gene family member 1, CGM1, CD66d), CEACAM4 (CEA gene family member 7, CGM7), CEA (carcinoembryonic antigen, CD66e), CEACAM6 (non-specific cross-reacting antigen, NCA, CD66c), CEACAM7 (CEA gene family member 2, CGM2), CEACAM8 (CEA gene family member 6, CGM6, CD66b). CEACAM receptors share a conserved N-terminal Ig variable ($Ig_v$)-like domain that is followed by 0–6 Ig constant ($Ig_c$)-like domains. CEACAM receptors are differentially expressed by various epithelial, endothelial and haematopoietic cells *in vivo* (Thompson *et al*., 1991; Prall *et al*., 1996; Beauchemin *et al*., 1999). CEACAM1, CEACAM3 and CEACAM4 are inserted into the cellular membrane via a carboxy-terminal transmembrane and cytoplasmic domain, whereas CEA, CEACAM6, CEACAM7 and CEACAM8 possess a GPI anchor instead. The level of glycosylation of CEACAM receptors may vary, depending upon their cell type and differentiation state, and multiple glycoforms of the same protein have been isolated.

In the present study, we analysed the interactions of Afa/Dr DAEC with the CEACAM receptors using an experimental model involving CEACAM-expressing epithelial cells. The results show that, although all the Afa/Dr adhesins bind and elicit recruitment of the CD55 (DAF) molecule, only a subfamily of Afa/Dr adhesins including Dr, F1845 and AfaE-III adhesins, is involved in adherence to CEA, CEACAM1 and CEACAM6 and the recruitment of CEA, CEACAM1, CEACAM3 and CEACAM6, thus indicating that these molecules play a role in the pathogenesis of Afa/Dr DAEC. We also demonstrated in CEA-expressing cells that the same subfamily of adhesins induces an activation of the Rho GTPase Cdc42, which in turn phosphorylates the actin-binding proteins, ezrin/radixin/moesin (ERM). These phenomena are involved in the observed elongated microvilli of the cell surface attaching to adhering bacteria in CEA-expressing cells, indicating that this Afa/Dr subfamily must elicit signalling pathways during epithelial cell infection.

## Results

### *The Afa/Dr-expressing bacteria recognize some CEACAMs as receptors*

In order to study the capacity of different Afa/Dr-expressing DAEC to bind onto CEACAMs, we first generated a set of recombinant bacterial strains expressing different Afa/Dr adhesins in *E. coli* strain AAEC185 that lack type 1 pili (Blomfield *et al*., 1991) (Table 1). Host cell targeting by Afa/Dr-expressing DAEC is species specific *in vitro*, and Chinese hamster ovary (CHO) cells thus provide a suitable negative background to examine the interaction of the different Afa/Dr adhesins with individual members of the CEACAM family. We used a panel of transfected CHO cell lines expressing each human recombinant CEACAM (CEACAM1, CEACAM3, CEACAM4, CEA, CEACAM6, CEACAM7 or CEACAM8) or containing the expression vector alone in order to test the recognition of CEACAMs by wild-type Afa/Dr DAEC and recombinant *E. coli* strains. Receptor expression levels in each cell line were confirmed by fluorescence-associated cell sorting (FACS) analysis (data not shown). A CD55-expressing CHO cell line was used as a control. *E. coli* strain AAEC185 lacking pili 1, used as a control, adhered poorly to all the cell lines expressing CEACAMs, and the background adherence of the Afa/Dr-expressing strains to a vector-transfected CHO cell line was negligible, indicating the specificity of the phenomenon. In contrast, as shown in Fig. 1 and Table 2, Dr-, F1845- and AfaE-III-positive recombinant *E. coli* strains, as well as their corresponding wild-type strains, adhered strongly to CHO-CEA cells and, to a lesser extent, to CHO-CEACAM1 and CHO-CEACAM6 cells, thus indicating that CEACAM recognition is mediated by these Afa/Dr adhesins. It was noticed that the adhesion level of adhering Afa/Dr-expressing strains to CHO-CEA cells was comparable to that observed when CD55-expressing CHO cells were used (data not shown), indicating that CEA constitutes a second important receptor for some members of Afa/Dr DAEC. Interestingly, both Dr-II- and AfaE-I-positive recombinant *E. coli* strains, which possess a weak homology when compared with Dr adhesin, adhered poorly to CHO-CEACAM1, CHO-CEA or CHO-CEACAM6 cells (Table 2) whereas, as expected, these strains adhered efficiently to CD55-expressing CHO cells. Altogether, these results indicate that CD55-binding Afa/Dr adhesins can be divided into two functionally distinct subfamilies (Table 2). In the Afa/Dr-I subfamily, comprising Dr, F1845 and AfaE-III, shared sequence homology is correlated with similar binding profiles to CEACAM receptors. In contrast, members of the Afa/Dr-II subfamily, including Dr-II and AfaE-I, fail to target CEACAM receptors in spite of recognizing CD55.

It has been reported previously that CD55 and CD66e (CEA) are recruited around adhering Afa/Dr DAEC bacte-

**Table 1.** Bacterial strains and recombinant plasmids used in this study.

| Strain | Plasmid | Vector | Characteristics | Reference |
|---|---|---|---|---|
| AAEC185 | | None | Type 1 pili deleted (Δ*fimB–H*) | Blomfield *et al.* (1991) |
| IH11128 | | None | Wild-type strain expressing Dr adhesin | Nowicki *et al.* (1988) |
| Dr | pCC90 | pACY177 | Strain AAEC185 harbouring plasmid pCC90 that encodes adhesin Dr from IH11128 | Carnoy and Moseley (1997); this study |
| Dr-D54G | pCC90-D54G | pACY177 | Strain AAEC185 harbouring plasmid pCC90-D54G that encodes adhesin Dr mutated in gene *draE*. Substitution of aspartic acid for glycine in position 54 | Carnoy and Moseley (1997); this study |
| Dr-D54C | pCC90-D54C | pACY177 | Strain AAEC185 harbouring plasmid pCC90-D54G that encodes adhesin Dr mutated in gene *draE*. Substitution of aspartic acid for cysteine in position 54 | Carnoy and Moseley (1997); this study |
| EC7372 | | None | Wild-type haemolytic strain expressing adhesin Dr-II (DAF 1) | Pham *et al.* (1997) |
| Dr-II | pTP411 | pBluescript II SK– | Strain AAEC185 harbouring plasmid pTP411 that encodes adhesin Dr-II (DAF 1) from EC7372 | Pham *et al.* (1997); this study |
| C1845 | | None | Wild-type strain expressing adhesin F1845 | Bilge *et al.* (1989) |
| F1845 | pSSS1 | pUC8 | Strain AAEC185 harbouring plasmid pSSS1 that encodes adhesin F1845 from C1845 | Bilge *et al.* (1989); this study |
| KS52 | | None | Wild-type strain expressing adhesin Afa-I | Labigne-Roussel *et al.* (1985) |
| AfaE-I | pILL14 | pBR322 | Strain AAEC185 harbouring plasmid pILL14 that encodes adhesin Afa-I from KS52 | Labigne-Roussel *et al.* (1985); this study |
| A30 | | None | Wild-type strain expressing adhesin Afa-III | Garcia *et al.* (1994) |
| AfaE-III | pILL1151 | pBR322 | Strain AAEC185 harbouring plasmid pILL1151 that encodes adhesin Afa-III from A30 | Garcia *et al.* (1994); this study |

ria (Guignot *et al.*, 2000). We then examined by indirect immunofluorescence and confocal laser scanning microscopy (CLSM) whether CEACAM receptors are recruited around adhering recombinant *E. coli* expressing the Dr, F1845 or AfaE-III adhesins and wild-type Afa/Dr DAEC bacteria, in infected, CEACAM-expressing CHO cells. After 1 h of infection, a strong recruitment of CEACAM1 and CEA around adhering bacteria was observed forming fine rings that completely marked the bacterial circumference (Fig. 2A and D). Micrographs in Fig. 2 show double immunofluorescence staining of bacteria labelled with an anti-Dr adhesin (observed in red in Fig. 2B and E) and CEACAM receptors (observed in green in Fig. 2A and D). CLSM analysis (0.5 μm optical sections) in Fig. 2C and F

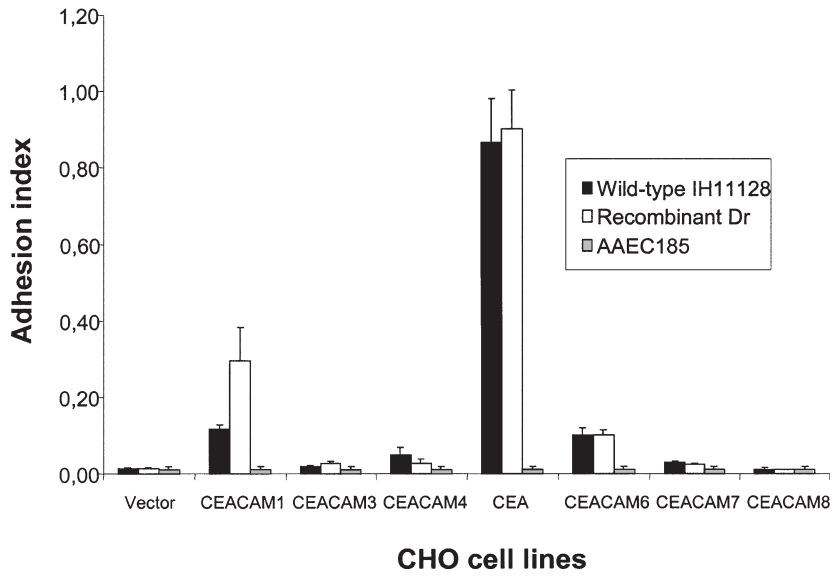


**Fig. 1.** Recognition of CEACAM receptors by Afa/Dr *E. coli*. CHO cell lines expressing each CEACAM were plated to confluency. Cell monolayers were inoculated with $5 \times 10^8$ bacteria $ml^{-1}$ strains IH11128 (wild-type strain expressing Dr adhesin), *E. coli* AAEC185 (control) and recombinant AAEC185 expressing Dr adhesin (Dr) and centrifuged to synchronize infections. After 1 h at 37°C, monolayers were processed to determine the cell-associated colony-forming units (CFU) as described in *Experimental procedures*. Indexes of cell-associated CFU per cell line were calculated on the basis of the higher cell association level (about $1.5 \times 10^7$ CFU $ml^{-1}$) obtained for Dr-positive strain in CHO-CEA in one of the experiments. Data shown are mean results ± SD of triplicate samples for each cell line. Data are representative of at least three independently performed experiments using IH11128, Dr recombinant or AAEC185 in each cell line.

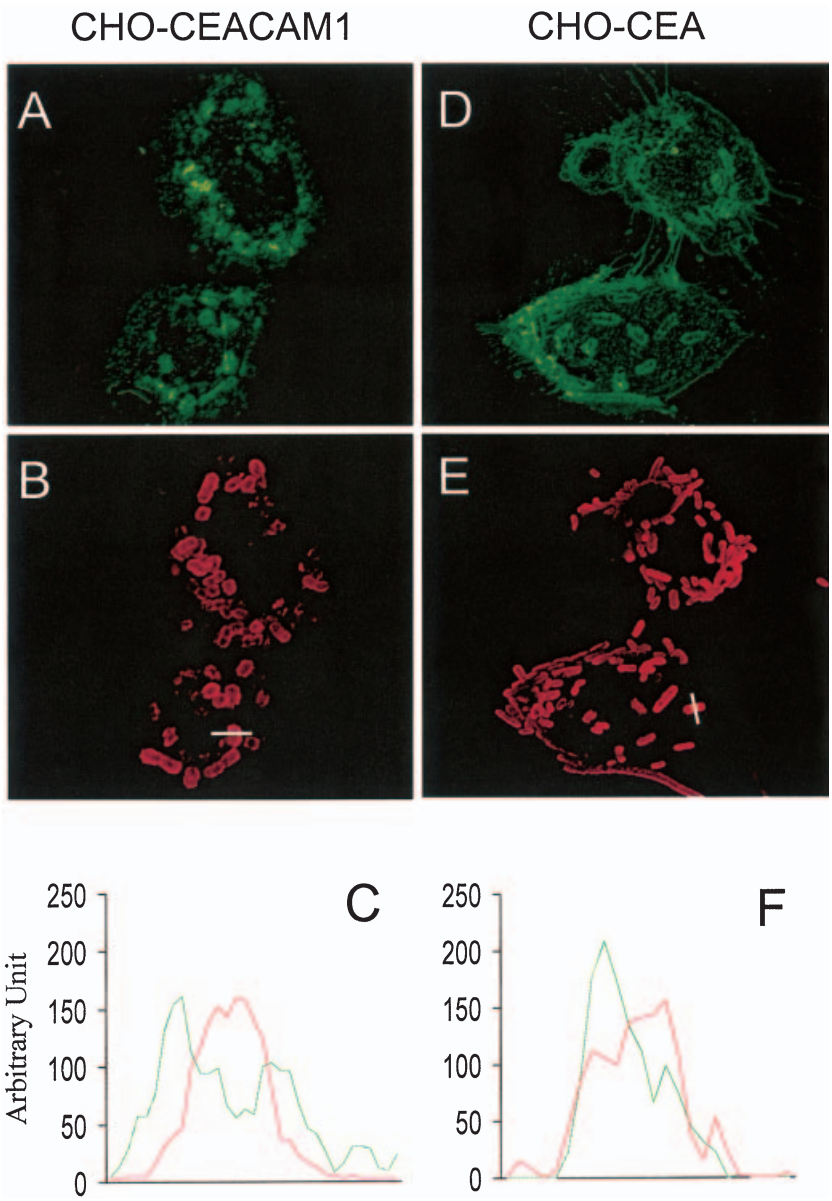


**Fig. 2.** Confocal projected sections showing adherent Dr-positive bacteria and recruitment of CEACAM1 and CEA in CHO-CEACAM cell lines. CHO cell lines expressing recombinant CEACAM1 (A, B, C) or CEA (D, E, F) receptors were infected with a Dr-positive strain for 1 h at a concentration of $1 \times 10^7$ bacteria $ml^{-1}$. After infection, samples were fixed and processed for double immunofluorescence labelling using anti-CEACAM antibody (green fluorescence in A and D) and anti-Dr adhesin antibody (red fluorescence in B and E). Optical sections (0.5 μm) representative of the apical part of the cells are shown. The fluorescence intensity for CEACAM (green) or Dr adhesin (red) was scanned following a transverse axis in Dr-positive adhering bacteria (white lane in B and E).
C and F. Scanning intensities of CEACAM (green curve) or Dr adhesin (red curve) in the optical section corresponding to (A), (B), (D) and (E). CEACAM1 and CEA co-localize with Dr adhesin expressed by the adhering bacteria (*x*-axes: bacterial transverse axis; *y*-axes: arbitrary fluorescence intensity units). It is important to note that no labelling was found around Dr-positive bacteria plated on a glass coverslip and subjected to immunofluorescence labelling with anti-CEACAM antibody (not shown).

**Table 2.** Adherence of Afa/Dr-expressing recombinant strains to CHO-CEACAM cell lines.

| | Expressed CEACAM | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Strain | None (vector) | 1 | 3 | 4 | 5 | 6 | 7 | 8 |
| AAEC185 | – | – | – | – | – | – | – | – |
| Afa/Dr-I subfamily | | | | | | | | |
| Dr | – | ++ | – | – | +++ | ++ | – | – |
| AfaE-III | – | ++ | – | – | +++ | ++ | – | – |
| F1845 | – | +++ | – | – | ++ | ++ | – | – |
| Afa/Dr-II subfamily | | | | | | | | |
| AfaE-I | – | – | – | – | + | – | – | – |
| Dr-II | – | – | – | – | – | – | – | – |

(–) no adhesion; (+, ++, +++) poor to very strong adhesion.

of the fluorescence intensity along a transverse axis of double-labelled bacteria (white lanes in Fig. 2B and E) shows a strong co-localization of the staining for Dr adhesin (red curves) and CEACAM1 or CEA (green curves). In contrast, no recruitment of CEACAM3, CEACAM4, CEACAM6, CEACAM7 or CEACAM8 was observed around rare randomly distributed adhering Dr-, F1845- or AfaE-III-positive bacteria (data not shown).

The role of the Afa/Dr-I subfamily of adhesins in eliciting recruitment of CEACAM1 and CEA in transfected CHO cells was also evidenced by testing AfaE-III- or bovine serum albumin (BSA)-coated carboxylated polystyrene beads previously used by Jouve *et al*. (1997). After incubation of each CHO-CEACAM cell line with coated beads, immunofluorescence was performed as indicated. A

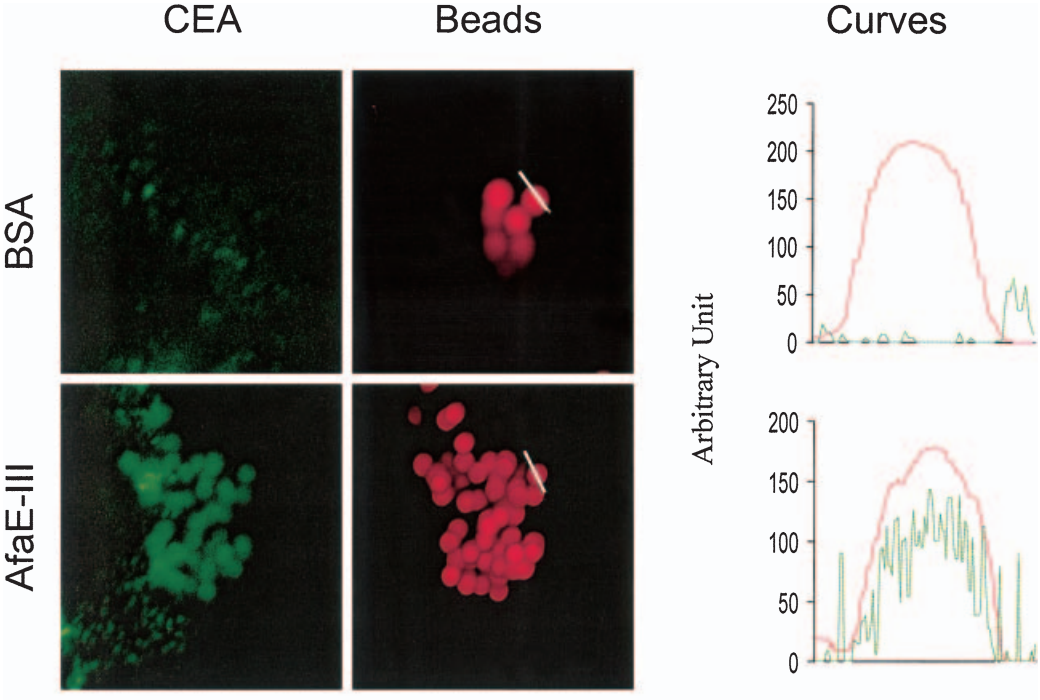


**Fig. 3.** CEA is recruited around AfaE-III-coated beads in CHO cells. After incubation of a CHO-CEA cell line with AfaE-III-coated or BSA-coated beads, immunofluorescence was performed using anti-CEACAM antibody as indicated in *Experimental procedures*. White arrows show dots of labelled CEA around AfaE-III adherent beads. The fluorescence intensity for CEA (green) or beads (red) was scanned following a transverse axis in a bead (white lanes). Curves show scanning intensities of CEA (green curve) or beads (red curve) in the optical sections (*x*-axes: bacterial transverse axis; *y*-axes: arbitrary fluorescence intensity units). Note a perfect co-localization of both green and red fluorescence.

recruitment of CEACAM1 (not shown) and CEA (Fig. 3) around adherent AfaE-III-coated beads was observed, whereas no recruitment with BSA-coated beads could be evidenced, thus indicating that CEACAM recruitment is an adhesin-dependent phenomenon.

We next examined whether CEACAMs were recruited around adhering Afa/Dr DAEC bacteria in HeLa cells that constitutively express CD55, which functions as a receptor for all the Afa/Dr adhesins (Nowicki *et al*., 1993; Pham *et al*., 1997). For this purpose, HeLa cells were transfected with CEACAM cDNAs. All the Afa/Dr DAEC strains expressing Dr, Dr-II, F1845, AfaE-I and AfaE-III adhesins adhered on to CEACAM1-, CEACAM3-, CEA-, CEACAM6- and CEACAM8-expressing HeLa cells as well as in HeLa cells containing the expression vector alone (not shown). Moreover, all the adhering Afa/Dr DAEC were lined by a CD55-positive labelling (not shown) in all these HeLa cell lines. Clustering of CEACAM receptors around Afa/Dr DAEC bacteria adhering on to CEACAM-expressing HeLa cells was examined (Fig. 4). Cells were infected for 1 h, and clustering of each CEACAM around bacteria was detected by indirect immunofluorescence labelling and CLSM. As expected, no CEACAM labelling was detected in HeLa cells harbouring plasmid alone, and no CEACAM recruitment was observed in CEACAM-expressing HeLa cells infected by control *E. coli* strain AAEC185 (data not shown). As observed above in CEACAM1- and CEA-expressing CHO cells, strong recruitment of CEACAM1 and CEA was observed around adhering recombinant an *E. coli* strain expressing Dr adhesin in CEACAM1- and CEA-expressing HeLa cells (data not shown). In contrast to what was observed in CEACAM-expressing CHO cells, a clustering of CEACAM3 and CEACAM6 was also observed around adhering Dr-positive bacteria (Fig. 4A). Interestingly, a similar pattern of recruitment of these CEACAM receptors was also observed when bacteria (recombinant and wild type) that express the other members of the Afa/Dr-I subfamily (F1845 and AfaE-III) were tested. In addition, a weak and heterogeneous mobilization of CEACAM8 around these adherent bacteria was also observed. In contrast, no recruitment of any CEACAM was elicited by Dr-II- or AfaE-I-expressing recombinant or wild-type bacteria (Fig. 4B).

### *Association within detergent-insoluble microdomains of CEACAMs acting or not as receptors for Afa/Dr adhesins*

Membrane lipid rafts have been widely implicated in the pathogenesis of infectious diseases (Rosenberger *et al*.,

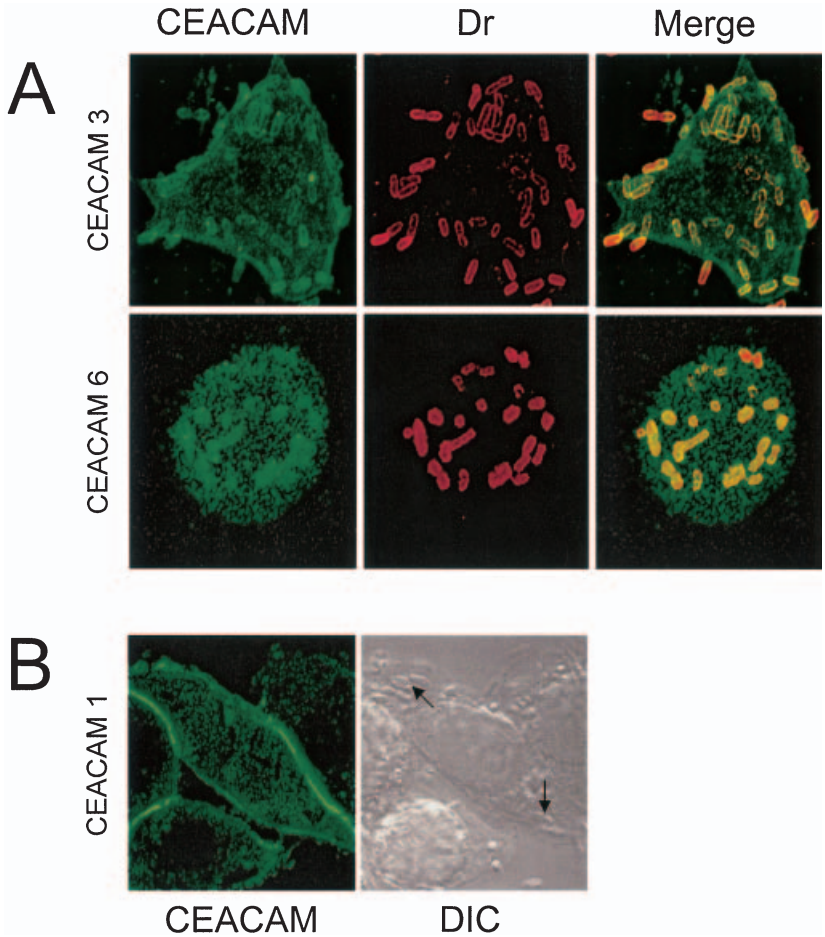


**Fig. 4.** Recruitment of CEACAM receptors around adhering Dr-positive bacteria infecting the CEACAM-expressing HeLa cells. Cells were infected and processed for immunolabelling as described in Fig. 2.
A. Confocal sections show CEACAM3 and CEACAM6 immunolabelling (green fluorescence), while red labelling corresponds to Dr adhesin. The superimposition of both immunofluorescences appears as yellow, showing that CEACAMs co-localize with Dr adhesin expressed by the adhering bacteria.
B. CEACAM1-transfected HeLa cells infected with recombinant Dr-II-positive strain and visualized using differential interference contrast (DIC). Arrows show adherent bacteria.

2000; Shin *et al*., 2000; van der Goot and Harder, 2001; Duncan *et al*., 2002; Pelkmans and Helenius, 2002). Consistent with the role of GPI-anchored proteins CD55 and CEA as receptors for Afa/Dr adhesins (Guignot *et al*., 2000), it has been reported previously that lipid rafts played a role in Afa/Dr DAEC pathogenicity (Goluszko *et al*., 1997; Guignot *et al*., 2001). We examined whether or not the CEACAM molecules recruited by infecting Afa/Dr DAEC are associated with detergent-insoluble microdomains in HeLa cells expressing both CD55 and CEACAMs.

We first examined whether the raft marker ganglioside GM1 (Fra *et al*., 1994; Brown and London, 2000) was recruited around adhering Dr-positive bacteria. For this purpose, GM1 was revealed by direct immunofluorescence labelling using fluorescein isothiocyanate (FITC)-conjugated cholera toxin subunit-B (CT-B) (Kenworthy *et al*., 2000). No CT-B labelling was detected when bacteria were plated on glass and processed with FITC-conjugated CT-b (data not shown). In HeLa cells infected with Dr-positive *E. coli*, a strong GM1 staining was observable around adhering bacteria, forming dense rings that completely marked the circumference of bacteria (Fig. 5A).

GPI-linked molecules are strongly associated with lipid rafts isolated biochemically according to their detergent insolubility (Brown and Rose, 1992; Fra *et al*., 1994; Kurzchalia and Parton, 1999; Brown and London, 2000; Simons and Toomre, 2000), whereas other membrane proteins, such as the transferrin receptor (TfR) do not co-purify (Rodgers and Rose, 1996). We first determined whether the GPI-anchored CEA and CEACAM6 and the transmembrane CEACAM1 and CEACAM3 are or are not constitutively associated with detergent-resistant microdomains in transfected HeLa cells. For this purpose, the cells were subjected or not to Triton X-100 extraction, and cell surface expression of CEACAM1, CEACAM3, CEA and CEACAM6 was examined by CLSM (Fig. 5B). As expected, cell surface expression of transmembrane CEACAM1 (Fig. 5B) and CEACAM3 (not shown) disappeared when the cells were subjected to Triton X-100 treatment. In contrast, cell surface expression of GPI-anchored CEA (Fig. 5B) and CEACAM6 (not shown) was not modified when the transfected cells were treated. An identical result was obtained with the GPI-anchored CD55 (data not shown). As a control experiment, the cell surface expression of the raft-associated

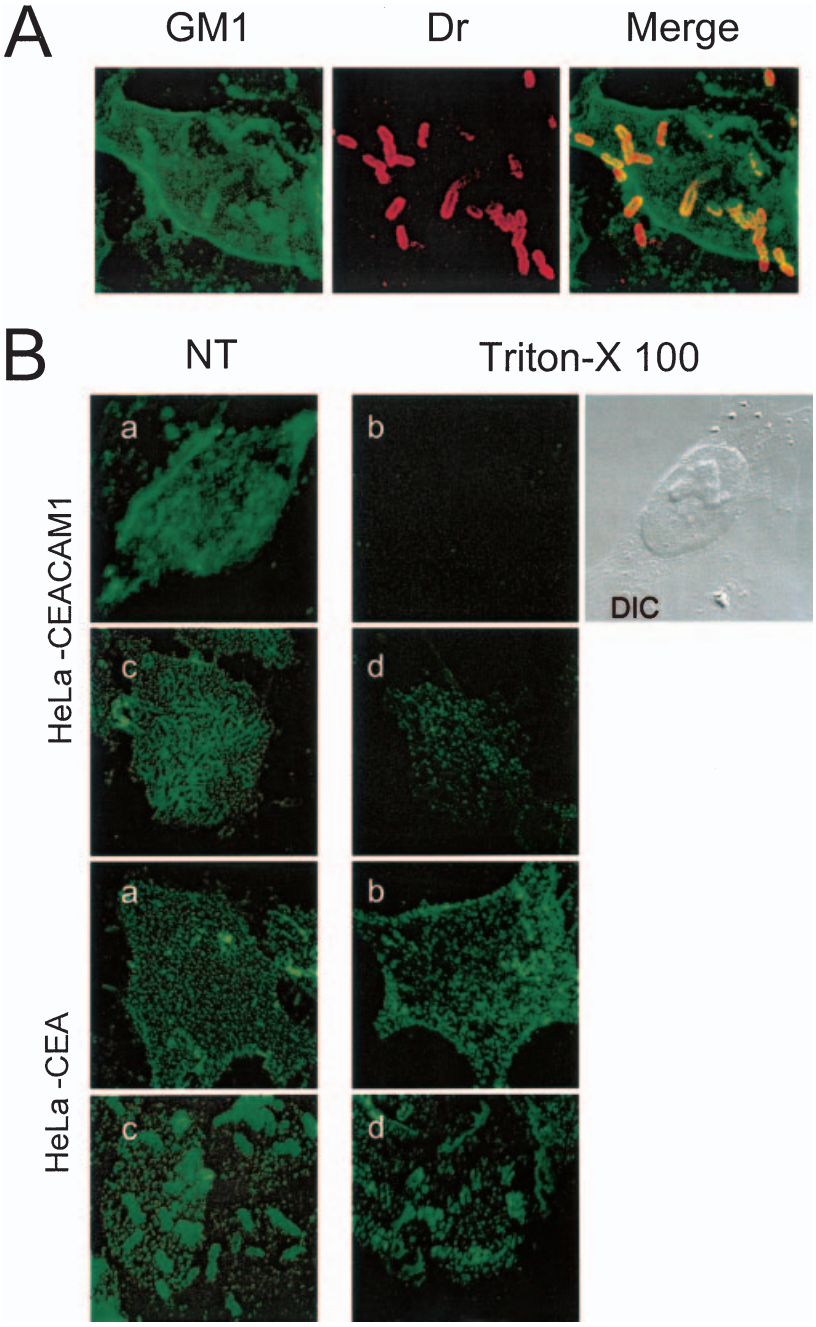


**Fig. 5.** Afa/Dr *E. coli* interacts with lipid rafts.
A. Recruitment of the raft marker GM1 around adhering Afa/Dr bacteria. Parental HeLa cells were infected with recombinant Dr-positive strain for 1 h at a concentration of $1 \times 10^7$ bacteria $ml^{-1}$. After infection, cells were treated with Triton X-100 as indicated in *Experimental procedures*. Samples were fixed and processed for double immunofluorescence labelling using CTB-FITC to label GM1 (green fluorescence) and anti-Dr adhesin antibody (red fluorescence). The superimposition of both immunofluorescences appears as yellow.
B. Confocal micrographs of HeLa-CEACAM1, HeLa-CEACAM3 and HeLa-CEA after Triton X-100 extraction in control cells and cells infected with recombinant Dr-positive strain. Cells were labelled with anti-CEACAM antibody before detergent extraction (NT) or after Triton X-100 treatment as indicated in *Experimental procedures*. For each CEACAM, (a) control untreated, (b) control Triton X-100-treated, (c) infected untreated and (d) infected Triton X-100-treated cells. Note the absence of green fluorescence after Triton X-100 extraction in HeLa-CEACAM1 cells. Corresponding cells in DIC panels are shown. In contrast, no modification of CEA labelling is observed in Triton X-100-treated HeLa-CEA uninfected cells. In Triton X-100-treated infected cells, no modification of CEA labelling is observed, while remaining CEACAM1 labelling around adhering bacteria is detected in infected HeLa-CEACAM1 cells. For all micrographs, projected optical sections (0.5 μm) are representative of the apical part of the cells.

molecule, ganglioside GM1, and the transmembrane protein, TfR (Rodgers and Rose, 1996), was examined in untreated and Triton X-100-treated HeLa cells. The GM1 labelling remained present when the vector-transfected HeLa cells were subjected to Triton X-100 treatment. In contrast, all the TfR labelling disappeared after treatment of the cells with Triton X-100 (data not shown). Altogether, these results show that Triton X-100 treatment fails to extract the molecules GM1, CEA and CEACAM6, but is efficient in extracting the molecules TfR, CEACAM1 and CEACAM3. This is consistent with the known GPI-anchored and transmembrane nature of these molecules respectively.

We next examined whether or not the clustered CEACAM receptors around adhering bacteria remain bacteria associated after Triton X-100 treatment (Fig. 5B). HeLa-CEACAM cell lines were infected for 1 h with Dr-positive bacteria, subjected to Triton X-100 treatment and then processed for immunofluorescence labelling and CLSM analysis. The results showed that the recruitment of the GPI-linked molecules, CEA (Fig. 5B) and CEACAM6, as well as CD55 (not shown), around adher-

ing bacteria was insensitive to detergent extraction. In contrast, the clustered CEACAM3 around adhering bacteria entirely disappeared after Triton X-100 treatment, as observed in uninfected cells (not shown). Surprisingly, a significant part of the bacteria-recruited transmembrane-linked CEACAM1 remained apparently observable as a punctuated labelling after Triton X-100 cell extraction at sites in which bacteria adhere (Fig. 5B), whereas the labelling totally disappeared in Triton X-100-treated uninfected cells. It should be noticed that this remaining labelling is quite different from that observed in infected cells without Triton X-100 extraction.

These combined results indicate that: (i) a mobilization of molecules within lipid rafts develops during Afa/Dr DAEC infection, based on the fact that the GPI-anchored CEA and CEACAM6 molecules and the raft-associated molecule, GM1, that were recruited around adhering Dr-positive bacteria, have the same extraction behaviour; (ii) a selective recruitment within lipid rafts of some transmembrane-linked molecules could take place during bacterial infection based on the fact that the transmembrane CEACAM1 remains associated in part with adhering Dr-positive bacteria after Triton X-100 extraction.

### *Recognition of CEACAMs by Afa/Dr-expressing bacteria is followed by a tight association of elongated microvilli-like extensions with adhering bacteria*

In order to examine the cellular consequence of the binding of Afa/Dr DAEC bacteria on to CEACAMs, we conducted experiments using scanning electron microscopy onto SEM of cultured CEACAM-expressing CHO cells infected with the Dr-expressing recombinant *E. coli* strains. We observed that, in CHO cells expressing CEA or CEACAM6, short microvilli-like extensions emanated from the cell surface (Fig. 6A). In infected cells, we observed that these microvilli-like extensions tightly associated with adhering bacteria. It is interesting to note that the microvilli-like extensions attached to the adhering bacteria appeared to be elongated compared with the short microvilli-like extensions present at the cell surface and without contact with the bacteria. In contrast, no attachment of microvilli-like extensions was triggered in infected CHO cell lines expressing the other CEACAM receptors. In particular, it is interesting to note that no attachment of microvilli-like extensions onto adhering bacteria was observed in CHO cells expressing CEACAM1, despite the fact that this molecule functions as a receptor for the Afa/Dr-I subfamily of adhesins.

In order to confirm the role of the Afa/Dr-I subfamily of adhesins in eliciting attachment of microvilli-like extensions, CHO-CEA cells were incubated with AfaE-III- or BSA-coated beads. As observed with Afa/Dr-expressing bacterial strains, AfaE-III-coated beads induced microvilli-like extensions whereas BSA-coated beads did not (Fig. 6B). This result, together with the above reported results, supports the hypothesis that the elongation of bacterial-attached microvilli-like extensions follows the recognition of CEA and CEACAM6 by the Afa/Dr-I adhesins.

We next investigated whether or not the elongated microvilli-like extensions attached to adhering Afa/Dr DAEC bacteria require an intact microfilament and/or microtubule networks. Treatment of both infected CEA- and CEACAM6-expressing CHO cells with the microtubule-disrupting drug nocodazole showed no modification in the attachment and size of the microvilli-like extensions (Fig. 6C, right), indicating that elongation of microvilli-like extensions following the recognition of these CEACAMs by Afa/Dr DAEC is not a microtubule-dependent event. We next determined the role of the F-actin cytoskeleton in the elongation of microvilli-like extensions following the recognition of CEACAM receptors by Afa/Dr adhesins. Treatment of the infected CEA- and CEACAM6-expressing CHO cells with the specific inhibitor of F-actin polymerization, cytochalasin D, showed a complete abolition of the elongated microvilli-like extensions entrapping adhering bacteria (Fig. 6C, left). Altogether, these results indicate that the Afa/Dr-elicited elongation of microvilli-like extensions following the recognition of CEACAM receptors is an F-actin cytoskeleton-related event.

We examined the cellular events after infection by Afa/Dr DAEC of epithelial cells expressing both CD55 and CEACAM molecules. Scanning electron microscopy of cultured vector-transfected HeLa cells showed that microvilli-like extensions emanating from the cell surface attach to the adhering Dr-positive *E. coli* (Fig. 7A). Demonstration that the recognition of CD55 is sufficient for the phenomenon to develop is given by the observation of elongated microvilli-like extensions attached onto Dr-positive *E. coli* in CD55-expressing CHO cells (Fig. 7B). At last, as reported above in CEACAM-transfected CHO cells, the Dr-induced microvilli elongation was blocked by cytochalasin treatment but not nocodazole (data not shown).

### *Point mutation in Dr adhesin at position 54 affects both CEACAM receptor recruitment and bacterial-elicited elongation of microvilli-like extensions*

It has been demonstrated previously that replacement of the aspartic acid residue at position 54 of the Dr adhesin by cysteine or glycine affects the mannose-resistant haemagglutination (MRHA), the type IV collagen binding and chloramphenicol sensitivity of binding, while retaining CD55-binding capability (Carnoy and Moseley, 1997). These D54 mutants lose CD55 and CEA clustering activity (Guignot *et al*., 2000). Moreover, it is interesting to note

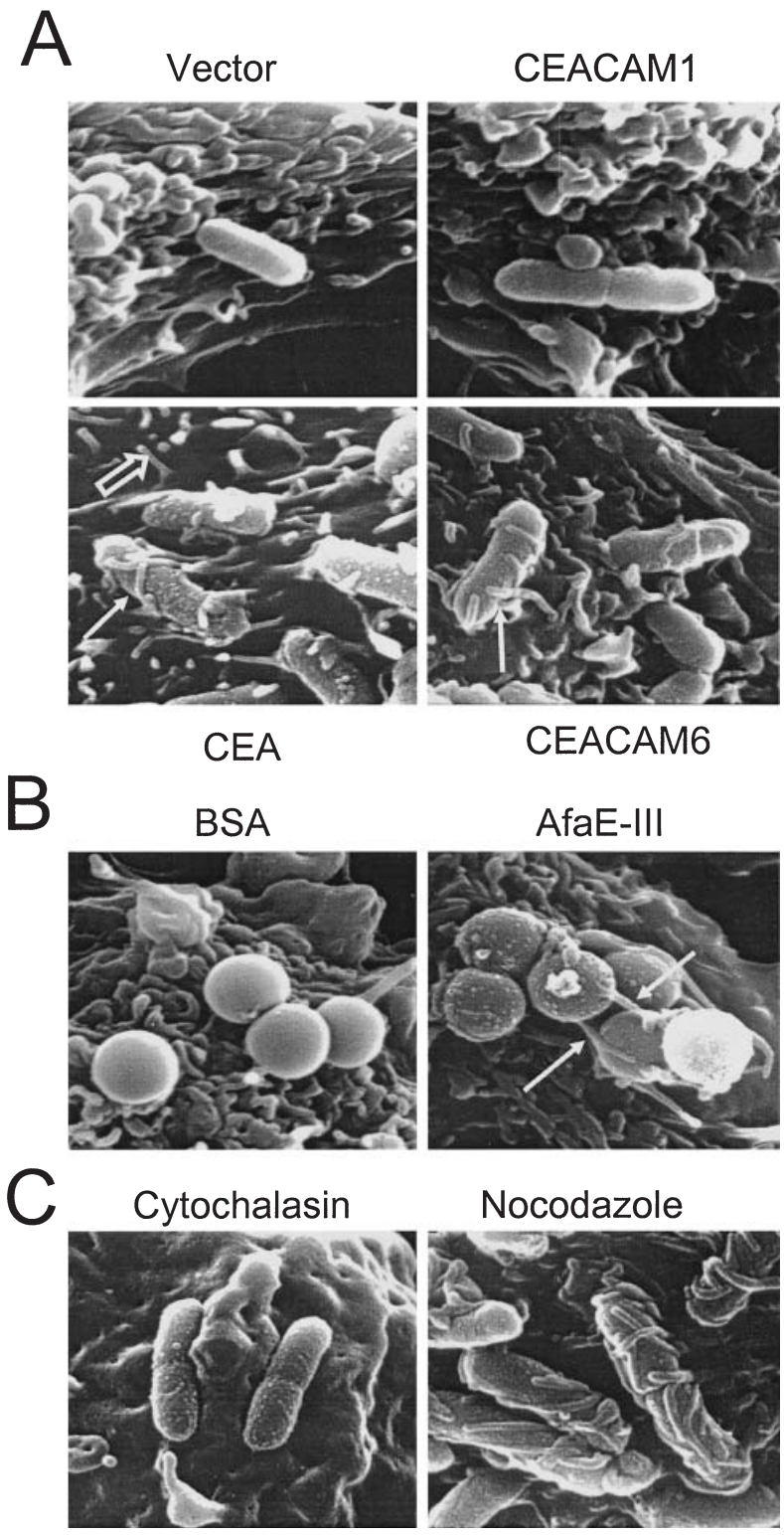


**Fig. 6.** Afa/Dr-expressing bacteria trigger ultrastructural modifications of the cell surface in CHO cells, depending on the expression of CEACAM receptors. Subconfluent monolayers of CHO (A) were infected with recombinant Dr-positive strain for 1 h at a concentration of $1 \times 10^7$ bacteria $ml^{-1}$. Scanning electron micrographs of infected CHO cells showing cellular protrusions extending from the cell surface of CHO-CEA and CEACAM6 tightly adhered to bacteria. No such protrusions were observed in CHO-CEACAM1 or CHO harbouring vector alone.
B. CHO-CEA cells incubated with BSA-coated or AfaE-III-coated beads for 1 h. Right: cellular microvilli extension adhering to AfaE-III-coated beads, while no such cellular protrusions were observed around BSA-coated beads. Images are representative of three independent experiments.
C. Cytochalasin D differentially inhibits Dr-mediated cellular membrane protrusions. After a 1 h preincubation period with either 1 $\mu g\ ml^{-1}$ cytochalasin D or 10 $\mu M$ nocodazole, cell lines were infected with recombinant Dr-positive strain for 1 h. Scanning electronic micrographs of cytochalasin D-treated CHO-CEA cells show the complete abrogation of Afa/Dr-dependent membrane extensions. Similar observations were obtained with CHO-CEACAM6 cells (not shown). Images are representative of three independent experiments.

that this amino acid residue is highly conserved among members of the Afa/Dr-I subfamily (Garcia *et al*., 1996). We thus investigated whether Dr-D54C and Dr-D54G adhesin mutants retain their capacity to adhere to CEACAM receptor-expressing cells and to elicit CEACAM recruitment and microvilli-like extensions. For this purpose, mutated D54C and D54G Dr adhesin were expressed in *E. coli* strain AAEC185 lacking expression

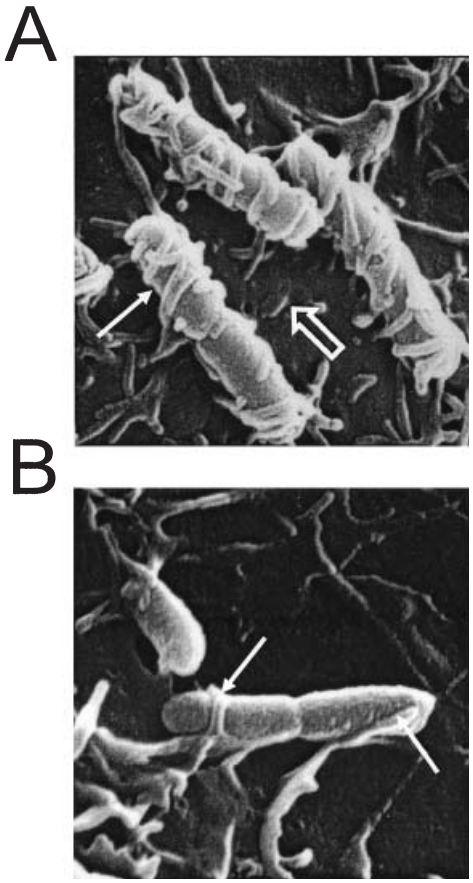


**Fig. 7.** Dr-positive *E. coli* trigger elongated microvilli formation in CD55-expressing cells.
A. Subconfluent HeLa cell monolayers (which express any CEACAM) were infected with Dr-positive strain for 1 h at a concentration of $1 \times 10^7$ bacteria $ml^{-1}$. Scanning electron micrographs of infected cells showing cellular protrusions interacting with adhering bacteria.
B. Observation of elongated microvilli interacting with adhering Dr-positive bacteria in CD55-expressing CHO cells. Images are representative of three independent experiments.

of type 1 pili. A dramatic decrease in the cell association of the Dr-D54G mutant was observed in CHO-CEACAM1, CHO-CEA or CHO-CEACAM6 cells compared with a recombinant *E. coli* strain expressing Dr adhesin (Fig. 8A). Similar results were obtained with the Dr-D54C mutant (data not shown). Recruitment of CEACAM receptors examined by indirect immunofluorescence and CLSM showed that both the remaining adherent Dr-D54C and D54G mutants did not elicit CEACAM recruitment (Fig. 8B). SEM examination of the cell surface of CHO-CEA or CHO-CEACAM6 cell lines infected with the mutant strains Dr-D54C and Dr-D54G revealed that no microvilli-like protrusions attached onto adherent Dr-D54C and D54G mutants (Fig. 8C). These combined results indicate that amino acid 54 is essential: (i) for the adhesin–CEACAM receptor interaction; (ii) for eliciting recruitment of these molecules at the site of bacterial adhesion; and (iii) for promoting the cytoskeleton-dependent cell protrusion entrapping adhering bacteria.

### *Dr-expressing bacteria trigger activation of Cdc42 Rho GTPase, which plays a role in microvilli-like protrusions mediated by CEA*

In order to gain insight into the signalling pathways involved in microvilli-like protrusions induced by Afa/Dr DAEC–CEA interaction, we attempted to identify elements known to induce cell actin reorganization. We first tested the effect of *Clostridium botulinum* C3 and *C. difficile* B toxins, which inactivate Rho GTPase proteins, in DAEC-elicited microvilli-like protrusions observed in CEA-expressing CHO cells. Cells pretreated for 2 h with *C. botulinum* TAT-C3 toxin fusion protein (TAT-C3) (Sauzeau *et al.*, 2001) and purified *C. difficile* b toxin (toxin B) (von Eichel-Streiber *et al.*, 1987) were infected for 1 h with a Dr-positive strain and then analysed by SEM. CEA-mediated microvilli-like protrusions were abrogated in the presence of toxin B (Fig. 9A) compared with untreated cells (not shown). Similar results were observed when cells were pretreated with TAT-C3 toxin (data not shown). These results suggest that Rho GTPases play a role in Dr-induced CEA-mediated cell protrusions. In order to determine whether Cdc42 and Rac Rho GTPases are implicated in this phenomenon, we specifically precipitated active Cdc42-GTP and Rac-GTP in CHO-CEA cells infected with a Dr-positive strain using glutatione-*S*-transferase (GST)–CRIB fusion proteins with each specific binding motif for Cdc42 (GST-WASP) and Rac (GST-PAK). Affinity precipitation of Cdc42-GTP in CHO-CEA cells revealed a twofold and threefold increased level of the protein at 30 and 60 min of cell infection, respectively, compared with uninfected cells (Fig. 9B and C). In contrast, no increase in Rac-GTP with time could be evidenced (not shown). It must be noted that increasing levels of Cdc42-GTP could be measured in spite of a high level of activation observed in serum-starved uninfected cells. These results clearly indicate that Dr-expressing bacteria activate Cdc42 GTPase, which in turn participates in microvilli elongation at the cell surface attached to adherent bacteria, and suggests a role for the receptor CEA in this phenomenon.

### *ERM are recruited around adherent bacteria and became phosphorylated*

As ERM play an important role in actin reorganization, and Rho GTPases act upstream activating ERM, we first tested, by immunofluorescence analysis, whether ERM were recruited around adherent DAEC in CEA-expressing CHO cells. As shown in Fig. 10A, ERM (green fluorescence) were observed around adherent bacteria and

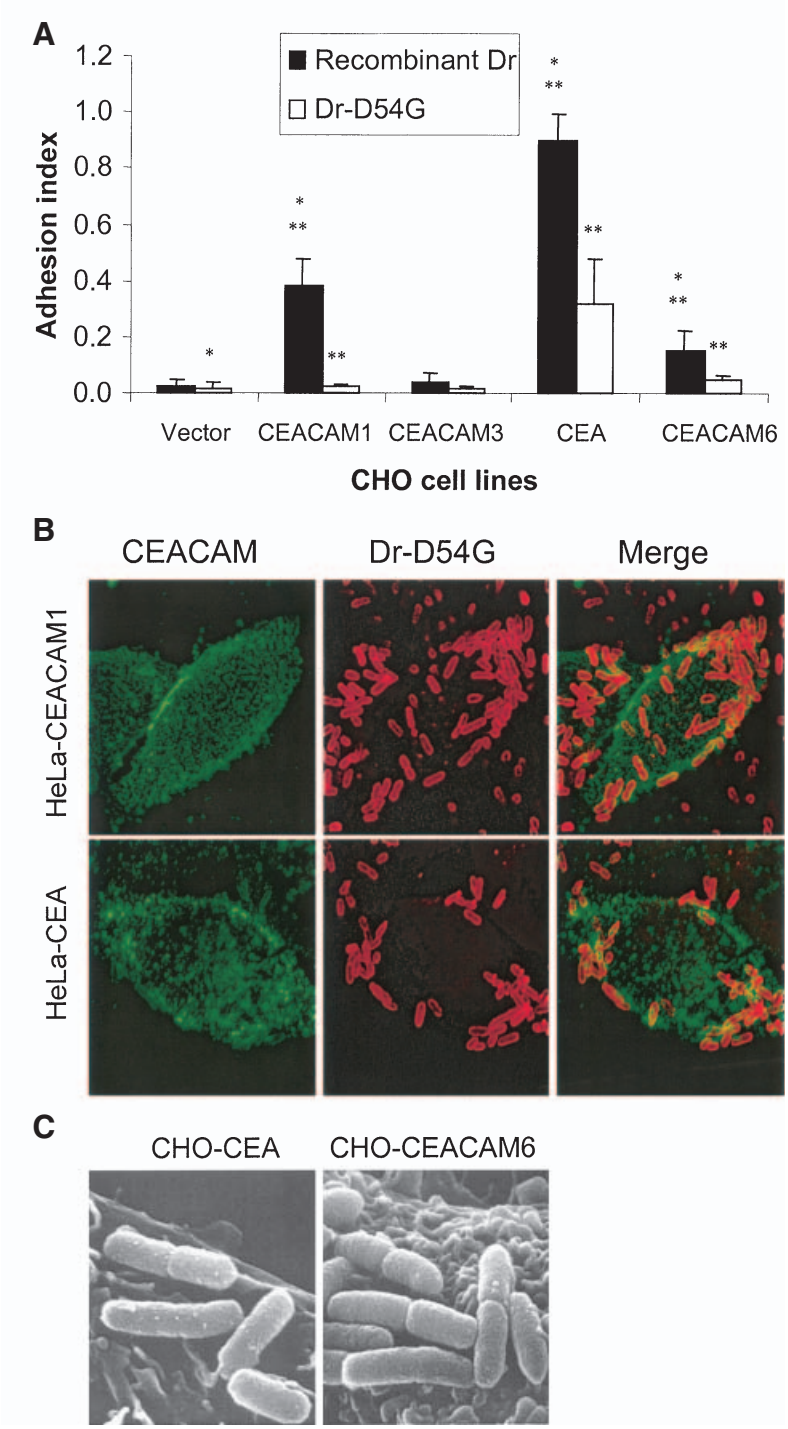


**Fig. 8.** D54G point mutation in Dr adhesin affects bacterial adhesion to CEACAM receptors, as well as CEACAM recruitment and bacterial-elicited cellular protrusions.
A. CHO cell lines expressing CEACAM1, CEACAM3, CEA or CEACAM6 or vector alone-containing CHO cells were plated to confluency. Cell monolayers were inoculated with recombinant Dr-positive *E. coli* and recombinant *E. coli* expressing mutated Dr-D54G ($5 \times 10^8$ bacteria $ml^{-1}$) and centrifuged to synchronize infections as in Fig. 1. After 1 h at 37°C, monolayers were processed to determine cell-associated colony-forming units (CFU) as described in *Experimental procedures*. Indexes of cell-associated CFU per cell line were calculated on the basis of the higher cell association level (about $1.5 \times 10^7$ CFU $ml^{-1}$) obtained for Dr-positive strain in CHO-CEA in one of the experiments. Data shown are mean results ± SD of triplicate samples for each cell line. Data are representative of at least three independently performed experiments using either Dr or Dr-D54G in each cell line. * and $^{**}P < 0.01$.
B. Confocal projected sections showing adherent Dr-D54G bacteria in HeLa-CEACAM1 and CEA cell lines. After infection, samples were fixed and processed for double immunofluorescence labelling using anti-CEACAM antibody (green fluorescence) and anti-Dr adhesin antibody (red fluorescence). The superimposition of both immunofluorescences is presented on the right. Optical sections (0.5 μm) representative of the apical part of the cells are shown.
C. Scanning electron micrographs of Dr-D54G-infected CHO-CEA and CEACAM6 cells showing the absence of cellular protrusions extending from the cell surface.

perfectly co-localized with CEA (red fluorescence). Interestingly, a similar pattern of fluorescence around bacteria was observed when phosphorylated ERM (p-ERM) were specifically labelled using an anti-p-ERM antibody that recognizes phosphorylated Thr-558 of moesin, phosphorylated Thr-567 of ezrin and phosphorylated Thr-564 of radixin (Fig. 10B). These results indicate that activated ERM accumulate around adhering bacteria, and suggest that this ERM activation might be associated with activation of Rho GTPases. To answer this question, we treated cells with GTPase inhibitors TAT-C3 and toxin B before infection and then tested the p-ERM and ERM labelling by immunofluorescence as described. The results in Fig. 10C clearly show a 50% decrease in p-ERM labelling around adhering bacteria in GTPase inhibitor-treated cells, thus indicating the involvement of Rho GTPases,

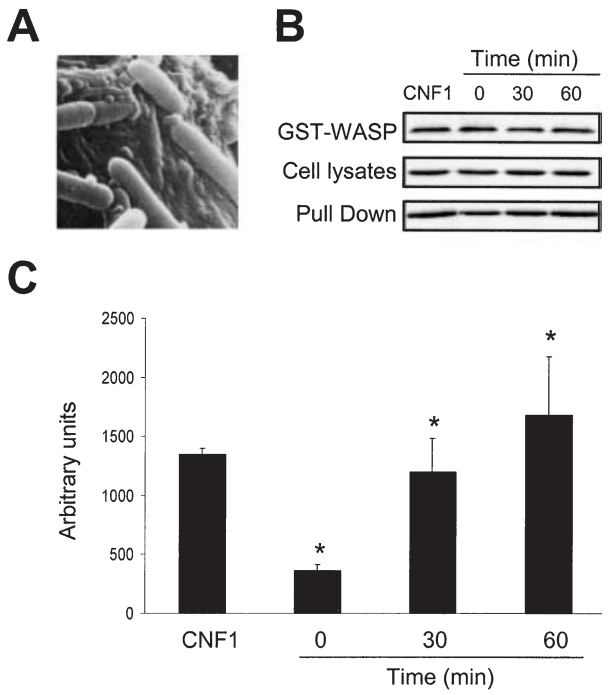


**Fig. 9.** Dr-positive *E. coli* activates Cdc42-GTP in CHO-CEA cells.
A. Inhibition of Rho GTPases affects Dr-dependent elongation of microvilli. Toxin B of *Clostridium difficile* inhibits Dr-mediated microvilli-like extensions. After a 2 h preincubation period with 100 ng ml$^{-1}$ toxin B of *C. difficile*, serum-starved CHO-CEA cells were infected with recombinant Dr-positive strain for 1 h. Scanning electronic micrograph shows the abrogation of Afa/Dr-dependent membrane extensions.
B. Serum-starved CHO-CEA cells were infected with Dr-positive strain for 30 and 60 min at a concentration of $1 \times 10^8$ bacteria ml$^{-1}$. Cells incubated with CNF1 for 2 h were used as a positive control. After incubation at 37°C, cells were processed to determine Cdc42 activation as described in *Experimental procedures* (pulldown experiments). Western blots are representative of three experiments.
C. Graphic shows measurement of blot densities from three pulldown experiments (means ± SD). *$P < 0.01$ compared with control (0).

and probably Cdc42, in activation of ERM during DAEC infection. In contrast, GTPase inhibitor treatment did not affect ERM recruitment around adhering bacteria, suggesting that this event is not dependent on Rho GTPases.

In order to confirm the ERM phosphorylation following DAEC infection, we analysed the phosphorylation of ERM after DAEC infection in CEA-expressing CHO cells by immunoblotting. As expected, a greatly increased level of p-ERM was observed at 30 and 60 min of infection compared with uninfected cells (Fig. 10D). Moreover, consistent with the immunofluorescence results, in similar experiments conducted in CHO-CEA cells pretreated for 2 h with GTPase inhibitors (toxin B and TAT-C3), we found a complete inhibition of time-increasing DAEC-induced ERM phosphorylation (Fig. 10D), thus confirming activation of ERM during DAEC infection in CEA-expressing cells.

## Discussion

We analysed here the interactions of Afa/Dr DAEC with members of the family of CEACAM receptors. Our results highlight the ability of Afa/Dr DAEC to recognize different CEACAMs, indicating that these bacteria have developed adhesins to target the CEACAM family, becoming capable of colonizing human mucosal epithelia. We first demonstrated that, even if all members of the Afa/Dr family of adhesins bind to CD55 (Nowicki *et al*., 1993; Pham *et al*., 1997), only a subfamily of these adhesins, named here Afa/Dr-I subfamily, which includes Dr, F1845 and AfaE-III adhesins, are involved in recognition of CEA, CEACAM1 and CEACAM6. In contrast, members of the Afa/Dr-II subfamily, which includes AfaE-I and Dr-II adhesins, bind poorly to CEACAMs although they recognize CD55 as a receptor. This differential CEACAM recognition by the Afa/Dr-I subfamily of adhesins suggests that these adhesins evolved independently in Afa/Dr DAEC bacteria to develop supplementary ways of host cell colonization. Consistent with this notion is the fact that, unlike Afa/Dr-II adhesin members (Labigne-Roussel *et al*., 1984; Pham *et al*., 1997), Afa/Dr-I adhesin members (Nowicki *et al*., 1987; Bilge *et al*., 1989; Le Bouguenec *et al*., 1993) share high homology in their primary peptide sequences.

The results presented here confirm and extend our previous report showing that CEA acts as a receptor for Afa/Dr DAEC (Guignot *et al*., 2000). The observation here that CEACAM1 is engaged as a receptor for Afa/Dr-I adhesin members is intriguing. It has been reported recently that gonococci Opa proteins engage CEACAM1 for signalling. For the invasion of epithelial cell lines via CEACAM1, a pathogen-directed reorganization of the actin cytoskeleton is not required (Billker *et al*., 2002), and a localized and transient accumulation of the class I PI3K product phosphatidylinositol 3,4,5-trisphosphate at sites of bacterial engulfment was observed (Booth *et al*., 2003). In addition, it has been reported recently that the cytoplasmic domain of CEACAM1 is associated with protein tyrosine kinases of the src family (Skubitz *et al*., 1995), as well as with the tyrosine phosphatases SHP-1 and SHP-2 (Huber *et al*., 1999). This same domain contains an ITIM (immune receptor tyrosine-based inhibitory motif)-inhibiting domain, which could be responsible for the inhibitory effects on cell growth (Chen *et al*., 2001a; Singer *et al*., 2000). When CEACAM1 recognized by *Neisseria gonorrhoeae* Opa proteins associates with the tyrosine phosphatases SHP-1 and SHP-2, which implicate the ITIM domain, activation and proliferation of CD4$^+$ T lymphocytes is suppressed (Boulton and Gray-Owen, 2002). Whether the engagement of CEACAM1 as a receptor for Afa/Dr-I adhesin members results in the same cell responses reported for gonococci infection remains to be demonstrated.

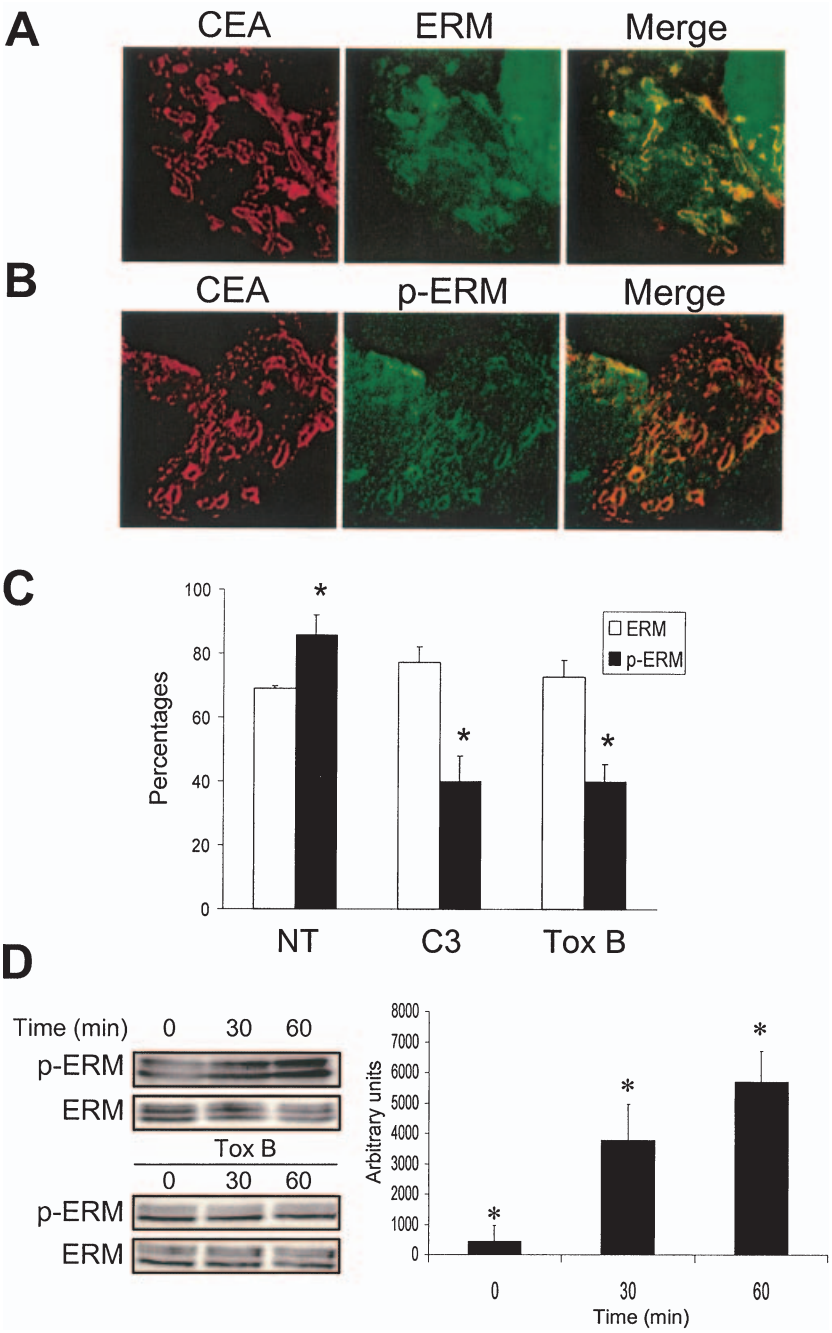


**Fig. 10.** ERM and p-ERM are recruited around adhering Dr-positive strain in CHO-CEA cells. Subconfluent monolayers were infected with Dr-positive strain for 1 h at a concentration of $1 \times 10^7$ bacteria ml$^{-1}$. After infection, cells were fixed and processed for double immunofluorescence labelling using anti-CEACAM antibody (red fluorescence) and anti-ERM (A) or phospho-ERM (B) (green fluorescence). The superimposition of both immunofluorescences appears as yellow. Adherent bacteria at the place of recruited molecules were visualized by transmission microscopy (not shown).
C. Recruitment of ERM and p-ERM was measured after incubating cells with GTPase blockers. Before infection, cells were treated for 2 h with 100 ng ml$^{-1}$ toxin b of *C. difficile* (Tox B) or 25 μg ml$^{-1}$ recombinant *C. botulinum* TAT-C3 toxin fusion protein (C3). After 1 h of infection, double immunofluorescence labelling was carried out as in (B). Percentage of green haloes present around adherent bacteria was determined in different fields. $^*P < 0.01$ compared with infected untreated (NT) cells.
D. Serum-starved CHO-CEA cells were infected with Dr-positive strain for 30 and 60 min at a concentration of $1 \times 10^8$ bacteria ml$^{-1}$. After incubation at 37°C, cells were processed to determine ERM and p-ERM as described in *Experimental procedures*. Similar experiments were conducted in CHO-CEA cells pretreated for 2 h with toxin B of *C. difficile* (Tox B) as indicated above. Western blots are representative of three experiments. Graphic shows measurement of blot densities (means ± SD) from three experiments in which increase in p-ERM was revealed. $^*P < 0.01$ compared with control (0).

Some pathogens involve lipid raft microdomains at the initial steps of host cell colonization (Rosenberger *et al.*, 2000; van der Goot and Harder, 2001; Duncan *et al.*, 2002). For example, the involvement of lipid raft microdomains in pathogenicity has been demonstrated for *Shigella* (Lafont *et al.*, 2002), FimH-expressing uropathogenic *E. coli* (Baorto *et al.*, 1997; Shin *et al.*, 2000), *Mycobacterium* spp. (Gatfield and Pieters, 2000), *Chlamydia trachomatis* (Norkin *et al.*, 2001) and *Salmonella* (Garner *et al.*, 2002). Proteins attached to membranes by a GPI anchor are known to localize mainly at the plasma membrane, where they partition predominantly in cholesterol-rich domains (Brown and London, 2000). We observed here the partition of GPI-anchored DAEC-binding molecules CD55, CEA and CEACAM6 within detergent-resistant lipid microdomains in uninfected cells, and then demonstrated that these molecules remain within these microdomains after their recruitment during bacterial adhesion. In contrast, transmembrane CEACAM1 and CEACAM3 did not resist detergent extraction, indicating their non-raft partition in resting uninfected cells. Interestingly, in Afa/Dr-infected cells, whereas CEACAM3

remained detergent sensitive as completely extracted, CEACAM1 became partially detergent resistant, indicating that this receptor became strongly associated with lipid rafts after cell infection. As CEACAM1 associates with actin (Schumann *et al*., 2001), the insolubility of this receptor in Triton X-100 could result from its association with the actin cytoskeleton. Several pathogens mobilizing lipid raft-associated molecules (Baorto *et al*., 1997; Shin *et al*., 2000; Garner *et al*., 2002; Lafont *et al*., 2002) use an actin-dependent mechanism for internalization (Knodler *et al*., 2001; Martinez and Hultgren, 2002). Like Afa/Dr DAEC (Goluszko *et al*., 1997; Guignot *et al*., 2001), it is interesting to note that no pathogen-directed reorganization of the actin cytoskeleton is required for gonococci invasion of epithelial cell lines via the GPI-anchored protein CEACAM6 (Billker *et al*., 2002). Lipid rafts present at the cell membrane function to provide a platform for the concentration of signalling molecules (Kurzchalia and Parton, 1999; Smart *et al*., 1999; Ilangumaran *et al*., 2000; Simons and Toomre, 2000). Interestingly, it has been observed previously that CD55, one receptor of the Afa/Dr adhesins (Nowicki *et al*., 1993), is raft associated and immunoprecipitates with the src-like protein tyrosine kinases p56lck and p59fyn (Stefanova *et al*., 1991; Shenoy-Scaria *et al*., 1992). The observation here that CEACAM, CEA and CEACAM6 associate together with a lipid raft marker around adhering bacteria expressing Afa/Dr-I adhesin members indicates that these GPI-anchored proteins are probably involved in cell signalling in targeted host cells.

Electron microscopy analysis of the Afa/Dr DAEC-infected CEACAM-expressing CHO cell lines allowed us to demonstrate that the recognition of CEA and CEACAM6, as well as CD55, but not CEACAM1, by Afa/Dr-I adhesin members is followed by elongation of microvilli-like extensions attached to the adhering bacteria. Interestingly, it has been observed previously that microvilli of the brush border in human intestinal cells become elongated after infection by Afa/Dr DAEC (Bernet-Camard *et al*., 1996). The role of the actin cytoskeleton in this cell response was highlighted by a biochemical approach using cytochalasin D, known to affect the F-actin microfilament network, and by the experiments conducted with the D54-Dr mutants. Elongation of microvilli-like extensions attached to the adhering bacteria was abolished when the infected cells expressing CEA or CEACAM6 were treated with the actin blocker cytochalasin D. In addition, consistent with previous results showing that D54-Dr mutants lose the actin-dependent cell response compared with Dr adhesin (Peiffer *et al*., 2000), we demonstrated the relevance of residue 54 in the Dr adhesin-elicited actin-dependent cell responses through the recognition of CEACAMs. Indeed, the results showed that, in infected CHO-CEA or CHO-CEACAM6 cells, the remaining adherent D54-Dr mutants showed no bacterial-attached microvilli-like extensions and no recruitment of CEACAMs. These combined results indicate that cell signalling following the recognition of CEA and CEACAM6, as well as CD55, by Afa/Dr-I adhesin members is involved in the actin-dependent elongation of microvilli-like extensions

The fact that Afa/Dr-I adhesin members are capable of binding to CEACAM receptors suggests that the engaged CEACAMs could be responsible for activation of cell signalling pathways involved in cellular responses during Afa/Dr DAEC infection. Indeed, the role of CEACAM receptors during bacterial infection in triggering cell responses has been demonstrated recently in elegant and well-conducted experiments that have given a comprehensive mechanism of pathogenicity for *Neisseria gonorrhoeae* and *Neisseria meningitidis*. In order to colonize diverse human mucosal epithelia, this human-specific, Gram-negative pathogen develops a combined strategy that involves the phase-variable expression of a large panel of adhesive proteins, including colony opacity-associated (Opa) proteins (Dehio *et al*., 2000; Merz and So, 2000). Four CEACAM receptors, CEACAM1, CEACAM3, CEA and CEACAM6, act as receptors for Opa proteins (Virji *et al*., 1996a,b; 1999; 2000; Bos *et al*., 1997; 1998; 1999; Popp *et al*., 1999; Virji, 2000). *Neisseria* that express CEACAM-binding Opa variants adhere to and invade human epithelial cell lines expressing recombinant and endogenous CEACAM receptors, and primary endothelial cells expressing CEACAM1 (Virji *et al*., 1996a,b; Chen *et al*., 1997; Gray-Owen *et al*., 1997a,b; Billker *et al*., 2000; 2002; Muenzner *et al*., 2000). In polarized T84 intestinal monolayers, CEACAM1, CEA and CEACAM6 mediate the invasion and subsequent transcytosis of gonococci by an intracellular route (Wang *et al*., 1998). In an *in vitro*-differentiated, neutrophil-like cell line, opsonization-independent phagocytosis of gonococci expressing the CEACAM-binding Opa requires the down-regulation of the host cell tyrosine phosphatase SHP-1, concomitant with increases in the tyrosine phosphorylation of several cellular proteins, the activation of src family tyrosine kinases and the small GTPase Rac (Hauck *et al*., 1999; 2000).

Recruitment of CEACAM3, which does not act as a receptor for Afa/Dr-I adhesin members, around adhering Afa/Dr DAEC bacteria suggests that the CEACAM3-associated cell signalling pathway could play a role in internalization of bacteria. Ligation of CEACAM3 by gonococci Opa proteins triggers a dramatic but localized reorganization of the host cell surface, leading to highly efficient actin-dependent engulfment of the bacteria (Billker *et al*., 2002; McCaw *et al*., 2003) in a process regulated by the small GTPases Rac1 and Cdc42, but not

by Rho (Billker *et al*., 2002). The cytoplasmic domain of CEACAM3 contains an ITAM (immune receptor tyrosine-based activating motif) reminiscent of that found within certain phagocytic Fc receptors, which could be involved in modifying cell function via Syk kinase and phospholipase C (Chen *et al*., 2001b). It has been demonstrated that two tyrosine residues of the ITAM in CEACAM3 are essential for internalization (Billker *et al*., 2002) and that phosphatidylinositol 3-kinases (PI3Ks) are needed in CEACAM3-mediated gonococcal uptake as well as for phagosomal maturation and acidification, which are required for optimal bacterial killing (Booth *et al*., 2003). However, it has been demonstrated recently that the CEACAM3-associated cell signalling pathway modulates actin-dependent cellular events, leading to highly efficient gonococci engulfment by a process regulated by the small GTPases Rac1 and Cdc42 (Billker *et al*., 2002). The fact that, unlike gonococci, Afa/Dr DAEC internalization is not actin dependent but is microtubule dependent (Goluszko *et al*., 1997; Guignot *et al*., 2001) raises the question of whether or not CEACAM3 plays a role in the microtubule-dependent internalization of Afa/Dr DAEC, and further experiments are needed to document this question.

Observation here that Afa/Dr-I adhesin members are capable of binding to CEA, recognition followed by F-actin-dependent elongation of microvilli-like extensions, suggests that engaged GPI-anchored receptor could be responsible for the activation of cell signalling pathways. Currently, no cell signalling pathway has been reported associated with CEA. It has been reported previously that the small G proteins superfamily that bind the GTP (GTPases) including the Ras, Rho, Rab, Sar1/Arf and Ran families, regulates the reorganization of the cell actin cytoskeleton. In particular, the formation of filopodia that resemble microvilli results from the localized elongation of the cell membrane that is controlled by Cdc42, a member of the Rho family (Castellano *et al*., 1999; Takai *et al*., 2001). Interestingly, it has been reported recently that bacterial recognition of CEACAM receptors by Opa proteins is followed by the activation of several small GTPases (Hauck *et al*., 1999; 2000; Billker *et al*., 2002). Here, we demonstrate for the first time the CEA-mediated activation of Cdc42 GTPase by Dr-positive bacteria. We provide evidence that this activation of Cdc42 seems to play a role in the activation of the actin-associated molecules ERM and, therefore, in DAEC-elicited microvilli-like elongation, as blockage of GTPases by means of Clostridia toxins inhibits phosphorylation of ERM as well as microvilli elongation in CEA-expressing cells. Moreover, as no activation of Rac in pulldown experiments or Rho in gel shift experiments (unpublished results) was observed, all these described phenomena could be attributed to activation of Cdc42 GTPase, according to previous reports showing a role for Cdc42 in the formation of microvilli-like structures (Gauthier-Rouviere *et al*., 1998; Eugene *et al*., 2002).

On the basis of the capacity of GTPase inhibitors to block ERM phosphorylation but not ERM recruitment, our data suggest that the first step in recruitment is not dependent on Rho GTPases. We thus hypothesize here that these events occur as follows: the recruitment of ERM molecules underneath adherent bacteria is followed by their Cdc42-mediated phosphorylation and subsequent actin nucleation, leading to the formation of the microvilli-like membrane protrusions in DAEC-infected epithelial cells. Otherwise, as Rho GTPases seem not to be involved in early steps of infection, other effectors leading to the movement of membrane-associated molecules, including CEACAM receptors and ERM, towards bacterial adherence sites could be involved. Therefore, further investigation will aim at identifying the signalling pathway involved in the recruitment of ERM proteins and adhesin receptors elicited by Afa/Dr adhesin-mediated adhesion of DAEC.

In conclusion, the results reported in the current study provide new insights into the mechanisms of Afa/Dr DAEC pathogenicity. We have identified a novel interaction between some Afa/Dr adhesins, the Afa/Dr-I adhesin members, and several CEACAM receptors present on distinct human target cells, particularly on intestinal epithelial cells and leucocytes. Targeting of CEACAMs would also be expected to lead to the host signalling mechanisms, which could result in a varied cell response. Our study also provides further evidence that Afa/Dr-I adhesin members promote recruitment of their different receptors by means of a probable association within lipid rafts. Further studies will be required to elucidate the mechanisms by which Afa/Dr DAEC elicits cell signalling cascades from these raft platforms during infection.

## Experimental procedures

### *Bacterial strains and growth conditions*

The *E. coli* strains used in this study and their origin are listed in Table 1. Recombinant Afa/Dr-expressing *E. coli* strains were obtained by transforming the *E. coli* AAEC185 strain (Blomfield *et al*., 1991), which lacks type 1 pili, with the recombinant plasmids that encode Dr, D54C and D54G mutants (Carnoy and Moseley, 1997), Dr-II (Pham *et al*., 1997), F1845 (Bilge *et al*., 1989), AfaE-I (Labigne-Roussel *et al*., 1985) and AfaE-III (Garcia *et al*., 1994) adhesins.

Stock cultures were maintained on 10% glycerol at −80°C. Before the experiments, bacterial strains were transferred onto fresh Luria-Bertani (LB) agar (Difco Laboratories) and incubated at 37°C for 24 h. For each experiment, bacteria were subcultured in LB broth at 37°C for 18 h with appropriate antibiotics. On the day of the experiment, bacteria were washed three times with sterile phosphate-buffered saline (PBS) and recovered with Hanks' balanced salt solution

(HBSS, Invitrogen). Bacterial cells were counted in a Salumbini chamber and adjusted to the desired concentration.

### *Cell lines and culture conditions*

Stably transfected HeLa cell lines expressing human recombinant CEACAMs or containing the expression vector alone (HeLa-SFFV.neo) were described previously (Nagel *et al*., 1993; Gray-Owen *et al*., 1997b). Selected populations of transfected Chinese hamster ovary (CHO) cell lines expressing human recombinant CEACAMs or containing the expression vector alone were obtained by lipofection of CHO K1 with different CEACAM cDNAs (or vector alone) in the self-replicating pCEP4 vector. Cells were selected for 2 weeks in 600 μg ml$^{-1}$ hygromycin B, and CEACAM-expressing cells were selected twice using M-450 Dynabeads coated with appropriate antibodies. CEACAM expression was detected in more than 80% of cells for each population as observed by FACS analysis (data not shown). Stably transfected CHO cells expressing human CD55 cDNA (DAF/A9) were kindly donated by D. Lublin (School of Medicine, Washington University, WA, USA) (Lublin and Coyne, 1991).

Transfected HeLa clones were grown in RPMI-1640 with L-glutamine (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen) and 500 μg ml$^{-1}$ G418 (Invitrogen) at 37°C in an atmosphere containing 5% $CO_2$. Transfected CHO cells were grown in DMEM-HamF12 with L-glutamine (Invitrogen) supplemented with 5% FCS and 100 μg ml$^{-1}$ hygromycin B (Invitrogen) at 37°C in an atmosphere containing 5% $CO_2$.

All cell lines were harvested from the flask with trypsin (0.5 mg ml$^{-1}$)/EDTA (0.2 mg ml$^{-1}$), washed once with medium and seeded into culture plates (TPP, ATGC Biotechnologie) at the desired cell densities and incubated at 37°C in an atmosphere containing 5% $CO_2$ before experiments.

### *Infection and treatment of cell lines*

Cells were grown in 24-well cell culture plates to confluency for plating assays or 60–70% confluency for immunofluorescence experiments on the day of infection. Cell culture in flasks to confluency was performed for cell lysates tested in pulldown and Western blot experiments. Monolayers were washed twice with PBS and then infected. Plates were then centrifuged at 120 *g* for 5 min to synchronize infections. During infection, cells were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. At the end of infection, monolayers were washed four times with sterile PBS to remove non-adhering bacteria and processed.

In order to determine the role of microfilament (MF) or microtubule (MT) network, depolymerizing drugs were used. Depolymerization of MF network was performed by culturing cells alone or with 1 μg ml$^{-1}$ cytochalasin D (Sigma) for 1 h before infection. MT depolymerization was achieved by using 10 μM nocodazole for 1 h before infection (Sigma).

Inhibition of Rho GTPases was obtained using recombinant *C. botulinum* TAT-C3 toxin fusion protein generously provided by J. Bertoglio (U-461 INSERM) and *C. difficile* B toxin provided by I. Just (Hanover Medical School, Germany). Cell monolayers were incubated with toxins for 2 h before infection.

### *Plating assay*

In order to determine cell-associated colony-forming units (CFU), infected monolayers ($5 \times 10^8$ bacteria ml$^{-1}$) were lysed with 1% saponin for 10 min. Bacteria were suspended by vigorous pipetting, and CFU in the lysates were determined by plating of serial dilutions.

### *Immunofluorescence and confocal microscopy*

Cell lines cultured on glass coverslips (24-well tissue culture plates) were fixed in 3% paraformaldehyde in PBS for 15 min at room temperature, washed three times with PBS, treated with 50 mM $NH_4Cl$ for 10 min for aldehyde function neutralization and then blocked with PBS containing 0.2% gelatin. Monolayers were incubated with primary antibodies diluted in PBS–gelatin for 1 h. After washing, cells were incubated for 45 min with the secondary antibodies diluted in PBS–gelatin. Finally, cells were washed three times in PBS, mounted in Vectashield (Citifluor Laboratories) and examined by conventional epifluorescence microscopy using a Leitz Aristoplan microscope (Leica) and confocal microscopy using a Zeiss confocal laser-scanning microscope 510 with its corresponding software (LSM version 2.5).

CEACAM clustering around adhering bacteria was detected on unpermeabilized cell monolayers by indirect immunofluorescence labelling using monoclonal antibodies (mAbs) D14HD11, which recognizes CEACAM1, CEACAM3, CEACAM4, CEA and CEACAM6, and 80H3, which recognizes CEACAM8. These antibodies were both generously provided by Dr Fritz Grunert, University of Freiburg, Germany. The CEACAM7-specific antibody was generously provided by Dr John Thompson (University of Freiburg, Germany). A FITC-conjugated anti-CD55 mAb (Bioatlantic) and an anti-transferrin receptor (Zymed) were used for labelling of these membrane molecules. Polyclonal anti-Dr antibody recognizing Dr, F1845 and AfaE-III adhesins was obtained from B. Nowicki (University of Texas, TX, USA). Polyclonal anti-p-ERM and anti-ERM (Santa Cruz Biotechnology) were used to detect the phosphorylated or not ezrin/radixin/moesin.

Alexa fluor-conjugated goat anti-mouse, chicken anti-goat and goat anti-rabbit antibodies (Molecular Probes) were used as secondary antibodies. Ganglioside GM1 labelling was performed using FITC-conjugated cholera toxin B subunit (CT-B, Sigma).

### *Detergent extraction of membrane proteins*

Monolayers of each cell line were cultured on glass coverslips in 24-well tissue culture plates. Cell infection was performed as indicated above. Infected or uninfected cells were washed four times with PBS to remove non-adhering bacteria and then incubated with 1% Triton X-100 for 5 min on ice before fixation in 3% paraformaldehyde. Preparations were processed for immunofluorescence as indicated above.

### *Electron microscopy*

Monolayers were prepared on glass coverslips in 24-well tissue culture plates. After infection, cells were fixed in 2.5%

glutaraldehyde in 0.1 M phosphate buffer for 30 min at room temperature. After washing, samples were post-fixed in 2% $OsO_4$ for 1 h and dehydrated in graded ethanol baths before critical-point drying with liquid $CO_2$. Coverslips were then coated with gold-palladium and observed with a Jeol JSM-840A scanning electronic microscope operated at 17 kV.

### *GST pulldown experiments*

The GST–WASP and GST–PAK fusion proteins containing the CRIB motif of WASP and PAK, respectively, were obtained as described previously (Haddad *et al.*, 2001). Affinity precipitation of Cdc42-GTP and Rac-GTP was performed as follows. Cell monolayers in flasks were washed twice with PBS and then cultured in serum-free medium for 5 h before infection. Flasks were inoculated with $1 \times 10^8$ bacteria $ml^{-1}$ and incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. At the end of infection, cells were washed with sterile and cold PBS to remove non-adhering bacteria and then lysed in solubilizing buffer containing 50 mM Tris, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$ and cocktail protease inhibitor (Sigma), pH 7.5, for 20 min at 4°C with gentle agitation. Cell lysates were centrifuged to remove insoluble materials. Protein concentration was determined by BC assay protein determination kit (Interchim). Purified GST fusion proteins were adsorbed (20 μg) onto glutathione-Sepharose beads (Amersham Pharmacia Biotech). Precleared lysates (250 μg) were rocked with coupled beads for 1 h at 4°C. Beads were washed four times with buffer containing 50 mM Tris, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$ and cocktail protease inhibitor (Sigma), pH 7.5, boiled in 4× Laemmli sample buffer. Bound Cdc42-GTP and Rac-GTP were analysed by Western blotting.

### *Preparation of cell lysates for detection of phosphorylated ERM*

Cell monolayers were cultured and infected as for GST pulldown experiments. At the end of infection, cells were washed with sterile and cold PBS to remove non-adhering bacteria and then lysed in buffer containing 50 mM Tris, 1% NP-40, 150 mM NaCl, 2 mM EDTA, 1 mM $Na_3VO_4$, 1 mM NaF, 6.25 mM NaPPi and cocktail protease inhibitor (Sigma), pH 7.5, for 30 min at 4°C with gentle agitation. After sonication, cell lysates were centrifuged to remove insoluble materials. Protein concentration was determined by BC assay protein determination kit (Interchim). Proteins (25 μg) were analysed by Western blotting.

### *Western blot*

Proteins were resolved by SDS–PAGE, and gels were transferred to polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech). For immunoblotting, membranes were washed with PBS 0.1% Tween, blocked in TBS (0.1% Tween, 3% BSA, 0.5% gelatin) and probed with specific antibodies overnight. Blots were then incubated with horseradish peroxidase-linked secondary antibody (Ozyme), followed by chemiluminescence detection, according to the manufacturer's instructions (Amersham Pharmacia Biotech). Autoradiographies were scanned and then quantified with SCION IMAGE software.

Polyclonal anti-Cdc42 (HS), anti-Rac1, anti-phospho-ERM and anti-ERM (Santa Cruz Biotechnology) were used to detect, respectively, the Rho GTPases and phosphorylated or not ezrin/radixin/moesin.

### *Statistical analysis*

All experiments were conducted in triplicate and repeated at least three times. Results were expressed as means ± standard deviation (SD). The statistical significance was determined by Student's *t*-test, and $P < 0.05$ was considered to be significant.

## Acknowledgements

We are grateful to R. Amsellem for her expert assistance in cell culture. We are also indebted to V. Nicolas for her kind help in confocal laser microscopy scanning analysis (Imagerie Cellulaire, IFR75-*ISIT*, Faculté de Pharmacie Paris XI) and M. Grasset (Service de Microscopie Electronique, Université Pierre et Marie Curie Paris VI) for kind help in SEM. We thank C. Le Bouguenec (Unité de Pathogénie Bactérienne des Muqueuses, Institut Pasteur, Paris) for kindly providing the wild-type strains expressing AfaE-I and AfaE-III, AfaE-I and AfaE-III recombinant, and AAEC185 *E. coli* strain, as well as AfaE-III-coated beads. We thank J. Bertoglio (Unité INSERM 461, Châtenay-Malabry) for kindly providing GST and TAT fusion proteins and for expert advice in pulldown experiments. We also thank I. Just (Institute of Toxicology, Hanover Medical School, Hanover, Germany) and P. Boquet (Unité INSERM 452, Faculté de Médecine, Nice) for providing bacterial toxins.

## References

Baorto, D.M., Gao, Z., Malaviya, R., Dustin, M.L., van der Merwe, A., Lublin, D.M., and Abraham, S.N. (1997) Survival of FimH-expressing enterobacteria in macrophages relies on glycolipid traffic. *Nature* **389:** 636–639.

Beauchemin, N., Draber, P., Dveksler, G., Gold, P., Gray-Owen, S., Grunert, F., *et al.* (1999) Redefined nomenclature for members of the carcinoembryonic antigen family. *Exp Cell Res* **252:** 243–249.

Beinke, C., Laarmann, S., Wachter, C., Karch, H., Greune, L., and Schmidt, M.A. (1998) Diffusely adhering *Escherichia coli* strains induce attaching and effacing phenotypes and secrete homologs of Esp proteins. *Infect Immun* **66:** 528–539.

Bernet-Camard, M.F., Coconnier, M.H., Hudault, S., and Servin, A.L. (1996) Pathogenicity of the diffusely adhering strain *Escherichia coli* C1845: F1845 adhesin–decay accelerating factor interaction, brush border microvillus injury, and actin disassembly in cultured human intestinal epithelial cells. *Infect Immun* **64:** 1918–1928.

Bilge, S.S., Clausen, C.R., Lau, W., and Moseley, S.L. (1989) Molecular characterization of a fimbrial adhesin, F1845, mediating diffuse adherence of diarrhea-associated

*Escherichia coli* to HEp-2 cells. *J Bacteriol* **171:** 4281–4289.

Bilge, S.S., Apostol, J.M., Jr, Fullner, K.J., and Moseley, S.L. (1993) Transcriptional organization of the F1845 fimbrial adhesin determinant of *Escherichia coli*. *Mol Microbiol* **7:** 993–1006.

Billker, O., Popp, A., Gray-Owen, S.D., and Meyer, T.F. (2000) The structural basis of CEACAM-receptor targeting by neisserial Opa proteins. *Trends Microbiol* **8:** 258–260; discussion 260–251.

Billker, O., Popp, A., Brinkmann, V., Wenig, G., Schneider, J., Caron, E., and Meyer, T.F. (2002) Distinct mechanisms of internalization of *Neisseria gonorrhoeae* by members of the CEACAM receptor family involving Rac1- and Cdc42-dependent and -independent pathways. *EMBO J* **21:** 560–571.

Blomfield, I.C., McClain, M.S., Princ, J.A., Calie, P.J., and Eisenstein, B.I. (1991) Type 1 fimbriation and fimE mutants of *Escherichia coli* K-12. *J Bacteriol* **173:** 5298–5307.

Booth, J.W., Telio, D., Liao, E.H., McCaw, S.E., Matsuo, T., Grinstein, S., and Gray-Owen, S.D. (2003) Phosphatidylinositol 3-kinases in carcinoembryonic antigen-related cellular adhesion molecule-mediated internalization of *Neisseria gonorrhoeae*. *J Biol Chem* **278:** 14037–14045.

Bos, M.P., Grunert, F., and Belland, R.J. (1997) Differential recognition of members of the carcinoembryonic antigen family by Opa variants of *Neisseria gonorrhoeae*. *Infect Immun* **65:** 2353–2361.

Bos, M.P., Kuroki, M., Krop-Watorek, A., Hogan, D., and Belland, R.J. (1998) CD66 receptor specificity exhibited by neisserial Opa variants is controlled by protein determinants in CD66 N-domains. *Proc Natl Acad Sci USA* **95:** 9584–9589.

Bos, M.P., Hogan, D., and Belland, R.J. (1999) Homologue scanning mutagenesis reveals CD66 receptor residues required for neisserial Opa protein binding. *J Exp Med* **190:** 331–340.

Boulton, I.C., and Gray-Owen, S.D. (2002) Neisserial binding to CEACAM1 arrests the activation and proliferation of CD4+ T lymphocytes. *Nature Immunol* **3:** 229–236.

Brown, D.A., and London, E. (2000) Structure and function of sphingolipid- and cholesterol-rich membrane rafts. *J Biol Chem* **275:** 17221–17224.

Brown, D.A., and Rose, J.K. (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* **68:** 533–544.

Carnoy, C., and Moseley, S.L. (1997) Mutational analysis of receptor binding mediated by the Dr family of *Escherichia coli* adhesins. *Mol Microbiol* **23:** 365–379.

Castellano, F., Montcourrier, P., Guillemot, J.C., Gouin, E., Machesky, L., Cossart, P., and Chavrier, P. (1999) Inducible recruitment of Cdc42 or WASP to a cell-surface receptor triggers actin polymerization and filopodium formation. *Curr Biol* **9:** 351–360.

Chen, T., Grunert, F., Medina-Marino, A., and Gotschlich, E.C. (1997) Several carcinoembryonic antigens (CD66) serve as receptors for gonococcal opacity proteins. *J Exp Med* **185:** 1557–1564.

Chen, T., Zimmermann, W., Parker, J., Chen, I., Maeda, A., and Bolland, S. (2001a) Biliary glycoprotein (BGPa, CD66a, CEACAM1) mediates inhibitory signals. *J Leukoc Biol* **70:** 335–340.

Chen, T., Bolland, S., Chen, I., Parker, J., Pantelic, M., Grunert, F., and Zimmermann, W. (2001b) The CGM1a (CEACAM3/CD66d)-mediated phagocytic pathway of *Neisseria gonorrhoeae* expressing opacity proteins is also the pathway to cell death. *J Biol Chem* **276:** 17413–17419.

Dehio, C., Gray-Owen, S.D., and Meyer, T.F. (2000) Host cell invasion by pathogenic *Neisseriae*. *Subcell Biochem* **33:** 61–96.

Duncan, M.J., Shin, J.S., and Abraham, S.N. (2002) Microbial entry through caveolae: variations on a theme. *Cell Microbiol* **4:** 783–791.

von Eichel-Streiber, C., Harperath, U., Bosse, D., and Hadding, U. (1987) Purification of two high molecular weight toxins of *Clostridium difficile* which are antigenically related. *Microb Pathog* **2:** 307–318.

Eugene, E., Hoffmann, I., Pujol, C., Couraud, P.O., Bourdoulous, S., and Nassif, X. (2002) Microvilli-like structures are associated with the internalization of virulent capsulated *Neisseria meningitidis* into vascular endothelial cells. *J Cell Sci* **115:** 1231–1241.

Fra, A.M., Williamson, E., Simons, K., and Parton, R.G. (1994) Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. *J Biol Chem* **269:** 30745–30748.

Garcia, M.I., Labigne, A., and Le Bouguenec, C. (1994) Nucleotide sequence of the afimbrial-adhesin-encoding afa-3 gene cluster and its translocation via flanking IS1 insertion sequences. *J Bacteriol* **176:** 7601–7613.

Garcia, M.I., Gounon, P., Courcoux, P., Labigne, A., and Le Bouguenec, C. (1996) The afimbrial adhesive sheath encoded by the afa-3 gene cluster of pathogenic *Escherichia coli* is composed of two adhesins. *Mol Microbiol* **19:** 683–693.

Garcia, M.I., Jouve, M., Nataro, J.P., Gounon, P., and Le Bouguenec, C. (2000) Characterization of the AfaD-like family of invasins encoded by pathogenic *Escherichia coli* associated with intestinal and extra- intestinal infections. *FEBS Lett* **479:** 111–117.

Garner, M.J., Hayward, R.D., and Koronakis, V. (2002) The *Salmonella* pathogenicity island 1 secretion system directs cellular cholesterol redistribution during mammalian cell entry and intracellular trafficking. *Cell Microbiol* **4:** 153–165.

Gatfield, J., and Pieters, J. (2000) Essential role for cholesterol in entry of mycobacteria into macrophages. *Science* **288:** 1647–1650.

Gauthier-Rouviere, C., Vignal, E., Meriane, M., Roux, P., Montcourier, P., and Fort, P. (1998) RhoG GTPase controls a pathway that independently activates Rac1 and Cdc42Hs. *Mol Biol Cell* **9:** 1379–1394.

Goluszko, P., Popov, V., Selvarangan, R., Nowicki, S., Pham, T., and Nowicki, B.J. (1997) Dr fimbriae operon of uropathogenic *Escherichia coli* mediate microtubule-dependent invasion to the HeLa epithelial cell line. *J Infect Dis* **176:** 158–167.

Goluszko, P., Selvarangan, R., Popov, V., Pham, T., Wen, J.W., and Singhal, J. (1999) Decay-accelerating factor and cytoskeleton redistribution pattern in HeLa cells infected

with recombinant *Escherichia coli* strains expressing Dr family of adhesins. *Infect Immun* **67:** 3989–3997.

van der Goot, F.G., and Harder, T. (2001) Raft membrane domains: from a liquid-ordered membrane phase to a site of pathogen attack. *Semin Immunol* **13:** 89–97.

Gray-Owen, S.D., Dehio, C., Haude, A., Grunert, F., and Meyer, T.F. (1997a) CD66 carcinoembryonic antigens mediate interactions between Opa-expressing *Neisseria gonorrhoeae* and human polymorphonuclear phagocytes. *EMBO J* **16:** 3435–3445.

Gray-Owen, S.D., Lorenzen, D.R., Haude, A., Meyer, T.F., and Dehio, C. (1997b) Differential Opa specificities for CD66 receptors influence tissue interactions and cellular response to *Neisseria gonorrhoeae*. *Mol Microbiol* **26:** 971–980.

Guignot, J., Peiffer, I., Bernet-Camard, M.F., Lublin, D.M., Carnoy, C., Moseley, S.L., and Servin, A.L. (2000) Recruitment of CD55 and CD66e brush border-associated glycosylphosphatidylinositol-anchored proteins by members of the Afa/Dr diffusely adhering family of *Escherichia coli* that infect the human polarized intestinal caco-2/TC7 cells. *Infect Immun* **68:** 3554–3563.

Guignot, J., Bernet-Camard, M.F., Poüs, C., Plançon, L., Le Bouguenec, C., and Servin, A.L. (2001) Polarized entry into human epithelial cells of uropathogenic Afa/Dr diffusely adhering *Escherichia coli* strain IH11128: evidence for alpha5beta1 integrin recognition, and subsequent internalization trough a pathway involving caveolae and dynamic unstable microtubules. *Infect Immun* **69:** 1856–1868.

Haddad, E., Zugaza, J.L., Louache, F., Debili, N., Crouin, C., Schwarz, K., *et al.* (2001) The interaction between Cdc42 and WASP is required for SDF-1-induced T-lymphocyte chemotaxis. *Blood* **97:** 33–38.

Hauck, C.R., Gulbins, E., Lang, F., and Meyer, T.F. (1999) Tyrosine phosphatase SHP-1 is involved in CD66-mediated phagocytosis of Opa52-expressing *Neisseria gonorrhoeae*. *Infect Immun* **67:** 5490–5494.

Hauck, C.R., Grassme, H., Bock, J., Jendrossek, V., Ferlinz, K., Meyer, T.F., and Gulbins, E. (2000) Acid sphingomyelinase is involved in CEACAM receptor-mediated phagocytosis of *Neisseria gonorrhoeae*. *FEBS Lett* **478:** 260–266.

Huber, M., Izzi, L., Grondin, P., Houde, C., Kunath, T., Veillette, A., and Beauchemin, N. (1999) The carboxyl-terminal region of biliary glycoprotein controls its tyrosine phosphorylation and association with protein-tyrosine phosphatases SHP-1 and SHP-2 in epithelial cells. *J Biol Chem* **274:** 335–344.

Ilangumaran, S., He, H.T., and Hoessli, D.C. (2000) Microdomains in lymphocyte signalling: beyond GPI-anchored proteins. *Immunol Today* **21:** 2–7.

Jouve, M., Garcia, M.I., Courcoux, P., Labigne, A., Gounon, P., and Le Bouguenec, C. (1997) Adhesion to and invasion of HeLa cells by pathogenic *Escherichia coli* carrying the afa-3 gene cluster are mediated by the AfaE and AfaD proteins, respectively. *Infect Immun* **65:** 4082–4089.

Kenworthy, A.K., Petranova, N., and Edidin, M. (2000) High-resolution FRET microscopy of cholera toxin B-subunit and GPI-anchored proteins in cell plasma membranes. *Mol Biol Cell* **11:** 1645–1655.

Kerneis, S., Bilge, S.S., Fourel, V., Chauviere, G., Coconnier, M.H., and Servin, A.L. (1991) Use of purified F1845 fimbrial adhesin to study localization and expression of receptors for diffusely adhering *Escherichia coli* during enterocytic differentiation of human colon carcinoma cell lines HT-29 and Caco-2 in culture. *Infect Immun* **59:** 4013–4018.

Kerneis, S., Gabastou, J.M., Bernet-Camard, M.F., Coconnier, M.H., Nowicki, B.J., and Servin, A.L. (1994) Human cultured intestinal cells express attachment sites for uropathogenic *Escherichia coli* bearing adhesins of the Dr adhesin family. *FEMS Microbiol Lett* **119:** 27–32.

Knodler, L.A., Celli, J., and Finlay, B.B. (2001) Pathogenic trickery: deception of host cell processes. *Nature Rev Mol Cell Biol* **2:** 578–588.

Kurzchalia, T.V., and Parton, R.G. (1999) Membrane microdomains and caveolae. *Curr Opin Cell Biol* **11:** 424–431.

Labigne-Roussel, A., and Falkow, S. (1988) Distribution and degree of heterogeneity of the afimbrial-adhesin-encoding operon (afa) among uropathogenic *Escherichia coli* isolates. *Infect Immun* **56:** 640–648.

Labigne-Roussel, A.F., Lark, D., Schoolnik, G., and Falkow, S. (1984) Cloning and expression of an afimbrial adhesin (AFA-I) responsible for P blood group-independent, mannose-resistant hemagglutination from a pyelonephritic *Escherichia coli* strain. *Infect Immun* **46:** 251–259.

Labigne-Roussel, A., Schmidt, M.A., Walz, W., and Falkow, S. (1985) Genetic organization of the afimbrial adhesin operon and nucleotide sequence from a uropathogenic *Escherichia coli* gene encoding an afimbrial adhesin. *J Bacteriol* **162:** 1285–1292.

Lafont, F., Tran Van Nhieu, G., Hanada, K., Sansonetti, P., and van der Goot, F.G. (2002) Initial steps of *Shigella* infection depend on the cholesterol/sphingolipid raft-mediated CD44–IpaB interaction. *EMBO J* **21:** 4449–4457.

Le Bouguenec, C., Garcia, M.I., Ouin, V., Desperrier, J.M., Gounon, P., and Labigne, A. (1993) Characterization of plasmid-borne *afa-3* gene clusters encoding afimbrial adhesins expressed by *Escherichia coli* strains associated with intestinal or urinary tract infections. *Infect Immun* **61:** 5106–5114.

Lublin, D.M., and Coyne, K.E. (1991) Phospholipid-anchored and transmembrane versions of either decay-accelerating factor or membrane cofactor protein show equal efficiency in protection from complement-mediated cell damage. *J Exp Med* **174:** 35–44.

McCaw, S.E., Schneider, J., Liao, E.H., Zimmermann, W., and Gray-Owen, S.D. (2003) Immunoreceptor tyrosine-based activation motif phosphorylation during engulfment of *Neisseria gonorrhoeae* by the neutrophil-restricted CEACAM3 (CD66d) receptor. *Mol Microbiol* **49:** 623–637.

Martinez, J.J., and Hultgren, S.J. (2002) Requirement of Rho-family GTPases in the invasion of type 1-piliated uropathogenic *Escherichia coli*. *Cell Microbiol* **4:** 19–28.

Merz, A.J., and So, M. (2000) Interactions of pathogenic *Neisseriae* with epithelial cell membranes. *Annu Rev Cell Dev Biol* **16:** 423–457.

Muenzner, P., Dehio, C., Fujiwara, T., Achtman, M., Meyer, T.F., and Gray-Owen, S.D. (2000) Carcinoembryonic antigen family receptor specificity of *Neisseria meningitidis* Opa variants influences adherence to and invasion of

proinflammatory cytokine-activated endothelial cells. *Infect Immun* **68:** 3601–3607.

Nagel, G., Grunert, F., Kuijpers, T.W., Watt, S.M., Thompson, J., and Zimmermann, W. (1993) Genomic organization, splice variants and expression of CGM1, a CD66-related member of the carcinoembryonic antigen gene family. *Eur J Biochem* **214:** 27–35.

Norkin, L.C., Wolfrom, S.A., and Stuart, E.S. (2001) Association of caveolin with *Chlamydia trachomatis* inclusions at early and late stages of infection. *Exp Cell Res* **266:** 229–238.

Nowicki, B., Barrish, J.P., Korhonen, T., Hull, R.A., and Hull, S.I. (1987) Molecular cloning of the *Escherichia coli* O75X adhesin. *Infect Immun* **55:** 3168–3173.

Nowicki, B., Moulds, J., Hull, R., and Hull, S. (1988) A hemagglutinin of uropathogenic *Escherichia coli* recognizes the Dr blood group antigen. *Infect Immun* **56:** 1057–1060.

Nowicki, B., Labigne, A., Moseley, S., Hull, R., Hull, S., and Moulds, J. (1990) The Dr hemagglutinin, afimbrial adhesins AFA-I and AFA-III, and F1845 fimbriae of uropathogenic and diarrhea-associated *Escherichia coli* belong to a family of hemagglutinins with Dr receptor recognition. *Infect Immun* **58:** 279–281.

Nowicki, B., Hart, A., Coyne, K.E., Lublin, D.M., and Nowicki, S. (1993) Short consensus repeat-3 domain of recombinant decay-accelerating factor is recognized by *Escherichia coli* recombinant Dr adhesin in a model of a cell–cell interaction. *J Exp Med* **178:** 2115–2121.

Nowicki, B., Selvarangan, R., and Nowicki, S. (2001) Family of *Escherichia coli* Dr adhesins: decay-accelerating factor receptor recognition and invasiveness. *J Infect Dis* **183 (**Suppl. 1): S24–S27.

Öbrink, B. (1997) CEA adhesion molecules: multifunctional proteins with signal-regulatory properties. *Curr Opin Cell Biol* **9:** 616–626.

Peiffer, I., Guignot, J., Barbat, A., Carnoy, C., Moseley, S.L., Nowicki, B., *et al.* (2000) Structural and functional lesions in brush border of human polarized intestinal Caco-2/TC7 cells infected by members of the Afa/Dr diffusely adhering family of *Escherichia coli*. *Infect Immun* **68:** 5979–5990.

Pelkmans, L., and Helenius, A. (2002) Endocytosis via caveolae. *Traffic* **3:** 311–320.

Pham, T.Q., Goluszko, P., Popov, V., Nowicki, S., and Nowicki, B.J. (1997) Molecular cloning and characterization of Dr-II, a nonfimbrial adhesin-I-like adhesin isolated from gestational pyelonephritis-associated *Escherichia coli* that binds to decay-accelerating factor. *Infect Immun* **65:** 4309–4318.

Popp, A., Dehio, C., Grunert, F., Meyer, T.F., and Gray-Owen, S.D. (1999) Molecular analysis of neisserial Opa protein interactions with the CEA family of receptors: identification of determinants contributing to the differential specificities of binding. *Cell Microbiol* **1:** 169–181.

Prall, F., Nollau, P., Neumaier, M., Haubeck, H.D., Drzeniek, Z., Helmchen, U., *et al.* (1996) CD66a (BGP), an adhesion molecule of the carcinoembryonic antigen family, is expressed in epithelium, endothelium, and myeloid cells in a wide range of normal human tissues. *J Histochem Cytochem* **44:** 35–41.

Rodgers, W., and Rose, J.K. (1996) Exclusion of CD45 inhibits activity of p56lck associated with glycolipid-enriched membrane domains. *J Cell Biol* **135:** 1515–1523.

Rosenberger, C.M., Brumell, J.H., and Finlay, B.B. (2000) Microbial pathogenesis: lipid rafts as pathogen portals. *Curr Biol* **10:** R823–R825.

Sauzeau, V., Le Mellionnec, E., Bertoglio, J., Scalbert, E., Pacaud, P., and Loirand, G. (2001) Human urotensin II-induced contraction and arterial smooth muscle cell proliferation are mediated by RhoA and Rho-kinase. *Circ Res* **88:** 1102–1104.

Schumann, D., Chen, C.J., Kaplan, B., and Shively, J.E. (2001) Carcinoembryonic antigen cell adhesion molecule 1 directly associates with cytoskeleton proteins actin and tropomyosin. *J Biol Chem* **276:** 47421–47433.

Shenoy-Scaria, A.M., Kwong, J., Fujita, T., Olszowy, M.W., Shaw, A.S., and Lublin, D.M. (1992) Signal transduction through decay-accelerating factor. Interaction of glycosyl-phosphatidylinositol anchor and protein tyrosine kinases p56lck and p59fyn 1. *J Immunol* **149:** 3535–3541.

Shin, J.S., Gao, Z., and Abraham, S.N. (2000) Involvement of cellular caveolae in bacterial entry into mast cells. *Science* **289:** 785–788.

Simons, K., and Toomre, D. (2000) Lipid rafts and signal transduction. *Nature Rev Mol Cell Biol* **1:** 31–39.

Singer, B.B., Scheffrahn, I., and Obrink, B. (2000) The tumor growth-inhibiting cell adhesion molecule CEACAM1 (C-CAM) is differently expressed in proliferating and quiescent epithelial cells and regulates cell proliferation. *Cancer Res* **60:** 1236–1244.

Skubitz, K.M., Campbell, K.D., Ahmed, K., and Skubitz, A.P. (1995) CD66 family members are associated with tyrosine kinase activity in human neutrophils. *J Immunol* **155:** 5382–5390.

Smart, E.J., Graf, G.A., McNiven, M.A., Sessa, W.C., Engelman, J.A., Scherer, P.E., *et al.* (1999) Caveolins, liquid-ordered domains, and signal transduction. *Mol Cell Biol* **19:** 7289–7304.

Stefanova, I., Horejsi, V., Ansotegui, I.J., Knapp, W., and Stockinger, H. (1991) GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science* **254:** 1016–1019.

Swanson, T.N., Bilge, S.S., Nowicki, B., and Moseley, S.L. (1991) Molecular structure of the Dr adhesin: nucleotide sequence and mapping of receptor-binding domain by use of fusion constructs. *Infect Immun* **59:** 261–268.

Takai, Y., Sasaki, T., and Matozaki, T. (2001) Small GTP-binding proteins. *Physiol Rev* **81:** 153–208.

Thompson, J.A., Grunert, F., and Zimmerman, W. (1991) Carcinoembryonic antigen family: molecular biology and clinical perspective. *J Clin Lab Anal* **5:** 344–366.

Van Loy, C.P., Sokurenko, E.V., Samudrala, R., and Moseley, S.L. (2002) Identification of amino acids in the Dr adhesin required for binding to decay-accelerating factor. *Mol Microbiol* **45:** 439–452.

Virji, M. (2000) The structural basis of CEACAM-receptor targeting by neisserial opa proteins: response. *Trends Microbiol* **8:** 260–261.

Virji, M., Makepeace, K., Ferguson, D.J., and Watt, S.M. (1996a) Carcinoembryonic antigens (CD66) on epithelial

cells and neutrophils are receptors for Opa proteins of pathogenic neisseriae. *Mol Microbiol* **22:** 941–950.

Virji, M., Watt, S.M., Barker, S., Makepeace, K., and Doyonnas, R. (1996b) The N-domain of the human CD66a adhesion molecule is a target for Opa proteins of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *Mol Microbiol* **22:** 929–939.

Virji, M., Evans, D., Hadfield, A., Grunert, F., Teixeira, A.M., and Watt, S.M. (1999) Critical determinants of host receptor targeting by *Neisseria meningitidis* and *Neisseria gonorrhoeae*: identification of Opa adhesiotopes on the N-domain of CD66 molecules. *Mol Microbiol* **34:** 538–551.

Virji, M., Evans, D., Griffith, J., Hill, D., Serino, L., Hadfield, A., and Watt, S.M. (2000) Carcinoembryonic antigens are targeted by diverse strains of typable and non-typable *Haemophilus influenzae*. *Mol Microbiol* **36:** 784–795.

Wang, J., Gray-Owen, S.D., Knorre, A., Meyer, T.F., and Dehio, C. (1998) Opa binding to cellular CD66 receptors mediates the transcellular traversal of *Neisseria gonorrhoeae* across polarized T84 epithelial cell monolayers. *Mol Microbiol* **30:** 657–671.

Zalewska, B., Piatek, R., Cieslinski, H., Nowicki, B., and Kur, J. (2001) Cloning, expression, and purification of the uropathogenic *Escherichia coli* invasin DraD. *Protein Expr Purif* **23:** 476–482.