Role of Src Kinases in Mobilization of Glycosylphosphatidylinositol-Anchored Decay-Accelerating Factor by Dr Fimbria-Positive Adhering Bacteria

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Diffusely adhering Escherichia coli (DAEC) belong to the sixth class of enterovirulent E. coli (29). Strains of DAEC expressing afimbrial (Afa) or fimbrial (Dr) adhesins have been subdivided into two classes, the typical Afa/Dr DAEC and the atypical Afa/Dr DAEC, each of which is further subdivided into two subclasses (52). The typical Afa/Dr DAEC class includes E. coli strains harboring AfaE-I, AfaE-II, AfaE-III, AfaE-V, Dr, Dr-II, F1845, and NFA-I adhesins, which (i) share the same genetic organization, (ii) are able to bind to human decay-accelerating factor (hDAF; CD55), and (iii) promote the mobilization of human hDAF around bacteria adhering to host cells. The typical subclass 1 strains, including strains harboring AfaE-I, AfaE-II, and F1845 adhesins, (i) bind to members of the human carinoembryonic antigen family (human carinoembryonic antigen cell adhesion molecules [hCEACAMs]; Afa/DrhCEA) and (ii) mobilize hCEACAMs around bacteria adhering to host cells. In contrast, typical subclass 2 strains, including strains harboring afimbrial AfaE-I and Dr-II adhesins, do not bind to hCEACAM members. All these adhesins are able to bind to human receptors but do not recognize the counterpart mouse, rat, or pig receptors (26). Typical Afa/Dr adhesins are encoded by a family of genes organized to form afa-dra-daa-related operons. All these operons are organized in a similar way and involve at least five genes (A to E). The last gene (E) encodes the major structural adhesin subunit. Structure-function analysis of the typical Dr and F1845 adhesins has shown that both the DraE and DaaE subunits function as adhesins (1, 24, 58, 62). The first receptor of typical Afa/Dr fimbriae to be identified was hDAF (41). Members of the CEACAM family, including CEACAM1-4L, CEACAM3, CEA, and CEACAM6, have recently been identified to be receptors of typical Dr and F1845 fimbriae (2, 23). Typical Afa/Dr fimbriae recognize the complement control protein 2 (CCP2) and CCP3 domains of hDAF (32, 41) and the N domain of CEACAMs (31). The DraE adhesin subunit has a distinct hCEACAM member binding site located primarily in its A, B, E, and D strands, located opposite the beta sheet encompassing the binding site for hDAF, indicating that the adhesin subunit can bind simultaneously to both receptors on the epithelial cell surface (31). Conformational receptor-binding studies have been conducted in order to identify the amino acid sequences in the major structural DraE and DaaE adhesin subunits engaged in receptor recognition (11, 30, 44, 45, 62).

DAF, a 70-kDa glycosphosphatidylinositol (GPI)-anchored protein, is attached to the outer leaflet of the cell membrane and localized in the lipid rafts (34). DAF is widely distributed on all blood cells and on endothelial and epithelial tissues. DAF contains a C-terminal GPI anchor, followed by a serine/threonine/proline-rich region, and is capped by four CCP domains (CCP1 to CCP4), which are characteristic of complement activation family protein regulators. The physiological role of DAF is to prevent complement cascade occurring at the level of the critical C3 convertase step, thus protecting against autologous cell lysis and tissue damage and, hence, it can contribute to preventing or modulating autoimmune disease and inflammation. Furthermore, the widespread expression of hDAF in malignant tumors suggests that it probably has several different functions, including that of promoting tumorogenesis, neoangiogenesis, invasive-
ness, cell motility, and immune modulation (36). Many bacterial and virus pathogens exploit the hDAF receptor as a vehicle for cell infection, and hDAF-associated signaling is exploited by virulent microorganisms to gain cell entry and for their infectivity (34). Signaling-dependent structural and functional alterations at the brush border of enterocyte-like cells (3, 43) and proinflammatory responses (4, 5, 9, 10) have been reported following interactions between Dr and F1845 fimbiae with hDAF. Moreover, Dr adhesion/hDAF interaction has been shown to promote lipid raft recruitment and F-actin network mobilization around bacteria (19, 23, 28). Interaction between Dr fimbiae and hDAF also allows bacteria to penetrate into host cells (17, 21, 22, 28, 33, 51).

For signaling purposes, hDAF associates with nonreceptor tyrosine kinases, including Src kinase family tyrosine kinases (SFKs) (25, 48, 53, 54, 56). The nine members of SFKs can be subdivided on the basis of their general pattern of expression. Yrk has been shown to be ubiquitously expressed only in chick- ens, Src, Fyn, and Yes are expressed in most human tissues; Blk, Fgr, Hck, Lck, and Lyn are found primarily in hematopoietic cells. Both Lck and Lyn have also been found in neu- rons (60). The SFKs are 52- to 62-kDa proteins composed of (i) an N-terminal Src homology 4 (SH4) domain containing a glycine in position 2, which is important for the addition of a myristic acid moiety and is involved in targeting SFKs to cell membranes, (ii) the unique region, (iii) the SH3 and SH2 protein-binding domains, (iv) the SH1 catalytic domain, and (v) the negative regulatory tail containing Tyr527 (8). SFKs regulate many cell functions, such as cell proliferation, cytoskeletal alterations, differentiation, survival, and migration (8). Moreover, the SFKs are considered to be oncogenic proteins, and their expression and activity are high in epithelial cancers (20).

In the present study, we investigated the role played by the Src kinases in the Dr fimbia-induced mobilization of hDAF around adhering E. coli expressing Dr fimbiae. We used the pyelonephritic Afa/Dr DAEC strain IH11128, which expresses Dr fimbiae. It belongs to the typical Afa/Dr DAEC subclass pyelonephritic Afa/Dr DAEC strain expressing Dr fimbriae. It belongs to the typical Afa/Dr DAEC subclass and has been shown to colonize both the human urinary and intestinal tracts and trigger proinflammatory responses (52). The pathogenesis of Afa/Dr DAEC is initiated by an initial adhesion step characterized by the recognition and mobilization of cellular receptors, including hDAF, around the bacteria. Here we show for the first time that activated Src kinases are necessary for the recruitment of hDAF around adhering Dr-positive bacteria, and we identify the c-Src kinases as being specifically involved in this process. We also show that the aspartic acids at position 54 in the DraE adhesin subunit and the CCP4 domain of hDAF are structural elements that regulate the Dr fimbia-induced hDAF mobilization and the activation of Src kinases. Finally, we demonstrate that the catalytic activity and the SH2 and SH3 protein-binding domains of Src kinases are crucial structural elements that contribute to the recruitment of hDAF around Dr-positive E. coli isolates. Taken together, these findings provide further insights into the molecular process underlying Afa/Dr DAEC adherence. Moreover, we demonstrated that Src kinases activated by Dr adhesion/hDAF interaction are involved in hCEACAM1-4L activation, suggesting that hDAF plays a central role in the organization of early events in response to infection with Afa/Dr DAEC.

MATERIALS AND METHODS

Reagents and antibodies. The anti-protease cocktail Triton X-100, methyl-β-cyclohexdrin (MCD), and cytochalasin D were purchased from Sigma. The Src kinase inhibitor PP2 and its inactive analog, PP3, were purchased from Calbi- ochem. The antibodies used were goat anti-hDAF polyclonal antibody (pAb) (AF2009) from R&D systems; mouse monoclonal antibody (MAb) 5D11, di- rected against the CCP4 domain of hDAF (Department of Pathology, Washing- ton University School of Medicine, St. Louis, MO); rabbit anti-phospho-Src Tyr416 pAb, which recognizes phospho-Tyr416 (mammals) or phospho-Tyr418 (human), and rabbit anti-Src pAb, purchased from Cell Signaling (Denvers, MA); mouse anti-c-Src MAb (Millipore); mouse anti-Yes MAb (BD Transduc- tion Laboratories); mouse anti-Fyn MAb and mouse anti-Lyn MAb (Exbio); rabbit antiaactin pAb (Sigma); antiphosphotyrosine pY20 MAb (BD Transduc- tion Laboratories); anti-CEACAM pAb (Dako Cytomation); anti-CEACAM MAb D14H1D1 (Genovac); and rabbit anti-Dr pAb (63), directed against the DraE adhesin subunit of Dr fimbiae (22). Fluorescein isothiocyanate (FITC)-labeled cholera toxin B subunit was from Sigma. The Alexa 488-labeled phalloi- din was from Invitrogen. For Western immunoblotting analysis, the secondary antibodies used were goat anti-mouse, goat anti-rabbit, and horse anti-goat conjugated with horseradish peroxidase (HRP) (Jackson Immunoresearch). For Western immunoblotting analysis after immunoprecipitation, we used rabbit or mouse IgG TrueBlot conjugated with HRP (E Bioscience) as secondary antibodies. The secondary antibodies used for immunofluorescence analysis were donkey anti- mouse FITC or Rhodamin Red-X (RRX); donkey anti-rabbit FITC, RRX, or Cy5; and donkey anti-goat tetramethyl rhodamine isocyanate (Jackson Immunoresearch Laboratories, Newmarket, United Kingdom).

Bacterial strains. The bacterial strains used are listed in Table 1. A Dr-positive (Dr+) strain with a mutated Dr adhesin subunit in position 54 (strain Dr+D54C) carried the pCC90 plasmid in which a point mutation in the draE gene was created by site-directed mutagenesis (Aasp54 replaced by cysteine) (11). The bacterial strains were stored at −80°C and maintained in 25% glycerol. For the experiments, the bacterial strains were transferred into fresh Difco LB broth at 37°C for 18 h. The Dr-positive and mutated Dr fimbiae Dr+D54C E. coli strains were grown in the presence of 100 μg/ml ampicillin. On the day that the cells were infected, the bacteria were washed twice with sterile phosphate-buffered saline (PBS) and recovered using an appropriate tissue culture medium.

### TABLE 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>AAEC185 (Dr-)</td>
<td></td>
<td>Nonpathogenic E. coli K-12, type 1 pilus deleted (ΔfimB to ΔfimH)</td>
<td>6</td>
</tr>
<tr>
<td>IH11128 (wt)</td>
<td>pCC90</td>
<td>Pyelonephritic Afa/Dr DAEC wild-type strain expressing Dr fimbriae</td>
<td>39</td>
</tr>
<tr>
<td>Dr+</td>
<td></td>
<td>Recombinant AAEC185 overexpressing Dr fimbiae</td>
<td>2</td>
</tr>
<tr>
<td>Dr+D54C</td>
<td>pCC90</td>
<td>Recombinant AAEC185 expressing mutant Dr fimbia with substitution of aspartic acid at position 54 by cysteine</td>
<td>11</td>
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Cell lines and culture conditions. HeLa cells constitutively expressing hDAF but no CEACAMs (7) were grown in RPMI 1640 medium with l-glutamine (Invitrogen) supplemented with 5% fetal bovine serum (FBS), 1% nonessential amino acids, and 1 mM sodium pyruvate (Invitrogen) at 37°C in an atmosphere containing 95% air–5% CO₂. The same HeLa cells transfected to stably express hCEACAM1-4L (HeLa-CAM1-4L cells) were cultured in the same medium supplemented with 100 μg/ml of hygromycin B (48). Chinese hamster ovary (CHO) cells stably expressing hDAF (CHO-DAF) or hDAF in which one of the four CCPs units had been deleted (CHO-hDAFΔCCP1, CHO-hDAFΔCCP2, CHO-hDAFΔCCP3, CHO-hDAFΔCCP4) (14) were cultured in Dulbecco modified Eagle medium–Ham’s F-12 medium with l-glutamine (Invitrogen) supplemented with 10% FBS and 400 μg/ml of G418 (Geneticin) at 37°C in an atmosphere containing 95% air–5% CO₂. Human embryonic kidney cells (HEK293) were cultured in minimal essential medium (Invitrogen) supplemented with 10% FBS at 37°C in an atmosphere containing 95% air–5% CO₂.

Cell infection and treatments. Cells growing in 6-well tissue culture plates were cultured without FBS for 24 h before being infected. They were infected by exposure for 15 min to bacterial strains at 1 × 10⁵ CFU/ml in the presence of 2% t-mannose to prevent type 1 pilus adhesion by the wild-type (wt) strain. At the times indicated, the infected cells were washed once with PBS-vanadate to remove any nonadhering bacteria. In some experiments, the cells were treated with the Src kinase inhibitor PP2 or its inactive analog, PP3 (10 μM for HeLa cells and 20 μM for CHO cells), 30 min before the infection. The lipid rafts were disrupted by incubating the cells with 2 mM MCD, a drug that extracts cholesterol from the plasma membrane, for 2 h before infection. In some cases, 0.3 mM water-soluble cholesterol (Sigma) was added to the MCD-treated cells for 1 h. F actin was depolymerized by exposure to 1 μg/ml cytochalasin D for 1 h before the infection. All the pharmacological agents were maintained during the infection; we checked that these treatments had no effect on the infection.

cDNA transfection. Cells were seeded in 24-well tissue culture plates. All green fluorescent protein (GFP)-Src kinase constructs were generously donated by Yoav I. Henis (Department of Neurobiochemistry, Georges S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel) and have been described elsewhere (49, 55). The myristoylation-defective GFP-Src kinase mutant (G2A) was a generous gift from Michael Way (Cell Motility Laboratory, London Research Institute, London, United Kingdom) (38). Transient transfections of CHO cells with GFP-Src kinase cDNAs were carried out using FuGENE HD reagent (Roche), according to the manufacturer’s protocol. The cells were either not infected or infected 24 h after the transfection. Transient transfections of HeLa cells with GFP-Src kinase cDNAs were carried out using Fugene HD reagent (Roche), according to the manufacturer’s protocol. The cells were either not infected or infected 24 h after the transfection.

siRNA transfection. HeLa cells seeded in 24-well tissue culture plates were transfected with 100 nM negative-control small interfering RNA (siRNA; Eurogentec) or specific hDAF siRNA (sense, 5′-GCUUUGGAAGGCCGUACAA-3′) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. The cells were either not infected or infected 24 h after the transfection. Transient transfections of HeLa cells with GFP-Src kinase cDNAs were carried out using Fugene HD reagent (Roche), according to the manufacturer’s protocol. The cells were either not infected or infected 24 h after the transfection.
FIG. 2. Mobilization of hDAF around adhering Dr-positive E. coli requires activated Src kinases. (A) Immunoblot analysis of hDAF, activated Src kinases (Src-Y418P), Src kinases (Src), and actin in the Triton X-100-extracted fraction (E) and the Triton X-100-resistant fraction (R) isolated from HeLa cells infected with IH11128 strain (wt), Dr-positive (Dr+) or Dr-negative (Dr−) E. coli, or not infected (NI). (Left panel) the different
fractures were analyzed by SDS-PAGE, followed by Western blotting with appropriate antibodies. (Right panel) Quantification of activated Src kinases (Src-Y418). (B) Knockdown expression of hDAF by specific siRNA in HeLa cells. The HeLa cells were transfected or not transfected with noncomplementing lentiviral shRNA expressing entry vector or with lentivirus containing the transgene of interest. Protein fractions were dissolved in the appropriate volume of Laemmli buffer and kept at 100°C for 5 min.

Immunoprecipitation. Confluent HeLa cells cultured in 6-well tissue culture were infected or not infected with Dr+ E. coli strain or strain H11128 (1 × 10^9 CFU/ml). The cells were washed twice with ice-cold PBS and then treated for 10 min at 4°C with an extraction buffer (25 mM HEPES, pH 7.5, 0.5% Triton X-100, 150 mM NaCl, 2 mM EDTA) containing protease and phosphatase inhibitors. Protein fractions were dissolved in the appropriate volume of Laemmli buffer and kept at 100°C for 5 min.

Immunofluorescence and confocal microscopy. For indirect immunofluorescence, the cells were cultured on glass coverslips (24-well plates). The noncomplementing cells were either not infected or infected with bacteria (1 × 10^9 CFU/ml) for 30 min. After infection, the monolayers were washed three times with PBS and then fixed with 3% paraformaldehyde in PBS for 15 min. The cells were treated with NH4Cl 50 mM in PBS for 10 min. For cytoplast protein examination, the cells were permeabilized using 0.2% Triton X-100 in PBS for 4 min, washed twice with PBS, and then saturated for 1 h with PBS containing 0.2% gelatin. For immunolocalization, the cells were incubated with appropriate primary antibodies diluted in PBS-0.2% gelatin for 45 min. The cells were washed three times and then incubated for 45 min with appropriate secondary antibodies diluted in PBS-0.2% gelatin. After they were washed three times with PBS, the coverslips were mounted in fluorescent mounting medium (DakoCytomation). Specimens were examined using an inverted Zeiss LSM-510 META confocal laser scanning microscope equipped with an argon laser (excitation wavelength, 488 nm) and two helium neon lasers (excitation wavelengths, 543 nm and 633 nm) using a Plan-Apochromat ×63 objective lens (numerical aperture, 1.40; oil immersion). Acquisitions were done using a sequential mode. FITC, rhodamine, and cyanine 5 fluorescences were collected with 505- to 550-nm band-pass, 560- to 635-nm band pass, and a 649- to 713-nm band pass emission filters, respectively. The pinhole diameter was set at 1.0 airy unit.

Image analysis and 3-dimensional (3D) reconstruction. To reconstruct the imaged area, we collected series of optical planes (z stacks) and rendered the volume in three dimensions to provide views of the image stack from different angles. The data sets were processed using Imaris (version 6.3) software (Bitplane AG).

Statistics. Data are expressed as a mean ± standard error of the means (SEM) of at least three experiments. The statistical significance was assessed by a Student’s t test.

RESULTS

hDAF mobilization around Dr-positive E. coli results in Src kinase activation. In this study, we used a specific clone of HeLa cells which expresses only one of the receptors for the Afa/Dr DAEC: hDAF. We used indirect immunofluorescence labeling of hDAF with an anti-hDAF antibody and of Dr fimbriae with an anti-Dr antibody, plus 3D image reconstruction of confocal microscopy images, to confirm previous observations showing that hDAF is mobilized in cells infected with Dr-positive bacteria (19, 23). HeLa cells infected with wt strain H11128 (which expresses Dr fimbriae) or with the recombinant E. coli K-12 strain AAEC185, which expresses recombinant Dr fimbriae (Dr-positive E. coli), showed that the positive labeling of Dr fimbriae and hDAF colocalized around adhering Dr-positive bacteria (Fig. 1A). This massive hDAF mobilization is directly induced by Dr fimbriae. Indeed, adherent E. coli K-12 strain AAEC185, which expresses type I pili as an adhesion factor but does not express Dr fimbriae, is not able to induce the mobilization of hDAF around bacteria in HeLa cells (see Fig. S1 in the supplemental material). Furthermore, 3D analysis showed that the hDAF immunolabeling decorating the adhering bacteria was clearly organized around them (Fig. 1B). Consistent with a previous observation (28), immunolabeling of hDAF and the revelation of lipid rafts by immunolabeling of the lipid raft-associated ganglioside M1 showed that the lipid rafts were mobilized around adhering Dr-positive E. coli and colocalized with the mobilized hDAF. We also showed that treatment with MCD, which abolishes the bacterial mobilization of ganglioside M1, does not affect that of hDAF (Fig. 1C). As a control, we observed that the addition of cholesterol restored the ganglioside M1 clustering around Dr-positive bacteria. Our findings suggest that transmembrane proteins and the actin-containing cortical cytoskeleton can organize lipids into short-lived nanoscale assemblies that can be assembled into larger domains under certain conditions and that many of the structural and functional properties of rafts require an intact actin cytoskeleton (12). In order to confirm that impairment of the integrity of lipid rafts does not affect the mobili-
zation of hDAF around adhering Dr-positive *E. coli*, we treated the cells with cytochalasin D, which is known to disrupt the F-actin network. Figure 1D shows that this treatment did not affect the mobilization of hDAF around adhering Dr-positive bacteria.

Several groups have reported that the signaling of GPI-anchored hDAF involves Src kinases (25, 53, 54, 56). Rougeaux et al. have recently reported that following Dr-positive *E. coli* infection, a time-dependent increase in the phosphorylation of Src occurs in cells that express hDAF but not hCEACAMs (48). We therefore investigated whether the mobilization of hDAF around adhering Dr-positive bacteria was related to the activation of Src kinases. Using detergent extraction followed by Western blot analysis, we analyzed the distribution of hDAF, Src kinases, and activated Src kinases in detergent-soluble and detergent-resistant fractions isolated from HeLa cells which had or had not been infected with bacterial strains which did or did not express Dr fimbriae. As expected, immunoblotting with an anti-hDAF antibody showed that hDAF was mainly present in the detergent-resistant fraction and that infection with Dr-positive *E. coli* did not modify the level or the distribution of hDAF compared to the levels or the distributions in uninfected cells and cells infected with a nonpathogenic *E. coli* strain (Fig. 2A). Immunoblotting with an antibody directed against activated Src kinases showed that they were mainly present in the detergent-resistant fraction of uninfected cells and of cells infected with a nonpathogenic *E. coli* strain (Fig. 2A). Infection of HeLa cells with wt strain IH11128 or recombinant Dr-positive *E. coli* resulted in a highly significant increase in the levels of activated Src kinases in the detergent-resistant fractions and the appearance of low levels of endogenous Src kinases, which were the same in the infected HEK293 cells treated with PP3 but not in HEK293 cells treated with PP2 (Fig. 2F). Collectively, these results suggest that the activation of Src kinases plays an important role in the mobilization of hDAF around adhering bacteria expressing Dr fimbriae. We had previously shown that lipid raft disorganization does not affect the mobilization of hDAF around Dr-positive bacteria (Fig. 1C). Src kinase activity was frequently associated with lipid rafts, but the disorganization of these lipid membrane microdomains using methyl-β-cyclodextrin did not affect the specific activation of Src kinases (see Fig. S2 in the supplemental material).

**Structural requirements for Src kinases to mobilize hDAF around Dr-positive *E. coli***. The SFKs are composed of several distinct functional regions, including a myristoylation motif, a unique region, an SH3 domain, an SH2 domain, a catalytic domain, and a C-terminal tail (47). We next investigated the structural requirements for Src kinases to mobilize hDAF around adhering Dr-positive bacteria. To do this, we used a series of GFP-tagged Src kinase mutants that modulate the properties of individual domains, activities, or motifs of the kinase, i.e., GFP-labeled constitutively activated Src kinase (Src-Y527F-GFP) or constitutively activated mutants of Src kinase: a kinase-dead mutant (Src-Y527F/K295M-GFP), an SH2-inactivated mutant (Src-Y527F/R175A-GFP), and an SH3-inactivated mutant (Src-Y527F/W118A-GFP) (55) (Fig. 3A). It should be noted that GFP-Src kinases had previously been shown to be regulated like the endogenous Src kinases.

**FIG. 3.** Kinase activity and the Src kinase protein-binding domains SH2 and SH3 are essential for hDAF to be mobilized around adhering Dr-positive *E. coli*. (A) Schematic representation of the Src kinase domains showing the positions of substituted amino acid in the GFP-Src kinase constructs. (B) Treatment of CHO cells transfected for the stable expression of hDAF (CHO-hDAF) with the Src kinase inhibitor PP2 resulted in inhibition of endogenous Src kinase phosphorylation or its inactive analog PP3. CHO-hDAF cells were infected with IH11128 (wt) or with Dr-positive *E. coli* (Dr+) or were not infected (NI). (C) CHO-hDAF cells were transfected with Src-wt-GFP or constitutively activated GFP-Src kinase (Src-Y527F-GFP). Cells were treated with PP2 or PP3 and were infected by Dr* E. coli* or not infected. Proteins were analyzed by SDS-PAGE and Western immunoblotted with appropriate antibodies to detect activated Src kinases (Src-Y418p), Src kinase proteins (Src), actin, or GFP. Immunoblots are representative of three independent experiments, and the bar represents the mean ± SEM.*P < 0.05 (Student’s t test) versus appropriate control cells. (D) Immunofluorescence analysis of CHO-hDAF treated with PP2 or PP3 or untreated and infected with Dr* E. coli*. Dr fimbriae (white) and hDAF (red) were stained using the J63 antibody and anti-hDAF AF2009 antibody, respectively. (E) hDAF was mobilized around Dr-positive *E. coli* adhering to CHO-hDAF cells transfected with constitutively activated GFP-Src kinase (Src-Y527F-GFP). No hDAF mobilization occurred around Dr-positive *E. coli* adhering to CHO-hDAF cells transfected with Src kinase mutants (Src-Y527F/K295M-GFP, Src-Y527F/W118A-GFP, Src-Y527F/R175A-GFP, Src-Y527/W118A-GFP). Before being infected, CHO-hDAF cells were treated with PP2, to inhibit the activation of endogenous Src kinases, and infected with Dr-positive *E. coli*. Transfected GFP-Src kinases were visualized in green. Dr fimbriae (white) and hDAF (red) were stained as described for panel D. (Under the micrographs) Intensity profiles of Dr* (gray) and hDAF (red) labeling. *, mismatches between the two intensity profiles. Micrographs are representative of three independent experiments.
(49) and to display lipid-like membrane association (55). We used CHO cells as a cellular model. As Dr adhesin is not able to recognize hamster receptors, CHO cells were transfected to stably express hDAF (14). We used experimental conditions under which the endogenous Src kinases were inactivated in CHO-hDAF cells by exposure to PP2 before the cell infection took place (Fig. 3B). PP2 treatment markedly reduced the mobilization of hDAF around Dr-positive bacteria adhering to CHO-hDAF cells compared to that around Dr-positive bacteria adhering to infected untreated and infected, PP3-treated CHO-hDAF cells (Fig. 3C). The faint hDAF-positive immunolabeling that was still observed in PP2-treated infected cells results from the basal level of hDAF mobilization that occurs in the absence of activation (Fig. 3C). We also confirmed that PP2 treatment inhibited the activity of the Src-wt-GFP but not that of the constitutively activated Src-Y527F-GFP (Fig. 3D). We then observed that cells expressing the constitutively activated Src-Y527F-GFP and exposed to PP2 displayed strong mobilization of hDAF around adhering Dr-positive E. coli and a complete absence of diffuse hDAF cell labeling (Fig. 3D). In contrast, the cells transfected to express kinase-dead Src-Y527F/K295M-GFP and exposed to PP2 displayed a basal level of mobilization of hDAF around adhering Dr-positive E. coli, indicating that the presence of a functional kinase domain is necessary for Dr fimbria-induced hDAF mobilization to occur (Fig. 3E). Rather surprisingly, we found that the mobilization of hDAF around adhering Dr-positive E. coli was abolished in cells expressing mutants in which either the SH2 or SH3 domain had been disrupted (Src-Y527F/R175A-GFP and Src-Y527F/W118A-GFP, respectively) and that the remaining labeling was equal to the basal level of bacterium-associated hDAF immunolabeling (Fig. 3E). Collectively, these results indicate that the kinase domain and the SH2 and SH3 protein-binding domains of Src kinases are all necessary for hDAF to be mobilized around Dr-positive E. coli.

**Mutation at aspartic acid 54 on the DraE adhesin subunit abolishes both hDAF mobilization and Src kinase activation.** Structure-function analysis of Dr adhesin has been performed using various different mutants (11, 30, 32, 44, 45, 61). One of them, the mutant strain Dr D54C (in which aspartic acid 54 is replaced by cysteine), retains hDAF-binding capability (11) but has lost the ability to mobilize hDAF around adhering Dr-positive bacteria (23). Interestingly, this mutant also fails to induce the signaling-dependent F-actin disassembly at the brush border of enterocyte-like Caco-2/TC7 cells (43). Indirect immunofluorescence of Dr fimbriae and hDAF and 3D analysis in Fig. 4A show that in contrast to Dr-positive E. coli-infected cells, the adhering Dr D54C mutant did not mobilize hDAF. We therefore investigated whether the cells infected with the Dr D54C mutant displayed activation of Src kinases. In Fig. 4B, the Western blot analysis of total and Y418-phosphorylated Src kinases in HeLa cells which were either not infected or infected with Dr-negative strain AAEC185, the Dr-positive E. coli strain, or the Dr D54C mutant shows that the increased level of phosphorylated Src kinases observed in cells infected with the Dr-positive E. coli strain did not occur in cells infected with the Dr D54C mutant. These results showed that the mutation at position D54 on the DraE subunits of Dr fimbriae is sufficient to abolish both the mobilization of hDAF

**FIG. 4. Recombinant E. coli expressing the Dr fimbriae mutated at position 54 of DraE adhesin subunit does not activate Src kinases and does not mobilize hDAF.** (A) Immunofluorescence analysis of hDAF mobilization around bacteria in HeLa cells infected with bacteria expressing Dr fimbriae (Dr) or Dr fimbriae mutated at position 54 (Dr+D54C). The white square indicates the cross-section used for 3D analysis. The AF2009 and J63 antibodies were used to detect hDAF and quantitate them (lower panel). Micrographs are representative of three independent experiments. Immunoblots are representative of three independent experiments. The value reported represents the mean ± SEM. *, P < 0.05 (Student t test) versus appropriate control cells.
around adhering Dr-positive bacteria and the activation of Src kinases.

**Deletion of the CCP4 domain of hDAF abolishes both hDAF mobilization and Src kinase activation.** We investigated the role played by each of the CCP domains of hDAF in its mobilization around adhering Dr-positive bacteria and activation of Src kinases. For this purpose, we used CHO-K1 cells stably expressing wild-type hDAF (CHO-hDAF) or hDAF with the CCP1, CCP2, CCP3, or CCP4 domain deleted (CHO-hDAFΔCCP1, CHO-hDAFΔCCP2, CHO-hDAFΔCCP3, and CHO-hDAFΔCCP4, respectively) (14). The CCP2 and CCP3 domains of hDAF have been identified as being necessary for the binding of the DraE subunit of Dr fimbriae (40). We did indeed observe that the adhesion of wt strain IH11128 and recombinant Dr-positive *E. coli* to CHO-hDAFΔCCP2 and CHO-hDAFΔCCP3 cells was entirely abolished and that the sparsely distributed Dr-positive bacteria still adhering were not decorated by positive hDAF immunolabeling (data not shown). In contrast, CHO-hDAFΔCCP1 and CHO-hDAFΔCCP4 cells showed the same levels of adhesion of Dr-positive bacteria as infected CHO-hDAF cells (data not shown). Using indirect immunofluorescence labeling of hDAF and Dr fimbriae and 3D reconstruction of confocal images, we investigated the mobilization of hDAF around Dr-positive *E. coli* adhering to CHO-hDAFΔCCP1 and CHO-hDAFΔCCP4 cells. The mobilization of hDAF observed around bacteria was the same for infected CHO-hDAFΔCCP1 cells as for CHO-hDAF cells (Fig. 5A). In contrast to the mobilization observed in infected CHO-hDAF or CHO-hDAFΔCCP1 cells, there was no mobilization of hDAF around Dr-positive *E. coli* adhering to CHO-hDAFΔCCP4 cells (Fig. 5A). 3D image analysis showed the persistence of the punctate, diffuse hDAF cell labeling in infected hDAFΔCCP4 cells that was not observable in CHO-hDAF cells due to hDAF mobilization around adhering bacteria (Fig. 5A). Western blot analysis showed an increase in the levels of activated Src kinases in CHO-hDAF and CHO-hDAFΔCCP1 cells infected with wt strain IH11128 or recombinant Dr-positive *E. coli* compared to those in cells that had not been infected. In contrast, the level of activated Src kinases was the same in infected CHO-hDAFΔCCP4 cells as in uninfected cells (Fig. 5B). Taken together, these results demonstrate the pivotal role of the CCP4 domain of hDAF in hDAF mobilization and the activation of Src kinases after cell infection with Dr-positive bacteria.

**Activated Src kinases were mobilized around adhering Dr-positive bacteria in the same way as hDAF.** The findings described above revealed the role of Src kinase activation in the mobilization of hDAF around adhering Dr-positive bacteria. We therefore went on to use indirect fluorescence microscopy to find out whether the activated Src kinases were mobilized around adhering Dr-positive bacteria. Uninfected HeLa cells immunolabeled with an antibody directed against activated Src kinases (Src-Y416P) and with an anti-hDAF antibody displayed diffuse positive labeling of activated Src kinases and hDAF, and the activated Src kinases were concentrated around the edges of the cells, which was consistent with the presence of filipodia (Fig. 6A). In cells infected with Dr-positive *E. coli*, we observed intense labeling of activated Src kinases around the adhering Dr-positive bacteria that colocalized with the mobilized hDAF (Fig. 6A). In order to confirm
this observation, CHO-hDAF cells were engineered to express Src-wt-GFP or constitutively activated Src-Y527F-GFP. Figure 6B showed that both Src-wt-GFP and Src-Y527F-GFP were recruited around adhering Dr-positive bacteria. In order to find out whether the recruitment of Src was hDAF signaling dependent, CHO-hDAFΔCCP4 cells were engineered to express Src-wt-GFP or constitutively activated Src-Y527F-GFP. As shown in Fig. 6B, the adhering Dr-positive bacteria were not decorated with Src-wt-GFP or Src-Y527F-GFP. We were able to confirm this finding, since there was no bacterial recruitment of either Src-wt-GFP or Src-Y527F-GFP in HeLa cells expressing Src-wt-GFP or constitutively activated Src-
Y527F-GFP and infected with the Dr" D54C mutant (Fig. 6C). Finally, we showed that in CHO-hDAF cells engineered to express myristoylation-defective GFP-Src kinase mutant G2A and infected with Dr-positive *E. coli*, there was no recruitment of the Src mutant around adhering bacteria (Fig. 6B). We also showed that there was no recruitment of the GFP-Src kinase G2A mutant around Dr-positive *E. coli* adhering to CHO-hDAFΔC4P4 cells or around the Dr" 54C mutant adhering to CHO-hDAF cells (Fig. 6B and C).

c-Src is specifically involved in the mobilization of hDAF around Dr-positive bacteria. We next identified the Src kinase involved in the hDAF clustering. SFK is composed of several members, including the kinases c-Src, Yes, Fyn, and Lyn, which are more specifically expressed in epithelial cells. We first investigated whether one or more of these four Src kinases were activated and recruited in the vicinity of hDAF in response to the infection of HeLa cells with Dr-positive *E. coli*. For this purpose, Fyn, Lyn, c-Src, and Yes were immunoprecipitated using specific monoclonal antibodies, and their active form (Y418P) was detected by Western blotting, as previously described (Fig. 7A1 to A4, top panels). The recruitment of each Src kinase was studied by immunofluorescence analysis and confocal microscopy (Fig. 7A1 to A4, lower panels). We observed that Fyn and Lyn were either activated or recruited in the vicinity of hDAF (Fig. 7A1 and A2), whereas c-Src and, to a lesser extent, Yes were phosphorylated at the Y418 position and colocalized with hDAF (Fig. 7A3 and A4) during infection.

To identify precisely which Src kinase was involved in hDAF clustering, we knocked down the expression of each kinase using specific siRNAs which inhibited at least 80 to 90% of the expression of c-Src, Yes, Fyn, and Lyn (Fig. 7B, top panels). Immunofluorescence analysis of hDAF clustering in infected HeLa cells followed by confocal microscopy then showed that only the down-expression of c-Src abolished the mobilization of hDAF around Dr" *E. coli* (Fig. 7B, lower panels). Taken together, these results strongly suggested the specific involvement of c-Src activation in hDAF clustering around Dr-positive bacteria.

hDAF-dependent Src kinase signaling is involved in hCEACAM1-4L activation during Dr-positive *E. coli* infection. Physiologically, several epithelial cells express hDAF and hCEACAM family receptors. It has been previously reported that hCEACAMs 1 and 6 and hCEA act as Afa/Dr DAEC receptors. Only the signalization induced by Afa/Dr DAEC hCEACAM1-4L is known. In particular, Rougeau et al. showed that the activation of hCEACAM1-4L by phosphorylation of its tyrosines 493 and 520 is dependent on Src kinases (48). Thus, we wondered whether a direct relationship exists between hDAF and hCEACAM1-4L signaling. To examine this, we used HeLa cells expressing hDAF and hCEACAM1-4L (HeLa-CAM1-4L cells) with or without infection. hCEACAM1-4L was immunoprecipitated, and tyrosine phosphorylation was analyzed by Western blotting. We confirmed that the inhibition of Src kinase signaling using PP2 inhibits the phosphorylation of hCEACAM1-4L induced by Dr-positive *E. coli* (Fig. 8A). Then we knocked down the expression of hDAF in HeLa-CAM1-4L cells using specific siRNAs. We observed that the activation of hCEACAM1-4L is specifically inhibited since the transfection with a control siRNA had no effect (Fig. 8B). Interestingly, we showed that the knockdown expression of c-Src and Yes in HeLa-CAM1-4L cells decreases the phosphorylation of hCEACAM1-4L on its tyrosine residues (Fig. 8C). These results strongly suggest that hDAF signaling induced by Dr adhesin regulates the activation of hCEACAM1-4L.

DISCUSSION

Many microbial pathogens use lipid raft microdomains in their pathogenicity processes (18). The cell entry of Afa/Dr DAEC isolates expressing typical Afa/DrDHAF and Afa/DrHCEACAM adhesins has been found to depend on the integrity of the lipid rafts (21, 22, 28). This dependency on lipid rafts has been attributed to the fact that the GPI-anchored hDAF, hCEA, and hCEACAM6 act as receptors for Afa/Dr DAEC cells expressing typical Afa/DrDHAF and Afa/DrHCEACAM fimbrii (2, 41) and are mobilized around Afa/Dr DAEC adhering bacteria (2, 19, 23). Moreover, the non-lipid-raft-associated transmembrane hCEACAM1-4L acting as a receptor for Afa/Dr DAEC expressing typical Afa/DrHCEACAM fimbrii is mobilized around adhering bacteria (2) and translocated within lipid rafts in infected cells to downregulate Dr fimbria-activated, hDAF-associated Src signaling (48). Curiously, results reported both here and previously (28) reveal that even though hDAF is associated with lipid rafts via its GPI anchor (34) and the raft markers ganglioside M1 and VIP21/caveolin are mobilized around adhering Dr-positive bacteria, the mobilization of hDAF occurs independently of lipid raft integrity. This could mean that only the extracellular domain of the GPI-anchored hDAF is needed for the Dr fimbria/hDAF interaction to occur, regardless of the membrane localization of the cell receptor. In support of this hypothesis, Guignot et al. (23) have shown that hDAF remains mobilized around Dr- and F1845-positive *E. coli* bacteria adhering to CHO cells expressing a transmembrane version of hDAF constructed with the transmembrane domain of the membrane cofactor protein (14). In addition, Lublin and Coyne (35) have observed that the transmembrane version of hDAF protects CHO transfectants against cytotoxicity as effectively as hDAF, suggesting that the GPI-anchored and transmembrane versions of a protein have the same degree of lateral mobility in the membrane. Supporting the pivotal role of the extracellular domain of hDAF, Guignot et al. (23) have observed that no mobilization occurs around Dr-positive bacteria adhering to cells expressing mutated hDAF in which the heavily O-glycosylated serine/threonine-rich (S/T) region has been deleted. This region acts as an important but nonspecific spacer that projects the hDAF functional domains above the plasma membrane (14).

The Src kinase activation that leads to the mobilization of hDAF around Dr-positive *E. coli* clearly depends on the structural features of Dr fimbriae, since we found that mutation D54C is sufficient to abolish Src kinase signaling and, consequently, hDAF clustering around *E. coli* expressing the mutated Dr fimbriae. Several distinct domains of the DraE adhesin subunit have been identified to be controlling adhesive functions. The binding of the DraE adhesin subunit to type IV collagen, in contrast to the other members of the Afa/Dr family which lack this adhesiveness, is mediated by domains located within the amino-terminal portion of the subunit (58). Individ-
eral amino acid changes at position 10, 63, 65, 75, 77, 79, or 131 of the DraE adhesin subunit all reduced the ability to bind hDAF but not collagen (62). Korotkova et al. (30) investigated Afa/Dr DAEC strains that form eight structural groups with a high level of amino acid sequence diversity between them and identified only three groups with synonymous mutations, indicating strong positive selection for amino acid replacements. Functional analysis of intragroup variants comprising the DraE adhesin subunit group has shown that point mutations result in distinctly different binding phenotypes, with a tendency toward increased affinity for hDAF, decreased sensitivity of hDAF binding to inhibition by chloramphenicol, and loss of binding capability to collagen, hCEACAM3, and hCEACAM6. Carnoy and Moseley (11) used site-directed mutagenesis to investigate members of the Afa/Dr family, including Afa-I, Afa-III, Dr, and F1845 fimbrae that bind to type IV collagen, and demonstrated that a negatively charged amino acid is required at position 54 of the DraE adhesin subunit to produce chloramphenicol-sensitive binding without modifying hDAF-binding capability. The draE mutant, in which cysteine replaces aspartic acid at position 54, displayed conserved hDAF-binding capacity (11) but failed to induce hDAF mobilization around adhering bacteria (23). This mutation affects Afa/Dr DAEC-induced brush border actin disassembly in polarized enterocyte-like cells (43) but does not affect the cell entry of Dr-positive E. coli (28). In contrast, this mutant as well as another mutant in which aspartic acid is substituted for glycine at position 54 showed a dramatic loss of binding to hCEACAM1, hCEA, and hCEACAM6, and these molecules failed to mobilize around the remaining adhering bacteria (2).

With regard to hDAF signaling, Shenoy-Scaria et al. (53, 54) have reported that the GPI anchor of hDAF is needed to transduce early activation events through its association with p56lck and p59fyn and that palmitoylation of the amino-termini cysteine residue(s), together with myristylation of the amino-terminal glycine residue, defines motifs that are important for the association between protein tyrosine kinases and the GPI-anchored protein. Our data reported here provide the first evidence that hDAF signaling also depends on the structural features of its extracellular domain. Indeed, we show that the CCP4 domain plays a pivotal role in signaling, since the specific deletion of this domain abolished the Dr fimbrin-induced activation of the Src kinases that control the mobilization of hDAF around adhering Dr-positive bacteria, whereas deletion of CCP1 did not. Coyne et al. (14) mapped the hDAF domains responsive to its complement regulatory function and have shown that the removal of CCP1 had no effect on hDAF function, whereas individual deletions of CCP2, CCP3, CCP4, or the S/T region totally abolished hDAF function. For the binding of Afa/Dr DAEC to host cells, Nowicki et al. (40) have reported that the deletion of CCP3 or a single-point substitution in the CCP3 domain (Ser165 to Leu) completely abolished the binding of Dr fimbrae to hDAF. Consistent with this loss of binding, Selvarangan et al. (51) observed that the deletion of CCP2 or CCP3 and the Ser165-to-Leu mutation in CCP3 resulted in the loss of the cell internalization of Dr-positive E. coli, whereas the deletion of CCP1 or CCP4 did not affect internalization.

We showed that activation of Src kinases is required for hDAF to be mobilized around Dr-positive E. coli, since no mobilization occurred when the cells were treated with the Src kinase inhibitor PP2. We confirmed the necessity of the presence of activated Src kinases, since no mobilization of hDAF was observed in cells overexpressing a mutant Src with an inactivated catalytic domain. The Src binding protein domains SH2 and SH3 are known to mediate the intramolecular interaction of the active form of Src kinases with several membrane-bound and cytosolic proteins (47). Investigating the role of SH2 and SH3, we found that even when the kinase was in its activated form, disruption of the SH2- or SH3-binding domain abolished the recruitment of hDAF around Dr-positive E. coli. It was noted that Shvartsman et al. (55) have observed that the SH2-binding domain, but not the SH3-binding domain, is required for the Src interaction with slower-diffusing membrane-associated proteins. However, it seems that the role of the SH2- and SH3-binding domains differed as a function of the Src kinase examined (59). For example, it has been established that the SH3 and SH2 domains are capable of directing specificity in substrate binding between Src and Yes (57). In addition, we showed that Src kinase myristylation plays a pivotal role in the Dr fimbrin-induced mobilization of Src kinases around adhering Dr-positive E. coli. Myristylation is known to be critical for the membrane association of Src kinases (46), and it also controls kinase activities (38, 42). We identified c-Src and, to a lesser extent, Yes as the Src kinases activated...
and recruited around adhering Dr-positive bacteria. However, we showed that only c-Src was involved in hDAF clustering. It is intriguing to speculate about the possible role of Dr fimbriae/hDAF-induced mobilization of activated Src kinases in downstream signaling and the cellular response(s). Evidence has recently been reported that viruses, bacteria, and parasites manipulate the tyrosine kinase-mediated signaling pathways of their hosts to achieve their efficient entry, replication, and exit during their infectious cycles (13, 16, 27, 37, 50). Concerning the Afa/Dr DAEC cell entry, several reports do not support the suggestion that Src kinases and F actin may be involved in the cell entry of Afa/Dr DAEC. Although actin is mobilized around Dr fimbria-expressing E. coli (19), the cell entry of Afa/Dr DAEC expressing typical Afa/DrhDAF or Afa/DrhCEACAM fimbriae mediated by hDAF, hCEA, or hCEACAM6 (22, 28) was not affected either by tyrosine kinase inhibitors or by disruption of the actin microfilaments by cytochalasin D. However, we report here for the first time that Src kinase signaling is involved in an early step during Afa/Dr DAEC infection. Indeed, hDAF clustering, which is rapidly mediated by Src kinase signaling, is probably a key step in Afa/Dr DAEC adherence to host cells. The Dr-induced activation of Src kinases could also be related to the previously observed hDAF-dependent proinflammatory responses. Indeed, the production of interleukin-8 (IL-8) and vascular endothelial growth factor has been found to be elevated in enterocyte-like cells as a result of the recognition of hDAF by Afa/DrhDAF fimbriae and the activation of mitogen-activated protein kinases (MAPKs) (4, 5, 9, 10, 15). Moreover, hDAF is well-known to prevent complement cascade occurring at the level of the critical C3 convertase step, thus protecting cells and tissues against complement-mediated damage. Hasan et al. reported that Dr adhesin/hDAF interaction decreases the protective effect of hDAF against the complement system by altering the regulation of the alternative pathway (24). The recruitment of hDAF observed could therefore play an important role in the dissemination of bacteria by increasing the sensitivity of cells to the autologous lysis induced by the complement system.

FIG. 8. hDAF-dependent Src kinase signaling is involved in hCEACAM1-4L activation during Dr-positive E. coli infection. HeLa cells constitutively expressing hDAF and transfected to stably expressed hCEACAM1-4L (HeLa-CAM1-4L) were not infected (NI) or infected for 1 h 30 min with Dr-positive E. coli (Dr+). hCEACAM1-4L was immunoprecipitated, and its expression and its tyrosine phosphorylation were analyzed by Western immunoblotting using specific antibodies directed against hCEACAMs and phosphotyrosine residues (pTyr). (A) HeLa-CAM1-4L cells were treated with Src kinase inhibitor PP2 or its inactive analog, PP3. (B) HeLa-CAM1-4L cells were transfected with siRNA control or specific for hDAF. (C) HeLa-CAM1-4L cells were transfected with siRNA control or specific for the kinase c-Src or Yes. Each immunoblot is representative of three independent experiments. Blots were quantified using Image J software. The value reported represents the mean ± SEM. *, P < 0.05 (Student’s t test) versus the appropriate control cells (A and B, lower panels; C, right panel).
In epithelial tissues, hDAF is coexpressed with other receptors of the IH1128 strain, including hCEACAM1, hCEACAM6, and hCEA. These receptors are also recruited around Afa/Dr DAEC (2). However, the signaling pathways associated with these hCEACAM family members in response to the infection with Afa/Dr DAEC are not characterized, with the exception of full-length hCEACAM1-4L (48). During infection, the full-length hCEACAM1-4L is translocated into lipid rafts and is phosphorylated on two cytoplasmic tyrosine residues (p-Tyr493 and p-Tyr520) by Src kinases. Phosphorylated hCEACAM1-4L functions as a negative regulator of hDAF-dependent Src kinase signaling. Here we showed that Src kinases are rapidly activated by Dr adhesin/hDAF interaction. Indeed, C-Src is necessary for the recruitment of hDAF around bacteria, and also, C-Src in combination with Yes is directly involved in the regulation of hCEACAM1-4L activation. The downregulation of hDAF signaling by activated hCEACAM1-4L suggests that hDAF is not implicated in the later cell responses to infection but that the hCEACAM1-4L signaling pathway takes over. Altogether, these results provide evidence that hDAF plays a central role in the early events of Afa/Dr DAEC pathogenesis, including bacterial adherence and the establishment of cellular responses.

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