Hydrophilic Domain II of *Escherichia coli* Dr Fimbriae Facilitates Cell Invasion

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Uropathogenic and diarrheal *Escherichia coli* strains expressing adhesins of the Dr family bind to decay-accelerating factor, invade epithelial cells, preferentially infect children and pregnant women, and may be associated with chronic or recurrent infections. Thus far, no fimbrial domain(s) that facilitates cell invasion has been identified. We used alanine scanning mutagenesis to replace selected amino acids in hydrophilic domain II of the structural fimbrial subunit DraE and evaluated recombinant mutant DraE for attachment, invasion, and intracellular compartmentalization. The mutation of amino acids V28, T31, G33, Q34, T36, and P40 of DraE reduced or abolished HeLa cell invasion but did not affect attachment. Electron micrographs showed a stepwise entry and fusion of vacuoles containing *Escherichia coli* mutants T36A and Q34A or corresponding beads with lysosomes, whereas vacuoles with wild-type Dr adhesin showed no fusion. Mutants T31A and Q34A, which were deficient in invasion, appeared to display a reduced capacity for clustering decay-accelerating factor. Our findings suggest that hydrophilic domain II may be involved in cell entry. These data are consistent with the interpretation that in HeLa cells the binding and invasion phenotypes of Dr fimbriae may be separated.

Urinary tract infections (UTIs) account for 7 to 8 million doctor's office visits a year (4, 25). The estimated annual cost of UTIs in the United States is approximately \$1.6 billion. About 90% of all UTIs are caused by *Escherichia coli* (15). *E. coli* colonizes and infects the host using surface appendages called fimbriae, pili, or adhesins that bind to receptors on the host cells. Among the most frequent colonization factors of uropathogenic *E. coli* are type 1 P fimbriae and the Dr family of adhesins with unusual uro-diarrheal virulence. About 50% of children with protracted diarrhea, 25% to 50% of children with cystitis, and 30% of pregnant women with pyelonephritis are infected with *E. coli* bearing Dr adhesins (15). Dr⁺ *E. coli* may increase the risk of recurrent UTIs (5). Infection with Dr⁺ *E. coli* may lead to chronic experimental pyelonephritis in C3H/HeJ mice (7). Dr⁺ *E. coli* is capable of long-term survival in human epithelial cells, and the fimbrial antigen may persist in the kidneys of experimental animals for several months (14). These observations are consistent with a hypothesis proposed in 1988 by Nowicki et al. about the association of *E. coli* Dr adhesins with chronic/recurrent UTIs (21).

The growing superfamily of Dr adhesins includes Dr hemagglutinin, Dr-II, Afa-I, -II, -III, and -IV, F1845, and several others of human and animal origin (20). Members of the Dr family recognize as their receptor the Dr(a) blood group antigen that was mapped to decay-accelerating factor (DAF)

(19). DAF (CD55) is a complement-regulatory protein that protects tissues from autologous complement-mediated damage. Dr⁺ *E. coli* has been the first example of a pathogen recognizing CD55 (17, 19). Recent studies have shown that echoviruses and coxsackieviruses bind to CD55 (2, 27). Closely related to DAF, CD46 acts as a receptor for *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Helicobacter pylori*, and *Streptococcus pyogenes* (13).

The DAF SCR-3 loop, the region between amino acids S155 and S165, is a critical binding site for Dr fimbriae (10, 17, 18). The structural adhesin (e.g., DraE), which forms the fimbrial polymer, determines the receptor-binding specificity of Dr adhesins (17). The interaction of *E. coli* bearing Dr adhesin with DAF leads to bacterial internalization (8). Mutation of the Dr operon abolishes bacterial attachment or invasion in cell lines and chronic nephritis in mice (7, 8). The receptor-binding site of the DraE fimbrial subunit appears to be located in the amino-terminal half, and amino acids 63 to 81 appear to be involved in DAF recognition (3, 29, 30).

The *dra* operon also encodes the DraD protein (adjacent to DraE), which is postulated to be an invasin (6). Recent studies of AfaD, a homolog of DraD, suggest that β 1 integrin may be involved in AfaD internalization (23).

We investigated hydrophilic domain II of the Dr adhesin and show here that the binding and invasion phenotypes of Dr fimbriae may be separated.

MATERIALS AND METHODS

Bacterial strains. All plasmids were maintained in *E. coli* XL1-Blue (Stratagene, La Jolla, Calif.). Bacteria were grown on Luria-Bertani agar with 100

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μg/ml ampicillin to maintain the plasmid. Restriction enzymes were obtained from Promega Corporation (Madison, Wis.).

Plasmids. Plasmid pCC90 contains the Dr operon with a deletion of regulatory genes upstream of *draB* (3) which results in a constant expression of Dr fimbriae. Plasmid pCC90-Δ*Sac* does not express Dr adhesin due to a 1.1-kb deletion that includes the 3' end of *draD* and the 5' end of *draE*. Plasmid pCC90-D54Stop does not express Dr adhesin due to a stop codon at amino acid 54 in *draE*.

Site-directed mutagenesis. Site-directed mutagenesis was performed using a QuikChange site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, Calif.). Synthetic oligonucleotides were purchased from MWG Biotech Inc. (Highpoint, NC). Plasmid pCC90 was used as the template to introduce mutations into *draE*. Mutations were confirmed by DNA sequencing at the Protein Chemistry Core Laboratory (University of Texas Medical Branch, Galveston, Tex.). Sequencing was performed with PE Biosystem 373XL automated DNA sequencers using a Big Dye Terminator cycle sequencing kit with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, Calif.).

Binding of Dr⁺ *E. coli* to DAF⁺ Chinese hamster ovary cells. *E. coli* cells expressing wild-type or mutant Dr fimbriae were added to culture plates containing DAF⁺ Chinese hamster ovary (CHO) cells grown on coverslips. After incubation for 2 h at 37°C, the cells were washed twice with phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde, and stained with Giemsa stain. The *E. coli* cells bound to DAF⁺ CHO cells were observed under a microscope. The number of bacteria bound to DAF⁺ CHO cells was determined by counting 30 CHO cells, and the result was expressed as the number of bacteria per DAF⁺ CHO cell. The experiments were performed in duplicate (26).

Invasion of DAF⁺ CHO cells by Dr⁺ *E. coli*. Recombinant CHO cells expressing DAF or HeLa cells were seeded in 24-well plates. *E. coli* cells expressing wild-type or mutant Dr fimbriae were added at a concentration of 5×10^7 /ml and incubated for 3 h at 37°C with 5% CO₂. The cells were washed and incubated with gentamicin (200 μg/ml) for 1 h. The monolayer of cells was washed twice with F12 medium and lysed in 100 μl of lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 2% Triton X-100, pH 7.5) as described previously (8). The intracellular bacteria were released with the lysis buffer, and aliquots were cultured overnight at 37°C with 5% CO₂. Invasion was expressed as the number of CFU per well. Each experiment was performed in triplicate.

Purification of Dr adhesin from *E. coli*. Dr adhesin was purified from *E. coli* by a heat shock procedure followed by 40% (wt/vol) ammonium sulfate precipitation. The suspension was centrifuged, and the pellet was resuspended in 40% (wt/vol) ammonium sulfate and incubated overnight at 4°C. Following dialysis against PBS, the collected fractions were passed through a Sepharose 4B column, from which Dr adhesin was eluted in the void volume. The protein concentrations in eluted samples were evaluated with a BCA protein assay (Pierce, Rockford, Illinois).

Hemagglutination of red blood cells. Purified Dr fimbriae at a concentration of 1 μg/ml or Dr⁺ *E. coli* was mixed with an equal volume of a 3% (vol/vol) suspension of human erythrocytes in PBS with 2% methyl-α-D-mannoside. Two-fold dilutions of *E. coli* or purified fimbriae were rotated for 5 min on ice, and the hemagglutination titer was recorded (16).

SDS-PAGE and Western blot analysis. Purified fimbrial proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis. SDS-PAGE was performed by the Laemmli method using a 15% polyacrylamide gel (12). Proteins were visualized by silver and Coomassie blue staining. Polyclonal rabbit antibodies to Dr adhesin were used for Western blot analysis to detect DraE, as described in our prior studies (22).

Coating of polystyrene beads with purified Dr adhesin. Polystyrene (latex) beads (Polybead 2.5% solid polystyrene beads with a 1.072-μm diameter; Polyscience Inc., Warrington, Pa.) were diluted 1:10, washed three times with 0.1 M borate buffer (pH 8.5), and resuspended in the same buffer. Purified Dr fimbrial protein or a Dr mutant (75 μg/ml) was diluted 1:10 in the suspension of polystyrene beads and mixed overnight at room temperature. Control beads were coated with bovine serum albumin (BSA, fraction V; Sigma Chemical Co., St. Louis, Mo.). The suspensions were spun down, resuspended in 1 ml of bovine serum albumin (10 mg/ml) in borate buffer, and incubated for 30 min at room temperature; the beads were then recovered by centrifugation. The procedure was repeated twice before the beads were finally resuspended in storage buffer (PBS containing 10 mg/ml bovine serum albumin and 5% glycerol) and stored at 4°C.

The biological activity of the fimbrial proteins and coated beads was confirmed by a hemagglutination assay. Each sample showed a similar hemagglutination titer, indicating the presence of similar concentrations of functional adhesin or its mutants on coated beads.

Adherence of beads coated with wild-type and mutated Dr fimbriae to HeLa

Domain II
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GFTPSGTTGTTKLTVTTEECQVRVGLTIVAKTRGQLTDAAP
GPVTVQALGCDARQVALKADTDNFEQGGKFFLISDNNRDKLY
VNIRPTDNSAWTTDNGVFYKNDVGSWGGIIGIYVDGQQTNT
PPGNYTLTLTGYYWAK
  
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FIG. 1. Sequence of amino acids (V28 to P40) in DraE domain II (in bold) targeted for alanine scanning mutagenesis. Amino acids replaced with alanine are pointed out with asterisks.

and DAF⁺ CHO cells. Latex beads coated with purified wild-type Dr fimbrial proteins at equivalent concentrations were used to assess the binding of the DAF receptor accumulated at the sites of adherence. Subconfluent HeLa or DAF⁺ CHO cell monolayers grown on glass coverslips were washed twice with PBS and subsequently incubated for 1 h with a bead suspension diluted 1:1,000 in appropriate culture medium supplemented with 10% fetal bovine serum. DAF⁺ CHO cells were used as a negative control. Cells were washed with PBS and fixed for 10 min with 4% formaldehyde. Coverslips were incubated for 45 min with 50 μl of rabbit anti-Dr polyclonal antibody diluted 1:100 in PBS, followed by two washings with PBS. Cells were then incubated with 50 μl of goat anti-rabbit antibody conjugated with Texas Red (Molecular Probes, Inc., Eugene, Oreg.) diluted 1:100 in PBS. Coverslips were mounted on microscopic slides with Cytoseal (Stephens Scientific, Riverdale, NJ) and examined under a Nikon Eclipse 600 fluorescence microscope (Nikon Inc., Melville, NY).

Recruitment of DAF (CD55) molecules to HeLa cells at sites of adherence of latex beads coated with purified Dr fimbrial proteins. The recruitment assay was designed to assess the DAF density at the sites of adherent beads coated with wild-type and mutated Dr proteins. Subconfluent HeLa cell monolayers were incubated with beads and processed for staining as in the binding assay. Coverslips were incubated for 45 min with 50 μl of an anti-CD55 monoclonal antibody (CALTAG Laboratories, Burlingame, Calif.) diluted 1:50 in PBS, followed by two washings with PBS. Cells were then incubated with 50 μl of goat anti-mouse antibody conjugated with Cy3 (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.) diluted 1:100 in PBS. Coverslips were examined with a fluorescence microscope. Images were acquired with a charge-coupled device camera (CoolSnap; Roper Scientific, Inc., Trenton, NJ). The DAF staining associated with different types of beads was evaluated with Metamorph software, version 5.0r7 (Universal Imaging Corporation, Downingtown, Pa.). The images of 15 randomly selected beads from each slide were framed into identical regions of 30 by 30 pixels, which entirely comprised a bead with a surrounding area. This approach allowed us to measure the intensity of DAF staining and to characterize the average intensity of staining associated with beads coated with wild-type and mutated Dr fimbrial proteins.

Electron microscopy. For transmission electron microscopy, cells were fixed with Ito's fixative and embedded in Poly/Bed 812 resin (Polysciences, Inc., Warrington, Pa.). Ultrathin sections were cut on a Sorvall MT-6000 ultramicrotome (RMC, Tucson, Ariz.), stained with aqueous uranyl acetate and lead citrate, and examined in a Philips 201 transmission electron microscope (Philips Electron Optics, Eindhoven, The Netherlands) at 60 kV.

Modeling of DraE hydrophilic domain II. For the preparation of figures showing DraE, we used previously identified coordinates and X-ray crystallographic data for the DraE structure which are available in the Protein Data Bank (23a). Figures were prepared using the programs AESOP, PyMol, and TOPDRAW.

RESULTS

Alanine scanning mutagenesis of hydrophilic domain II of DraE. Amino acids V28, T31, G33, Q34, L35, T36, and P40 of hydrophilic domain II of DraE were replaced with alanine by site-directed mutagenesis (Fig. 1), and the mutations were confirmed by DNA sequencing. *E. coli* expressing the mutant Dr adhesin or purified adhesins were used in the experiments described below.

Alanine substitutions in hydrophilic domain II do not affect binding to cellular receptor DAF. *E. coli* expressing wild-type Dr adhesin or the Dr mutants was first quantitatively assayed for the agglutination of red blood cells. Hemagglutination by Dr⁺ *E. coli* is the result of binding to DAF on the surfaces of

FIG. 2. Binding of *E. coli* Dr mutants to DAF. White bars, DAF⁺ CHO cells; black bars, DAF⁻ CHO cells. Note that all mutants, with one exception, bound to DAF⁺ but not to DAF⁻ CHO cells. The binding of all mutants to SCR-1 and SCR-4 deletion mutants was similar to that observed for the intact DAF, and conversely, the lack of binding to SCR-2 and SCR-3 mutants was identical to that in DAF-negative CHO cells (identical binding was not depicted for clarity).

red blood cells. All but one mutant showed hemagglutination titers of 1:64 to 1:128, comparable with that of wild-type Dr adhesin (1:128). The *E. coli* mutant L37A was negative for hemagglutination.

To further study the binding of *E. coli* expressing mutant Dr adhesins to DAF, we performed a cell binding assay using recombinant CHO cells expressing human DAF. The results showed that mutants V28A, T31A, G33A, Q34A, T36A, and P40A bind DAF comparably to wild-type Dr adhesin but that mutant L35A does not (Fig. 2). Control Dr⁺ *E. coli* did not bind to DAF⁻ CHO cells, and Dr⁻ *E. coli* did not bind to DAF⁺ CHO cells. These results indicate that alanine substi-

tutions for amino acids in hydrophilic domain II did not affect the binding of the Dr adhesin to DAF.

Expression of Dr adhesin was comparable for wild-type and mutant *E. coli* strains. A Western blot analysis of whole-cell lysates (not shown) or purified fimbriae showed that the Dr adhesin is expressed at equitable levels in the V28A, T31A, G33A, Q34A, T36A, and P40A mutants compared with the wild type (Fig. 3). The L35A mutant did not express Dr adhesin in these immunoblots. These results are consistent with the hemagglutination titers and attachment data and indicate that the differences in invasion between the mutants were not due to differences in expression.

Binding patterns of mutant Dr adhesins are similar to that of wild-type Dr adhesin. *E. coli* strains expressing mutated Dr adhesins bound DAF⁺ CHO cells comparably to the wild type, but the mutants either did not invade DAF⁺ cells or invaded them at a very low frequency. To confirm that the binding specificities of the mutated Dr adhesins were unchanged, we tested the binding of *E. coli* expressing the mutated Dr adhesins to CHO cells expressing DAF with deletions in each of the four SCR domains. The results indicated that wild-type Dr adhesin, as well as the adhesins from mutants V28A, T31A, G33A, Q34A, T36A, and P40A, binds to DAF with deletions of SCR-1 and SCR-4 but not to DAF with deletions of SCR-2 and SCR-3 (Fig. 2). A quantitative analysis showed similar rates of binding for the mutant adhesins and wild-type Dr adhesin. These results affirm that the lack of invasion was not due to a change in receptor-binding specificity.

Alanine scanning mutagenesis of amino acids in the domain from V28 to P40 impedes cell invasion by Dr⁺ *E. coli*. The *E. coli* mutants were tested on two types of cells, namely, recombinant DAF⁺ CHO and HeLa cells, which express abundant DAF. The data show that mutation of the targeted amino acids in Dr adhesin hinders the invasion of recombinant DAF⁺ CHO cells by Dr⁺ *E. coli* (Fig. 4). *E. coli* expressing wild-type Dr adhesin invaded cells at rates of 2,500 to 4,000 CFU/well; with the V28A, G33A, and Q34A mutants, however, invasion was reduced 25- to 40-fold. Invasion was totally abolished in

FIG. 3. Visualization of Dr adhesins expressed by recombinant *Escherichia coli*/pCC90 and its mutants by Coomassie blue staining (A) and Western blotting with rabbit anti-Dr serum (B). All preparations were isolated from the same amount of bacteria (optical density at 600 nm, 0.6). Note that Dr and Dr mutant proteins migrate with the 14.3-kDa marker. Lanes: M, molecular weight markers; 1, *E. coli*/pCC90; 2, V28A; 3, T31A; 4, G33A; 5, Q34A; 6, L35A; 7, T36A; 8, P40A; 9, D54Stop; 10, SacI.

FIG. 4. Mutation of target amino acids of domain II hinders invasion. (A) Invasion of DAF⁺ CHO cells (white bars) and DAF⁻ CHO cells (black bars). (B) Invasion of HeLa cells.

the T31A, T36A, and P40A mutants. The background level of invasion of T31, T36, and P40 mutants was similar to that observed with fimbria-negative control Dr⁻ *E. coli*.

In a further study, we reduced the bacterial inoculum from 2×10^8 to 5×10^7 . The reduction of the inoculum size decreased the rate of invasion and allowed the elimination of a 15% background (not shown) of DAF-independent, presumably nonspecific, internalization. The reduction of nonspecific invasion was important for further evaluation of the potential differences between mutants and for objective analysis by electron microscopy.

With HeLa cells (Fig. 4B), invasion was reduced 14-fold for the V28A, G33A, Q34A, and P40A mutants and was totally abolished for the T31A and T36A mutants. Thus, the cell invasion patterns were generally similar for CHO and HeLa cell types. In the control experiments, there was no invasion of DAF⁻ CHO cells, and Dr⁻ *E. coli* did not invade DAF⁺ CHO cells or HeLa cells. The results indicate that amino acids V28, T31, G33, Q34, T36, and P40 play a role in cell invasion.

The contribution of each amino acid to the process of cell entry was further evaluated by electron microscopy. Dr⁺ *E. coli* strains with T31A, G33A (Fig. 5B and C), and P40A mutations bound to the surfaces of DAF⁺ CHO cells in large numbers, but there was no evidence of cell invasion. Conversely, mutant V28A showed bacteria that appeared to be in the early stages of invaginating the cell membrane. Mutant Q34A was internalized in a loose vacuole containing more than one bacterium, with the morphological feature of phagolysosomal fusion (Fig. 5D). A similar pattern of internalization was found for mutant T36A. A vacuole containing *E. coli*/pCC90 with wild-type Dr fimbriae did not show morphological features of fusion with a lysosomal compartment (Fig. 5A).

FIG. 5. Electron micrographs of cell invasion by *E. coli* expressing wild-type and mutant Dr adhesins. Panel A shows *E. coli*/pCC90 internalized in a tight vacuole (arrows). Panels B and C show *E. coli* mutants T31A and G33A (arrows) binding to but failing to invade DAF⁺ CHO cells. Panel D shows multiple bacteria of the mutant Q34A (arrows) internalized in a loose vacuole with the morphological features of phagolysosomal fusion (arrowheads). Panel E shows that *E. coli*/pCC90 does not bind or invade DAF⁻ CHO cells.

FIG. 6. Electron micrographs showing invasion of DAF⁺ CHO cells by beads coated with wild-type and mutant Dr adhesins. Panels A, B, and C show binding, engulfment, and internalization, respectively, of latex beads (arrows) coated with wild-type adhesin. Inside the cell, the beads are in a tight vacuole with no features of fusion with a lysosome. Panel D shows beads coated with the mutant Dr adhesin Q34A (arrows) inside vacuoles containing vesicular material (arrowheads). This might suggest fusion with the phagolysosomal compartment. Panel E shows that latex beads coated with BSA did not bind DAF⁺ CHO. Note that this control experiment indicated the specificity of the binding assay.

Binding and cell invasion by beads coated with recombinant mutant Dr fimbriae. To further characterize the properties of purified adhesins and dissect the possible impact of other *dra* gene products expressed in *E. coli* on the invasion process, latex beads were coated with equal quantities of purified mutant Dr fimbriae and tested for binding and invasion. Both control and mutant Dr adhesin-coated beads bound DAF⁺ CHO and HeLa cells. There was no noticeable difference in the average numbers of beads associated with DAF⁺ cells between the wild-type and mutant Dr adhesins (data not shown); these results corroborated the data with *E. coli* showing that the replacement of amino acids in domain II did not affect DAF binding.

Dr adhesin-coated beads were tested for the ability to invade DAF⁺ CHO cells. Beads coated with Dr adhesin and bound to DAF⁺ CHO cells were engulfed with the involvement of cellular microvilli and internalized into characteristic tight vacuoles with no phagolysosomal fusion (Fig. 6A to C). Both the number of DAF⁺ CHO cells with internalized beads and the number of beads inside each cell were reduced when the amino acids in domain II were mutated. For example, beads coated with proteins with Q34A (Fig. 6D) and T36A mutations poorly invaded the cells, and those that were internalized were confined to the vacuoles with features of lysosomal fusion. Latex beads coated with an equivalent concentration of BSA did not bind DAF⁺ CHO cells (Fig. 6E).

FIG. 7. Immunofluorescence staining of DAF on HeLa cells incubated with latex beads coated with purified Dr fimbrial protein and a mutated protein with a T31A substitution. (A) Intense fluorescence around beads coated with wild-type Dr protein (arrows) is interpreted as an increased accumulation of DAF molecules. (B) In contrast, mutant T31A displays low DAF accumulation manifested as weak fluorescence (arrows).

Mutation in the hydrophilic region of *draE* results in a decrease in the amount of DAF accumulated at the sites of adherence. Our previous studies have shown that the accumulation (clustering) of glycosylphosphatidylinositol (GPI)-anchored DAF at the sites of bacterial adherence is a characteristic feature of uropathogenic and diarrheagenic *E. coli* bearing Dr and Afa adhesins (9). We attempted to evaluate whether mutations of the DraE protein within hydrophilic domain II affected the amount or pattern of DAF receptor clustered at the sites of bacterial adherence. HeLa cell monolayers were incubated with DraE-coated beads and then immunostained for DAF. Certain mutations in *draE* resulted in decreased amounts of DAF accumulated at the areas adjacent to latex beads coated with purified mutated proteins (Fig. 7A and B). The average densities of DAF staining at the sites adjacent to adherent beads coated with mutated proteins with the substitutions T31A, Q34A, and T36A were significantly lower ($P < 0.05$; Student's *t* test) than that of the control (Fig. 8). This finding indicates that while mutations do not affect the amount of expressed adhesin and the binding to DAF⁺ CHO or HeLa cells, they may impair the capacity of mutated proteins to recruit the DAF receptor to the sites of adherence.

FIG. 8. Densities of DAF staining at sites of adherence of latex beads coated with DraE or mutated DraE proteins. Asterisks indicate statistically significant differences ($P < 0.05$).

FIG. 9. Atomic structure of DraE depicting hydrophilic domain II and mutations affecting invasion. Panel A is a ribbon representation of DraE, with "domain II" colored in orange. The position of chloramphenicol binding is shown in blue. Strands B, D, E, and G are labeled. Panel B is an additional view of DraE. The individual mutants affecting adhesin binding are shown as orange spheres (centered on the alpha carbon of the residue in question). Panel C is a topology diagram of the DraE subunits in polymeric form. In the top subunit, domain II is shown in orange and superimposed with chloramphenicol (blue). Panels A and B were rendered using PyMOL, and panel C was created using the program TOPDRAW.

Molecular model of DraE and mutants of hydrophilic domain II. Our recent study resulted in the solution of the molecular structure of the fimbrial subunit DraE (1, 23a). We now depict the location of hydrophilic domain II and the amino acids at which a substitution to alanine affected invasion. As shown in Fig. 9, the mutants affecting invasion were localized to the lower end of loop B (domain II). This loop is about 10 Å from the central part of DraE (strands B and E), which is required for binding to DAF.

DISCUSSION

This study shows for the first time that attachment and invasion phenotypes displayed by recombinant Dr adhesin in

HeLa cells might be separated by mutation in hydrophilic region II. Receptor binding and HeLa cell invasion phenotypes appear to involve distinct domains of DraE in which specific amino acids play functionally dominant roles. While the binding domains of DraE may contain amino acids located in the central part of the DraE subunit, localized to strands B and E, the invasive region may involve amino acids located in the lower part of the DraE molecule (1, 13, 14).

The exact mechanism(s) of invasiveness of *E. coli* bearing Dr and Afa adhesins is not yet understood. There are at least two working hypotheses addressing these issues. The original hypothesis proposed by Le Bouguenec et al. is that AfaE, a product of the gene *afaE*, is an adhesin and that AfaD is an

invasin (11). The extended hypothesis, proposed by our group, is that DraE/AfaE possess properties of both adhesins and invasins. To test our hypothesis that DraE has invasive properties, we tested whether mutants in the hydrophilic domain II might adversely affect fimbria-receptor interactions and thus interfere with internalization. Our data are consistent with this hypothesis.

A recent collaboration between four groups led by S. Lea, S. Matthews, C. Le Bouguenec, and our group resulted in the solution of the crystal structure of DraE/AfaE (1). We have used the molecular model of DraE to localize hydrophilic domain II. Computer modeling showed that mutations affecting invasion were localized to the lower end of DraE loop B, which is 10 Å apart from the central region of DraE involved in DAF binding. The preliminary finding proposed in the collaborative work was that the DraE/AfaE fimbrial (filamentous) fiber may carry a tip protein, DraD (1). A structure analysis of the invasive epitope of DraE, however, does not appear to suggest that DraD interacts with DraE at this location. Therefore, we further lean towards our hypothesis that DraE is both an adhesin and an invasin. However, the questions of how DraE may interact with DraD and to what extent DraE may cooperate with DraD for invasion remain to be addressed.

The second part of the study was more subjective and involved the interpretation of electron microscopy and semi-quantitative clustering data. Electron microscopy examination of the mutants revealed that they may fall into three distinct groups that presumably participate in the early, mid, and late events of invasion. Dr⁺ *E. coli* strains with the mutations T31A, G33A, and P40A were bound to the host cell surface in large numbers but showed no evidence of cell invasion, suggesting that T31, G33, and P40 may be involved in the early events of invasion. On the other hand, mutant V28A showed bacteria that were in the early stages of engulfment by cellular microvilli but were unable to enter the cell. One can speculate that V28 might be required for the completion of cell invasion/formation of a vacuole or just to enhance the invasion rather than facilitate it. Mutants Q34A and T36A demonstrated decreased invasion rates. An alternative explanation could be that these mutants had decreased intracellular survival. The second explanation was supported by the fact that the internalized mutants were found in vacuoles containing more than one bacterium and having morphological features of phagolysosomal fusion. Similar fusion was observed with latex beads coated with purified fimbrial protein mutants, suggesting the role of domain II in the invasion and/or survival process. Our previous work has shown that a DAF construct with a CD46 anchor replaces GPI anchor-directed bacteria in loose vacuoles, followed by lysosomal fusion (26). Mutations of amino acids in domain II of the fimbriae induced comparable changes.

An immunofluorescence study showed that mutation in domain II resulted in less accumulation of DAF molecules at the sites of adherence of beads coated with mutated proteins than that with the wild-type control. Although ligand-specific receptor clustering is known to trigger signaling cascades, it is unclear whether DAF clustering is required to initiate the entry of Dr⁺ *E. coli* into epithelial cells. Therefore, we are particularly careful about proposing a unified mechanism of entry

mediated by Dr fimbriae. The invasion assay is based on counting intracellular bacteria remaining after gentamicin treatment; therefore, invasion at 3 h represents both the invasion process and intracellular killing. Further studies are necessary to precisely dissect these issues and compare them to the related processes observed with other bacterial species.

Certain microorganisms such as *Mycobacterium* spp. are able to avoid intracellular killing. This phenomenon is attributed to the failure of mycobacterial phagosomes to undergo fusion with lysosomes (33). It is also generally believed that microorganisms targeting the components of lipid rafts (such as GPI-anchored proteins) as their cellular receptors avoid fusion with a lysosomal compartment (24). Type 1 fimbriated *E. coli* strains that recognize GPI-anchored CD48 can avoid lysosomal fusion, but the type 1 fimbrial and CD48 receptor epitopes involved are not known (28). Overall, our data show that both hydrophilic domain II of Dr adhesin (this study) and the type of DAF receptor anchorage (28) are essential for the entry of *E. coli* and bacteria into the endocytic pathway, which appears to be morphologically different from the destructive phagolysosomal fusion pathway.

The model described here may be relevant to other members of the Dr superfamily and could also serve as a model for multiple bacteria, viruses, and parasites exploiting DAF and related tissue receptors. Further studies on the molecular mechanisms of internalization and direction of pathogens to a phagolysosomal environment may contribute to novel therapeutic strategies aimed at modulating the fate of intracellular microorganisms.

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