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Truncated Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 Intimin (EaeA) Fusion Proteins Promote Adherence of EHEC Strains to HEp-2 Cells

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Intimin, the product of the *eaeA* gene in enterohemorrhagic *Escherichia coli* O157:H7 (EHEC), is required for intimate adherence of these organisms to tissue culture cells and formation of the attaching and effacing lesion in the gnotobiotic pig. Because of the importance of intimin in the pathogenesis of EHEC O157:H7 infection in this animal model, we began a structure-function analysis of EaeA. For this purpose, we constructed amino-terminal fusions of the intimin protein with six histidine residues to form two independent fusions. The longer fusion, RIHisEae, contained 900 of the 935 predicted amino acids and included all but the extreme amino terminus. The second fusion, RVHdHisEae, consisted of the carboxyl two-thirds of the protein. Purified extracts of either construct enhanced binding of wild-type 86-24 to HEp-2 cells and conferred HEp-2 cell adherence on 86-24*eae*Δ*10*, an *eaeA* deletion mutant, and B2F1, an EHEC O91:H21 *eaeA* mutant strain. When 86-24*eae*Δ*10* was transformed with either of the plasmids encoding the intimin fusion proteins, the transformant behaved like the wild-type parent strain and displayed localized adherence to HEp-2 cells, with positive fluorescent-actin staining. In addition, polyclonal antisera raised against RIHisEae reacted with both fusion constructs and recognized an outer membrane protein of the same mass as intimin (97 kDa) in EHEC and enteropathogenic *E. coli* but not *E. coli* K-12. The intimin-specific antisera also blocked adherence of EHEC to HEp-2 cells. Thus, intimin (i) is a 97-kDa outer membrane protein in EHEC that serves as a requisite adhesin for attachment of the bacteria to epithelial cells, even when the protein is truncated by one-third at its amino terminus and (ii) can be added exogenously to specifically facilitate HEp-2 cell adherence of EHEC but not *E. coli* K-12.

Enterohemorrhagic *Escherichia coli* (EHEC) is responsible for a spectrum of illnesses in humans, including nonbloody diarrhea, hemorrhagic colitis (HC), and the hemolytic uremic syndrome (36, 45). EHEC is recognized as the leading cause of infectious bloody diarrhea in the United States as well as acute renal failure among U.S. and Canadian children (3). The primary characteristics of EHEC that have been linked to the organism's virulence are (i) production of the Shiga-like toxins (reviewed in references 33 and 46), (ii) presence of the 60-MDa plasmid (9, 18, 19, 47, 50), and (iii) the capacity to produce an attaching and effacing (A/E) intestinal lesion in experimental animals (7, 35, 49).

Several bacterial components have been implicated in intimate adherence of EHEC to epithelial cells and the capacity of the organism to cause the A/E lesion in vivo. These factors include (i) a product(s) of the aforementioned 60-MDa plasmid (termed pO157 in reference 47); (ii) a 94-kDa outer membrane protein (OMP) required for EHEC adherence to Henle 407 cells (41, 42); (iii) intimin, the product of the chromosomal *eaeA* gene (5, 6, 29); and (iv) products of two genes that have been insertionally inactivated by Tn*phoA* (6). Evidence that the first three components are distinct is as follows. First, Dytoc et al. (6) demonstrated that the factor(s) encoded by pO157 is different from the chromosomally encoded *eaeA* product and the 94-kDa OMP by showing that plasmid-cured EHEC O157:H7 strains react with antisera to the 94-kDa protein. Second, Sherman et al. (41) reported that antibodies that recognize the 94-kDa OMP block adherence of EHEC strains to epithelial cells in culture, but these same antibodies do not recognize intimin (6). That none of the factors described above are capable of conferring epithelial cell adherence when transformed independently into an *E. coli* K-12 background suggests that multiple EHEC determinants are required for adherence of the organism to epithelial cells in culture.

Although the mechanism(s) by which EHEC attach to the intestinal epithelium and cause the A/E lesion are not fully understood, we have shown that intimin is one essential component for attachment of EHEC O157:H7 strains to epithelial cells in culture and for formation of the A/E lesion in vivo (5, 29). Specifically, we demonstrated that an in-frame mutation in the *eaeA* locus of EHEC strain 86-24 (O157:H7) ablates HEp-2 cell adherence and A/E lesion formation in the gnotobiotic piglet. Full adherence and fluorescent-actin staining (FAS) activity is restored to the mutant by transformation with a plasmid that encodes the wild-type copy of *eaeA* (29). Because of this critical role for the *eaeA* gene product of EHEC O157:H7 in the initial steps of EHEC pathogenesis, we sought to begin an examination of the relationship between intimin structure and its function as an adhesin.

In the present study, six histidine (6/X/His) residues were fused to the amino terminus of intimin to create two independent truncated fusion proteins. These fusion proteins were used as tools to begin to define the mechanisms by which intimin promotes EHEC adherence to epithelial cells.

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TABLE 1. Strains and plasmids used in this study

E. coli strain or plasmid	Relevant characteristic(s)	Source (reference)
Strains		
86-24	Wild-type EHEC O157:H7	P. Tarr (11)
B2F1	Wild-type EHEC O91:H21	B. Rowe (16)
86-24*eae*Δ*10*	86-24 *eaeA* deletion mutant	29
EDL933	Wild-type O157:H7	K. Wachsmuth (37)
933cu	Plasmid-cured derivative of EDL933	19
E2348/69	Wild-type EPEC O127:H6	24
DH5α	Cloning host, adherence-negative control	BRL
XL1-Blue	Cloning host	Stratagene
M15	Expression host	Qiagen
SG13009	Expression host	Qiagen
2442	$O8^-$ (rough)	32
2443	$O8^+$ (smooth)	31
HS	Nonpathogenic normal-flora isolate, O9:H4	24
Plasmids		
pEB310	PCR-derived *eaeA* clone from EHEC 86-24 in $pBRKS^-$ in orientation of P_{T7}	29
pQE32	Histidine fusion cloning vector	Qiagen
pEB312	6XHis::*eaeA*, source of RVHdHisEae	This study
pEB313	6XHis::*eaeA*, source of RIHisEae	This study
pQE16	6XHis::*dhfrs*, source of HisDHFR	Qiagen
pREP4	*lacI*^{q}	Qiagen

MATERIALS AND METHODS

Cells, bacterial strains, and plasmids. HEp-2, human laryngeal epithelial cells (ATCC CCL23), and HCT-8, human ileocecal epithelial cells (ATCC CCL244), were obtained from the American Type Culture Collection, Rockville, Md. Eukaryotic cell cultures were maintained by serial passage in complete Eagle's minimal essential medium (EMEM) (BioWhittaker, Walkersville, Md.) (10% fetal calf serum, 20 mM L-glutamine, 100 μg of gentamicin per ml, 100 U of penicillin G).

The bacterial strains and plasmids used in this study are described in Table 1. EHEC O157:H7 strain 86-24 was isolated in 1986 from a patient in Seattle, Wash. (11). Strain 933 (EHEC O157:H7) was isolated from a patient in the 1982 outbreak of HC (37). *E. coli* K-12 strain DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used as a negative control in the adherence assays. The *eaeA* gene from 86-24 was cloned into plasmid vector $pBRKS^-$ (40). Briefly, $pBRKS^-$ is a pBR328-based vector that includes the multicloning site from pBluescriptKS$^-$ (Stratagene) as well as the promoter of β-galactosidase (P_{lac}) in one orientation and the T7 phage promoter (P_{T7}) in the opposite orientation (40). The cloning vectors used to construct the histidine-intimin fusion proteins were obtained from Qiagen, Inc. (Chatsworth, Calif.). The fusion constructs were expressed in an M15 or SG13009 background which had been transformed with pREP4 (described in Table 1).

Media, enzymes, biochemicals, and radionuclides. Bacterial strains were routinely grown on Luria broth (LB) (10 g of tryptone, 5 g of yeast extract, 5 g of sodium chloride) or MacConkey (Difco Laboratories, Detroit, Mich.) or LB agar. When appropriate, antibiotics (Sigma Chemical Co., St. Louis, Mo.) were added to the medium at the following final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 25 μg/ml; and tetracycline, 12.5 μg/ml. These concentrations were occasionally varied for a specific assay. To screen clones that contained DNA inserts which inactivated β-galactosidase, the gratuitous inducer of the *lac* operon, IPTG (isopropyl-β-D-thiogalactopyranoside) and the colorimetric substrate X-Gal (5-bromo-4-chloroindolyl-β-D-galactoside) were added at 100 mM and 20 μg/ml, respectively, to solidified LB agar. Restriction endonucleases and calf intestinal alkaline phosphatase were purchased from Bethesda Research Laboratories, Boehringer Mannheim Biochemicals (Indianapolis, Ind.), or New England BioLabs (Beverly, Mass.). Enzymes were used according to the particular manufacturer's instructions unless otherwise stated. Radionuclides were purchased from New England Nuclear Research Products (Boston, Mass.).

DNA manipulations. Cloning procedures and plasmid manipulations were carried out essentially according to the procedures described by Maniatis et al. (27). Plasmid DNA was isolated by alkaline lysis (27) for screening or by use of Qiagen plasmid isolation columns (Qiagen, Inc.) for large-scale preparations. Plasmids were moved into the appropriate host strains by $CaCl_2$ transformation (26) or electroporation (43).

Adherence assays. Adherence of *E. coli* to either HEp-2 or HCT-8 cells was assessed by a modification of the method of Cravioto et al. (4) that was described earlier (30). Only results with the HEp-2 cells are presented, but similar adherence data were obtained with the HCT-8 cells. The extent and phenotype of adherence of a particular bacterial strain to these cells were assessed by microscopic observation rather than quantitative bacterial counts because in preliminary studies the former method was a more sensitive estimate of phenotypic difference between strains.

To assess the effect of anti-intimin antibodies on EHEC adherence, mouse or rabbit anti-intimin antisera (or preimmune sera as controls) were added to the bacteria suspended in 1 ml of adherence medium, and the bacterium-antiserum mixture was incubated at 37°C for 30 min prior to infection of the HEp-2 cells. Antisera were present in the adherence medium throughout the assay. To evaluate the effect of exogenously added intimin fusion proteins on bacterial HEp-2 cell adherence, the purified His-intimin fusion protein was diluted in adherence assay medium and added to the epithelial cell monolayer. The HEp-2 cells were incubated in the presence of the fusion protein for 30 min at 37°C prior to the addition of bacteria. The presence of the fusion protein was maintained throughout the standard 6-h adherence assay. In separate experiments, the bacteria were preincubated with the fusion protein before addition to the HEp-2 cell monolayer. The results were indistinguishable whether the fusion protein was maintained in the medium or preincubated with the bacteria. Other specific manipulations of the adherence assay are discussed in Results.

Expression and visualization of native intimin proteins. The *eaeA* gene was cloned into vector $pBRKS^-$ in the orientation of the T7 RNA polymerase-dependent promoter (P_{T7}) to allow expression of the *eaeA* gene product as described by Tabor and Richardson (44). Proteins encoded by pEB310 and under P_{T7} control were selectively expressed in DH5α which contained plasmid pGP1-2, a plasmid that encodes a temperature-inducible copy of the T7 RNA polymerase (44). The protein products were pulse-labeled with [^{35}S]methionine, and whole-cell lysates or fractionated cell components were separated by discontinuous denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 4% stacking gel with 10% separating gel) (20) on a Mini Protean II slab cell apparatus according to the instruction manual provided by the manufacturer (Bio-Rad). Proteins that had incorporated the [^{35}S]methionine were visualized by autoradiography.

Expression and purification of His-intimin fusion proteins. To construct RIHisEae, the *Eco*RI-*Hin*dIII fragment from pEB310 (29), which includes most of the *eaeA* gene, was inserted into the *Sma*I-*Hin*dIII sites of vector pQE32. Specifically, the sticky end generated by *Eco*RI digestion of pEB310 was filled in with nucleotides to create a blunt end which, in turn, was ligated into the *Sma*I site of pQE32. The construct that expressed RVHdHisEae was created by ligation of the *Eco*RV-*Hin*dIII fragment from pEB310 into the *Sma*I-*Hin*dIII sites of pQE32. The His-intimin fusion proteins were expressed (as described below), denatured with urea according to the manufacturer's protocol, and purified to homogeneity over Ni-NTA resin (nickel-nitrilotriacetic acid agarose) (Qiagen, Inc.) as shown schematically in Fig. 1. For large-scale purification of the His-intimin fusions, log-phase cultures (0.25 to 1 liter) of either *E. coli* MG15(pREP4, pEB312) or *E. coli* MG15(pREP4, pEB313) in LB were induced with 0.1 mM IPTG for 2 h at 37°C with vigorous shaking and the bacteria were harvested by centrifugation. Bacterial cells that expressed the His-intimin fusion proteins were lysed by addition of 5 ml of buffer A (6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, 0.01 M Tris [pH 8.0]) per g (wet weight) of the bacterial pellet and gently stirred for 1 h at room temperature. The slurry was centrifuged at 10,000 × *g* for 15 min at 4°C to remove bacterial debris. The supernatant was mixed with 2 ml of Ni-NTA resin preequilibrated in buffer A and gently stirred at room temperature. The resin was washed twice with buffer A and then loaded into a 1.6-cm-diameter column. The column was washed extensively with buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris [pH 8.0]) and then multiple times with buffer C (buffer B at pH 6.3). To determine the protein content of the washes, the A_{280} of column fractions was monitored. One-milliliter fractions of the fusion proteins were eluted in buffer C supplemented with 250 mM imidazole. Samples of the eluted fractions that contained the fusion protein were separated by SDS–10% PAGE, and the protein bands were visualized by Coomassie blue staining of the gel. Urea was removed from the eluted fractions, and the proteins were renatured by exhaustive dialysis against 10 mM phosphate-buffered saline (PBS), pH 7.4.

For the small-scale purification procedure, 25-ml cultures of *E. coli* MG15(pREP4) that contained either plasmid pEB312, pEB313, or pQE16 were induced with 0.1 mM IPTG, and the expressed fusion proteins were processed as

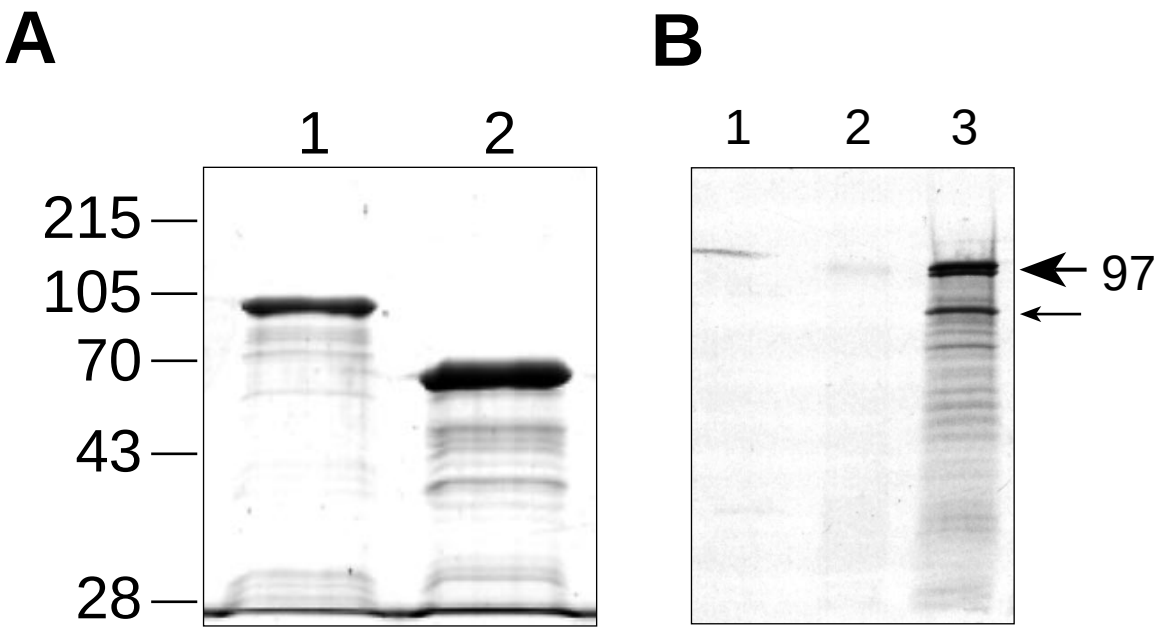

FIG. 1. (A) Purified His-intimin fusion proteins were separated by SDS-PAGE and stained with Coomassie blue. RIHisEae is 101 kDa, and RVHdHisEae is 65 kDa. (B) Autoradiograph of the [^{35}S]methionine-labeled product of the EHEC *eaeA* locus expressed from the T7 RNA polymerase-dependent promoter. Lane 1, DH5α(pGP1-2, pBRKS$^-$); lane 2, ^{14}C-labeled carbonic anhydrase marker (M_r = 97,400); lane 3, DH5α(pGP1-2, pEB310). The large arrow points to the doublet thought to be intimin; the smaller arrow indicates a minor degradation product of intimin at M_r = 80,000.

recommended by the manufacturer's instructions over Ni-NTA spin columns (Qiagen). Approximately 100 to 200 μg of either RVHdHisEae, RIHisEae, or His:DHFRS per ml, respectively, was obtained by this small-scale purification method.

Immunization of mice and rabbits. Four female BALB/cJ mice (Jackson Laboratory, Bar Harbor, Maine) were prebled and then injected intraperitoneally with 25 μg of RIHisEae fusion protein in TiterMax adjuvant (Vaxcel, Inc., Norcross, Ga.) three times over a 6-week period. Blood samples were obtained by nicking the tail veins of the mice. When the serum sample from each mouse contained a high titer of anti-intimin antibodies, all animals were sacrificed by exsanguination and the sera were collected.

Two male New Zealand White rabbits (HRP, Inc., Denver, Pa.) were injected intramuscularly at four sites with 100 μg (total) of RIHisEae in TiterMax and then given two booster injections with 100 μg of the protein per ml in PBS at 3-week intervals. Anti-intimin antibody titers of the mouse or rabbit antisera were assessed by the enzyme-linked immunosorbent assay (ELISA) described below.

ELISA to detect anti-intimin antibodies. Fifty nanograms of RIHisEae (or RVHdHisEae, where specified) was plated onto polystyrene 96-well microtiter plates as an antigen substrate to test the reactivity of rabbit or mouse sera against intimin. After an overnight incubation at 4°C, the coated plates were washed with PBS and unoccupied sites on the wells were blocked with 3% bovine serum albumin in 10 mM PBS, pH 7.4. Two-hour incubations with primary and secondary antibodies (either horseradish peroxidase-conjugated goat anti-mouse [Boehringer Mannheim Biochemicals] or donkey anti-rabbit [Amersham Corp., Arlington Heights, Ill.] when appropriate] were carried out at room temperature with PBS washes between each step. TMB (3′,3′,5′,5′-tetramethylbenzidine) peroxidase (Bio-Rad) was added to each well as the substrate. After 10 to 30 min of incubation, the enzymatic reaction was terminated by the addition of 1 N H_2SO_4, which caused the mixture to turn yellow. The intensity of the yellow color was monitored on a Titertek Multiscan MC microliter plate reader (Flow Laboratories, McLean, Va.). The optical density values were plotted for each sample. The anti-intimin titer was defined as an A_{450} value 0.2 U above the negative control value, which was usually the value obtained with normal sera.

Bacterial membrane fractionation. Bacterial membrane fractionation was done essentially according to the method of Achtman et al. (1). Overnight cultures of bacteria were subcultured into fresh LB for 2 h at 37°C. Pelleted bacteria were resuspended in 10 mM Tris with 100 μg of PMSF (phenylmethylsulfonyl fluoride) per ml and sonically disrupted. The bacterial lysates were subjected to low-speed centrifugation to remove intact cells, and the resultant supernatant was centrifuged at 100,000 × *g* for 1 h to harvest the bacterial membranes. OMPs were extracted by precipitation with 1.67% sarcosyl in 11.1 mM Tris, pH 7.6, at room temperature and then pelleted by centrifugation at 4°C. The sarcosyl precipitate was solubilized in Tris-PMSF, quantitated by the Bradford method (BCA Quantification Kit; Pierce), and subjected to SDS-PAGE followed by immunoblot analysis (described below).

Immunoblotting. Proteins separated by SDS-PAGE were electroblotted onto nitrocellulose according to the method of Towbin et al. (48). The membranes were blocked with 5% nonfat dried milk (Carnation Company, Los Angeles, Calif.) in Tris-buffered saline, pH 7.2, with 0.1% (vol/vol) Tween 20 (TBS-T), washed, and then incubated with either mouse or rabbit anti-intimin serum (the concentration of antiserum is indicated in each experiment). The membranes were washed three times with TBS-T and then overlaid with a 1:5,000 dilution of either horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Boehringer Mannheim Biochemicals), donkey anti-rabbit immunoglobulin (Amersham), or sheep anti-human immunoglobulin (Amersham). Antigen-antibody complexes were visualized by chemiluminescence with the ECL Western blotting (immunoblotting) detection kit (Amersham).

Agglutination tests. The capacity of the purified fusion proteins to agglutinate wild-type EHEC (either 10 μl of a saturated broth culture or a single colony from an agar plate) was tested by mixing 5 μg of purified fusion protein with *E. coli* O157:H7 strain 86-24. The capacities of antisera raised against the intimin fusion proteins to agglutinate EHEC strain 86-24 were assessed by slide agglutination tests with neat sera or sera diluted 1:10 in PBS. Agglutination with rabbit anti-O157:H7 sera (Difco) was used to confirm the serotype of *E. coli* O157:H7 strains used throughout this study.

RESULTS

Expression of the *eaeA* product under control of the T7 promoter. We first sought to evaluate EHEC intimin at the protein level by analysis of the native protein. Plasmid pEB310 contains the wild-type *eaeA* locus cloned from EHEC O157:H7 strain 86-24 in an orientation such that the T7 RNA polymerase-dependent promoter (P_{T7}) can drive transcription of the gene (the construction of pEB310 was previously described in reference 29). The intimin protein encoded by pEB310 was overexpressed in a background that included a temperature-inducible copy of the T7 RNA polymerase gene on plasmid pGP1-2. Three major products of the cloned insert were observed (Fig. 2). The major bands were a doublet at 97 kDa and a singlet at approximately 80,000 Da. The majority of the intimin expressed by this method remained associated with the bacterial membrane fraction even after sonic disruption of the

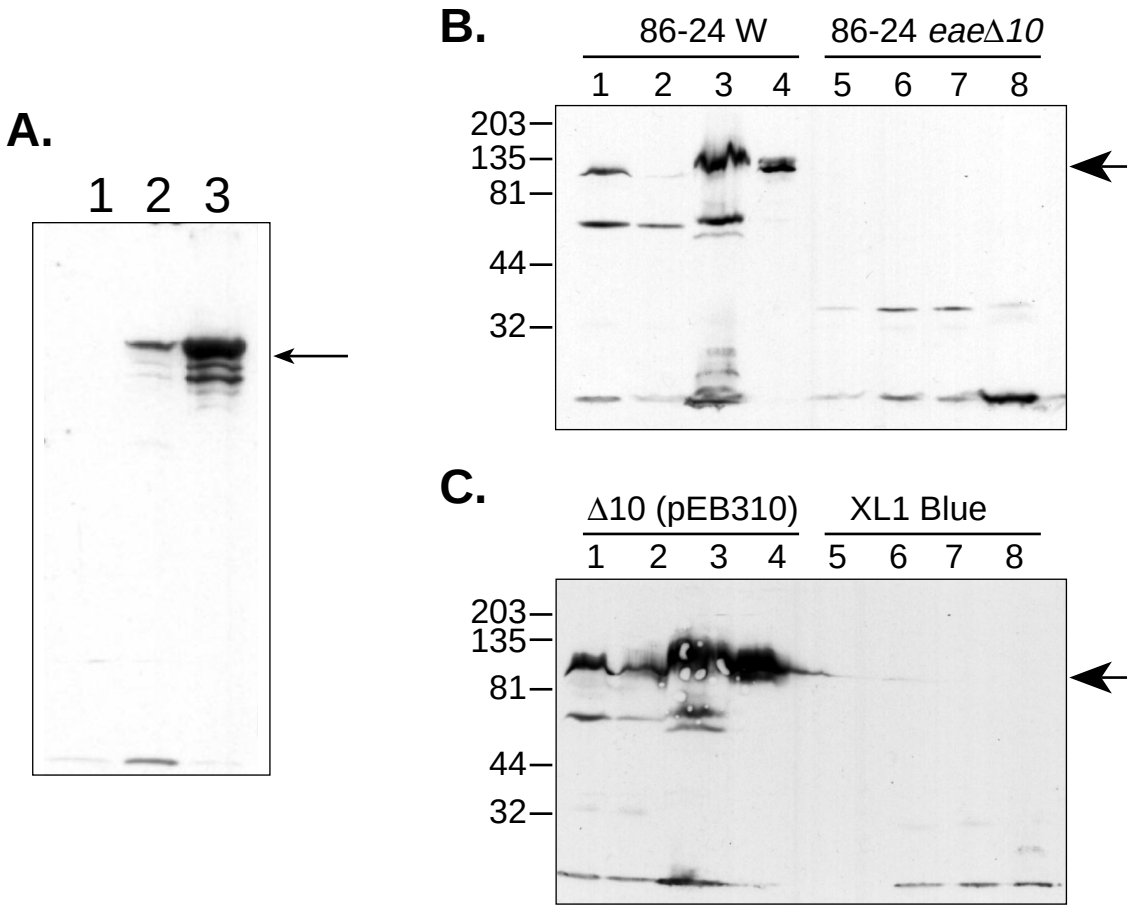

FIG. 2. (A) Reactivity of mouse anti-intimin antibodies (1:2,500) against intimin expressed from plasmid pEB310. Lane 1, DH5α(pGP1-2, pBRKS$^-$) membrane-associated fraction; lane 2, DH5α(pGP1-2, pEB310) supernatant fraction from sonically disrupted cells; lane 3, DH5α(pGP1-2, pEB310) membrane-associated fraction. The arrow indicates the 97-kDa protein, intimin. (B and C) Immunoblots of mouse anti-intimin antisera against bacterial fractions from wild-type 86-24, the *eaeA* mutant with and without plasmid pEB310, or the K-12 strain XL1-Blue. Lanes: 1 and 5, low-speed supernatant; 2 and 6, high-speed supernatant; 3 and 7, high-speed pellet fraction; 4 and 8, sarcosyl precipitate from the high-speed pellet. Molecular weight markers (in thousands) are to the left; the arrow indicates intimin.

host bacterium and addition of mild detergent to the extraction buffer.

Construction and purification of intimin fusion proteins. The insolubility of the intimin protein, combined with the abundance of other native *E. coli* protein species in the 97-kDa range (as seen by Coomassie blue staining of such proteins separated by SDS-PAGE gels [data not shown]), made purification of the native protein difficult. For these reasons, we sought a fusion protein approach for rapid and easy purification of intimin. The 6XHis fusion protein system of Qiagen, Inc., was chosen to create His-intimin fusions because (i) a small peptide is added to the amino-terminal end of the protein rather than a bulky protein like the maltose binding protein (MBP); (ii) the 6XHis tag binds tightly to a nickel affinity matrix, which facilitates one-step purification of the fusion protein for further studies; and (iii) the expression system is designed to maintain tight control of the 6XHis fusion proteins to prevent any possible lethal effects of the recombinant protein on the *E. coli* host strain.

Two fusions were constructed as outlined in Materials and Methods. The longer fusion, RIHisEae, was ~101 kDa and encoded 900 of 935 predicted amino acids (Fig. 1). This construction resulted in an in-frame fusion of the 6XHis tag with intimin that had been truncated by 35 amino acids at its amino terminus. These 35 residues were deleted to remove any potential signal sequence that might lead to cleavage of the His tag from the intimin. Such a possibility is suggested by analogy with the mature intimin from EPEC E2348/69, which begins 39 residues from the first methionine residue and, thus, appears to be cleaved or processed (10). The shorter fusion, RVHdHisEae, was ~65 kDa and encoded 604 amino acids of the carboxyl two-thirds of the wild-type intimin protein. As with the P_{T7}-expressed intimin, each of the fusion proteins remained primarily in the insoluble pellet after sonic disruption of the host *E. coli* (data not shown). Therefore, 8 M urea was included in all of the extraction buffers to allow solubilization of the fusion proteins from the pellet. Both His-intimin fusion constructs were purified to homogeneity over nickel affinity columns (Fig. 1). Neither of the fusion proteins agglutinated wild-type 86-24 (data not shown).

Antisera to His-intimin fusion proteins. Intimin-specific antibodies were produced in both mice and rabbits that had been immunized with purified RIHisEae fusion protein. The anti-Eae antibody titers for all sera were high (>10,000 for the rabbit sera and 1,300 to 5,000 for the mouse sera). The antisera raised in mice and rabbits recognized both fusion proteins by Western blotting (data not shown) as well as intimin expressed under the control of the T7 promoter in pEB310 (representative mouse anti-intimin serum data are shown in Fig. 3A).

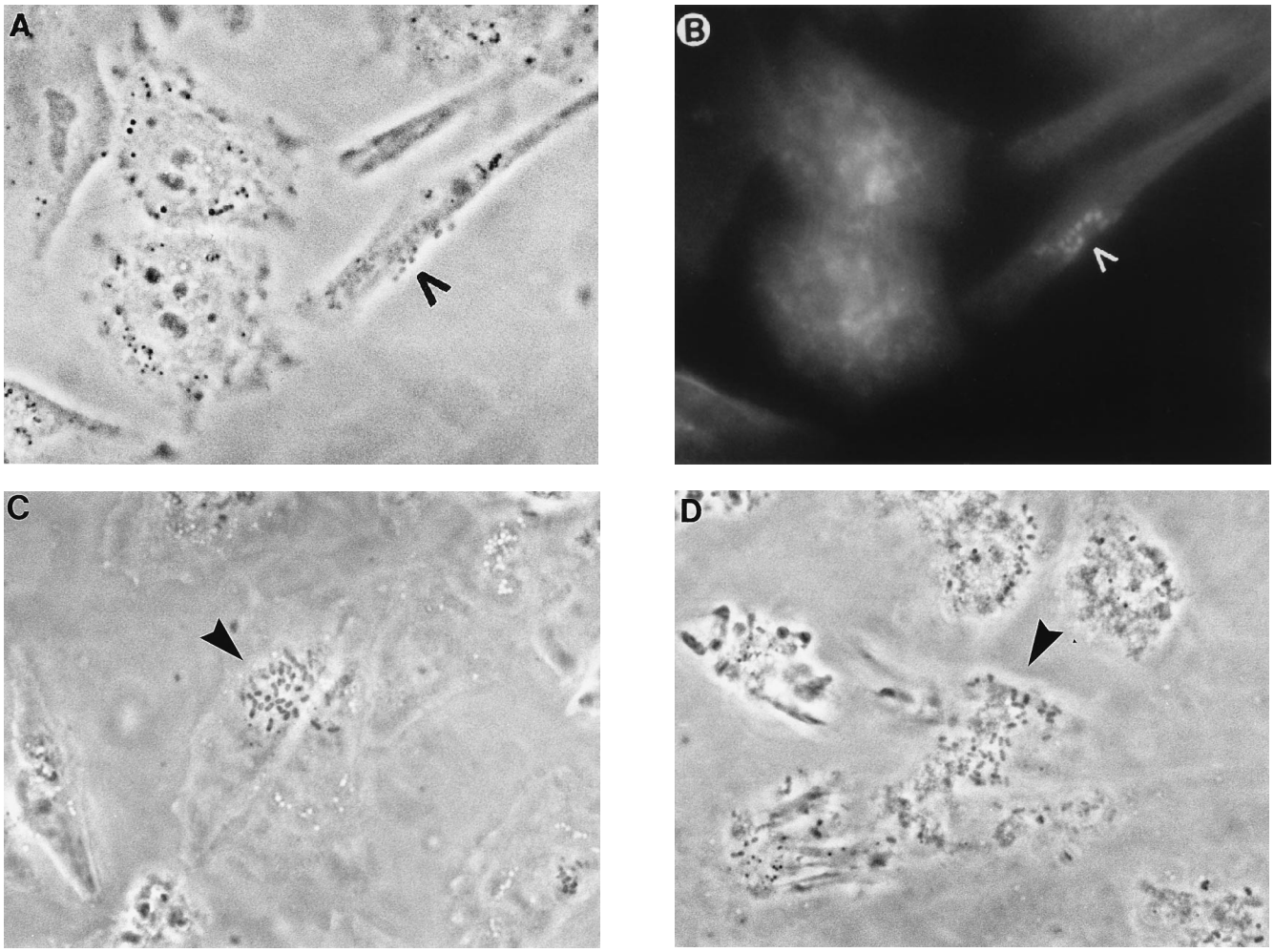

FIG. 3. Microcolonies (indicated by the arrows) exhibited by wild-type 86-24 on HEp-2 cells in the presence of 20 ng of the histidine fusion protein. (A) One microscopic field with FAS; (B) the same field viewed by phase microscopy. In the presence of 2 μg (C) (phase contrast) or 20 μg (D) (phase contrast) of RIHisEae, microcolonies (indicated by the arrowheads) were larger and a greater proportion of the HEp-2 cell surface was covered by bacteria.

Prebleed sera from the mice and rabbits did not recognize the fusion proteins (immunoblot not shown).

Recognition of 97-kDa bacterial OMP by anti-intimin sera. Antibodies to the intimin fusion protein raised in mice recognized a 97-kDa protein in the sarcosyl-insoluble fraction of extracts from 86-24 and 86-24*eae*Δ*10*(pEB310) (Fig. 3B and C). An OMP of approximately 97 kDa expressed by EHEC O157:H7 strains 933 and 933cu as well as by EPEC strain E2348/69 reacted with anti-intimin sera (data not shown). The mouse anti-intimin sera did not recognize any proteins in outer membrane extracts from the *E. coli* K-12 XL-1Blue (Fig. 3C) or the O91:H21 *eaeA* mutant isolate, B2F1 (data not shown), that were unique to these strains. From these data, we conclude that EHEC intimin is a 97-kDa OMP expressed by bacterial strains that carry the *eaeA* locus and that the intimins expressed by EHEC and EPEC are immunologically related. The anti-intimin sera also recognized a smaller-molecular-weight protein in the fraction of bacteria that included both the cytoplasmic and periplasmic contents. This 50-kDa species was present only in preparations that had been stored at 4°C or those preparations that had gone through at least one freeze-thaw cycle. Therefore, we believe that this 50-kDa protein is a degradation product of RIHisEae.

Recognition of EHEC proteins by HC patient sera. Tested convalescent-phase immune sera from HC patients ($n = 4$; kindly provided by T. Barrett, Centers for Disease Control and Prevention, Atlanta, Ga.) reacted with P_{T7}-expressed intimin as well as other *E. coli* proteins in a Western immunoblot (data not shown). To decrease reactivity of the HC patients' sera with nonintimin *E. coli* proteins contained in the expression system, samples of sera were adsorbed with whole-cell extracts of DH5α transformed with pGP1-2 and pBRKS$^-$ (the expression system). After adsorption, the convalescent-phase HC patient sera recognized intimin and a few other *E. coli* proteins but no longer reacted with proteins expressed from pEB310 other than intimin or the vector control (data not shown). Commercially available pooled normal human sera also recognized RIHisEae, but two other individual samples of naive human sera did not. These findings suggest that intimin-expressing bacteria may infect a higher portion of individuals than is currently recognized.

Blocking of HEp-2 cell adherence by anti-intimin antibodies. The anti-intimin antibodies raised in mice blocked adherence of strain 86-24 to HEp-2 cells in a dilution-dependent manner when preincubated with the bacteria for 30 min prior to the infection of the monolayer (data not shown). At a

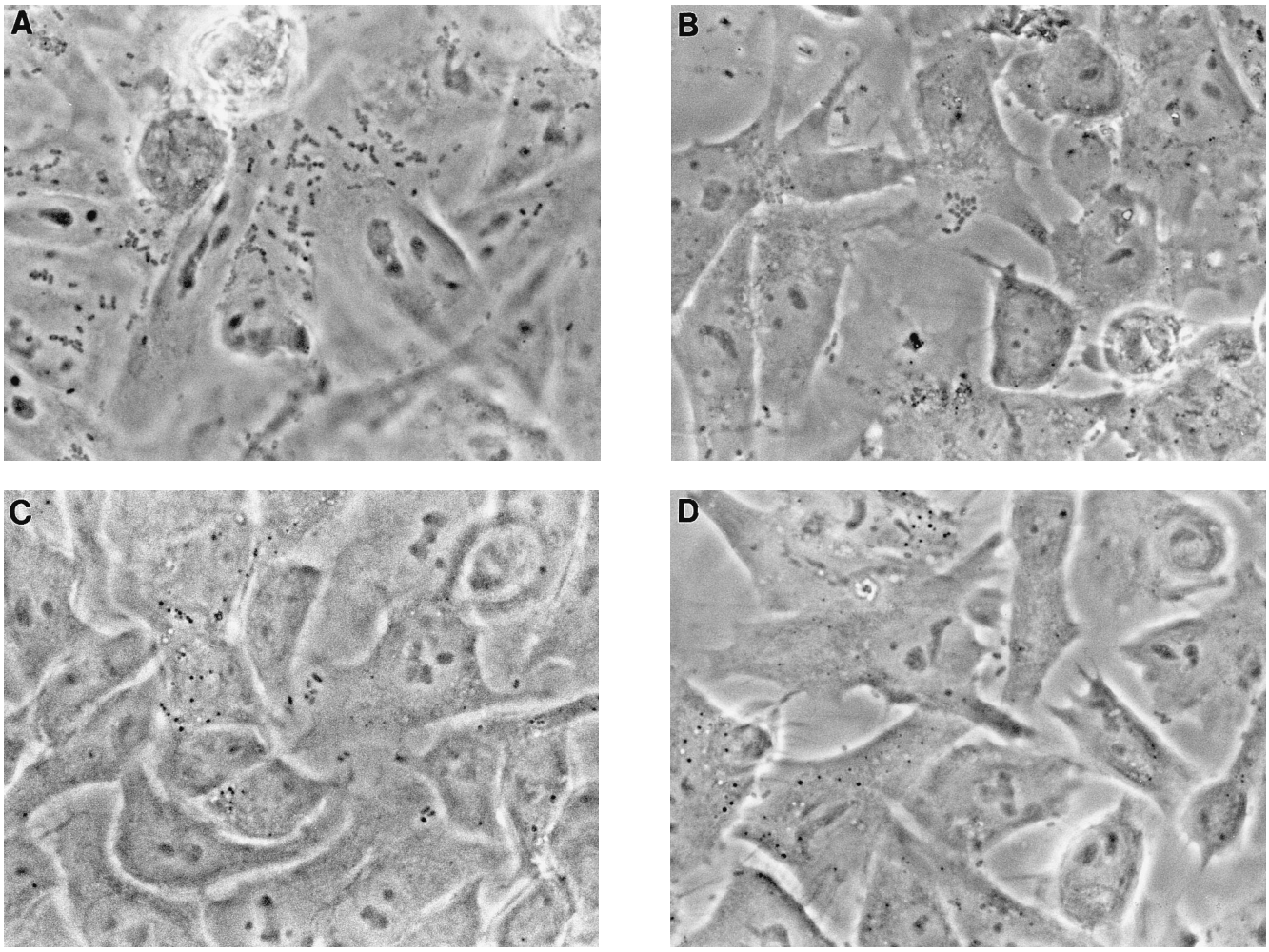

FIG. 4. Histidine-intimin fusion proteins render mutant 86-24*eae*Δ*10* adherent to HEp-2 cells. Adherence assays with the in-frame deletion mutant strain conducted in the presence of 1 μg of RIHisEae (A) or 2 μg of RVHdHisEae (B) resulted in microcolony formation by the bacteria. (C) EHEC O91:H21 strain B2F1 was rendered adherent to HEp-2 cells in the presence of 2 μg of RVHdHisEae. (D) The *E. coli* K-12 control strain DH5α failed to bind HEp-2 cells in the presence of 1 μg of RIHisEae.

1:1,000 dilution of the anti-intimin sera, partial blocking of bacterial adherence was observed. As the concentration of anti-intimin antibodies increased to a 1:10 dilution, total blocking of EHEC 86-24 adherence to HEp-2 cells was achieved. With preimmune sera, partial interference with adherence was seen at dilutions up to 1:100. Neither the mouse nor the rabbit anti-intimin sera agglutinated wild-type 86-24 when tested neat or diluted 1:10 (data not shown).

Adherence of wild-type EHEC in the presence of His-intimin fusion proteins. Both of the fusion proteins were tested for the capacity to block binding of 86-24 to HEp-2 or HCT-8 cells in vitro. We hypothesized that if intimin acts as a primary EHEC adhesin, then preincubation of the eukaryotic cells with excess His-intimin should occupy most or all of the receptor binding sites available on the HEp-2 cell surface for intimin. This hypothetical receptor saturation should block further adherence by the wild-type bacteria. To test this theory, we incubated HEp-2 cell monolayers with 20 ng to 20 μg of RIHisEae for 30 min prior to the addition of 86-24. The presence of the fusion protein was maintained throughout at least the first 3 h of the adherence assay. At the end of 6 h, the infected monolayers were washed extensively, stained with fluorescein isothiocyanate-phalloidin, and observed by either phase-contrast or fluorescence microscopy. The results are presented in Fig. 4. Contrary to our expectation that the fusion proteins would block adherence, the fusions enhanced binding of EHEC 86-24 to HEp-2 cells. The size of the EHEC 86-24 microcolony as well as the total number of HEp-2 cells with adherent microcolonies increased as the concentration of RIHisEae increased. A similar amino-terminal fusion of six histidine residues to mouse dihydrofolate reductase (His-DHFR) did not enhance the adherence of 86-24. At high doses (20 to 200 μg), both the fusion protein and the control His-DHFR caused the HEp-2 cells to show aberrant appendages and processes which were FAS positive even in the absence of bacteria (data not shown). Therefore, we chose 1 to 2 μg of the fusion proteins as the optimal dose for further studies to avoid nonspecific effects on the cells.

Next, we asked whether the fusion protein-mediated enhancement of adherence of smooth EHEC 86-24 but not rough *E. coli* DH5α to HEp-2 cells could be a consequence of the histidine residues added to the intimin proteins nonspecifically associating with the lipopolysaccharide (LPS). To test this possibility, an isogenic pair of O8 strains and a normal-flora *E. coli*

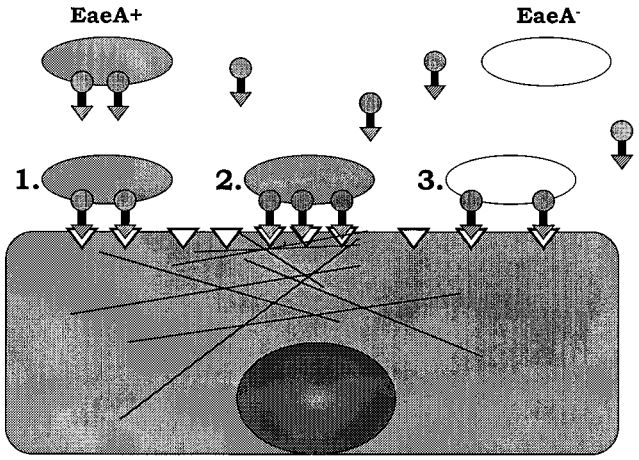

FIG. 5. Model of intimin interaction with the bacterium and the epithelial cell. Intimin (⊶) is depicted as a molecule with distinct ends; one end interacts with the bacterium (circle), and the other end interacts with the eukaryotic cell (triangle). (1) Wild-type bacteria express intimin as an OMP. Intimin interacts with a putative receptor on the eukaryotic cell which results in the condensation and rearrangement of cytoskeletal components (lines within epithelial cell). (2) When there is exogenous intimin added (in this studtype *eaeA*$^+$ EHEC [stippled ovals]), the LA/FAS phenotype is enhanced. (3) When the his-intimin fusions are added to EHEC bacteria that do not express intimin (i.e., 86-24*eae*Δ*10* or B2F1, shown here as open ovals), the protein interacts with both the bacterium and the eukaryotic cell to form a "bridge." Bridge formation confers the LA phenotype to 86-24*eae*Δ*10* and B2F1 but not the FAS phenotype.

strain (HS, serogroup O9:H4) were assayed in the HEp-2 cell adherence assay in the presence or absence of exogenous RIHisEae or RVHdHisEae. Strains 2442 (O8$^-$) and 2443 (O8$^+$) adhered diffusely to HEp-2 cells in the absence or presence of the His-intimin fusion proteins (data not shown). The smooth HS strain was nonadherent to HEp-2 cells whether or not exogenous His-intimin was present (data not shown). Thus, intimin-promoted HEp-2 cell attachment appears to be independent of the presence or serotype of LPS on the surface of the infecting bacterium.

Adherence of *eaeA*-negative strains in the presence of His-intimin fusion proteins. When added exogenously to HEp-2 cells, RIHisEae and RVHdHisEae complemented the HEp-2-cell binding defect of 86-24*eae*Δ*10* (Fig. 4). Similar concentrations of RIHisEae were unable to render *E. coli* K-12 strain DH5α adherent to HEp-2 cells (Fig. 4). Moreover, the mutant, 86-24*eae*Δ*10*, became localized adherence (LA)/FAS positive on HEp-2 cells when transformed with either plasmid pEB312 or pEB313, which encode the His-intimin fusion proteins. The enhancement with exogenous RIHisEae and RVHdHisEae of wild-type EHEC adherence to HEp-2 cells and the complementation of 86-24*eae*Δ*10* for binding to HEp-2 cells suggest that intimin is capable of interaction with both the bacteria and the epithelial cells.

EHEC strain B2F1 (O91:H21, *eaeA* negative) does not bind to HEp-2 or HCT-8 cells in an LA/FAS manner and does not cause the A/E lesion in the gnotobiotic piglet intestine (29). When B2F1 was preincubated with 2 μg of RVHdHisEae, the bacteria were rendered adherent to HEp-2 cells; i.e., the B2F1 bacteria were observed singly and in small clusters on the apical surface of the HEp-2 cells (Fig. 4). However, this adherence did not result in the rearrangement of the eukaryotic cytoskeleton as assessed by FAS. No B2F1 or 86-24*eae*Δ*10* organisms adherent to HEp-2 cells were seen in the presence of 2 μg of His-DHFR (data not shown). Furthermore, plasmid pQE16, which encodes the His-DHFR fusion, did not complement 86-24*eae*Δ*10* for localized adherence or FAS (data not shown).

DISCUSSION

In this study, we purified EHEC intimin by adding six histidine residues to the amino terminus of truncated forms of the protein and purified the fusion protein in a single step over a nickel affinity column. The purified fusion protein was used to raise antisera that recognized native intimin expressed by EHEC and enteropathogenic *E. coli* (EPEC), and the antisera blocked binding of wild-type EHEC to epithelial cells in culture. Furthermore, we demonstrated that EHEC O157:H7 intimin, a 97-kDa OMP, can mediate binding of EHEC strains to HEp-2 cells when expressed endogenously (as a native or fusion protein) as well as when added exogenously as a purified histidine fusion protein. Lastly, we showed that the amino-terminal third of EHEC intimin is not required to mediate adherence to HEp-2 cells, as evidenced by the complementation of the mutant with construct RVHdHisEae.

One model to explain intimin-mediated EHEC adherence of wild-type EHEC to HEp-2 cells is shown in Fig. 5. In this model, the intimin protein acts as a bridge between the bacterium and the epithelial cells with two distinct ends: one that interacts with the putative receptor on the eukaryotic cell surface and another end that interacts with the outer membrane of the bacterium. The observation that neither RIHisEae nor RVHdHisEae agglutinated wild-type 86-24 suggests that intimin does not promote bridge formation between the bacteria. Therefore, according to our model, addition of RIHisEae to the 86-24–HEp-2 cell milieu caused the formation of additional bacterial-epithelial cell bridges, which led to larger microcolonies on the HEp-2 cells (Fig. 5, part 2).

When the fusion proteins were added to 86-24*eae*Δ*10*, a nonadherent mutant that does not express intact intimin, we speculate that the fusion proteins stuck to the mutant and became the bridge to the HEp-2 cells (Fig. 5, part 3). This adherence-promoting effect was fusion protein concentration dependent. Consistent with this model, the intimin fusion proteins rendered B2F1, a naturally *eaeA*-negative EHEC isolate that does not bind HEp-2 cells in an LA/FAS manner, capable of binding HEp-2 cells in small clusters. Thus, exogenous complementation for adherence by the intimin fusion proteins was EHEC specific. Neither of the fusion proteins altered the HEp-2 cell adherence phenotype of the non-EHEC *E. coli* (e.g., *E. coli* K-12, normal-flora HS, O8$^+$, or O8$^-$).

Several possibilities can be proposed to explain the weak or negative FAS activity observed with both the exogenously complemented deletion mutant and the exogenously complemented B2F1 strain. First, there may have been an imprecise association of the intimin protein with the bacterium or the epithelial cell when the protein was added exogenously. Second, the entire intimin molecule may be required for the FAS phenotype (neither of the fusion proteins described contained the full predicted amino acid sequence). Third, the lack of FAS activity may be due to an uncharacterized effect of the histidines on the amino terminus of the intimin protein. Or fourth, accessory proteins or factors required for FAS activity by the bacteria may not be present in this O91:H21 strain. The recent description of the LEE locus of A/E bacterial species (28) supports the latter hypothesis. This 35-kb chromosomal region encodes several loci that are required for LA and/or FAS activity of the A/E-causing bacterial species (EHEC, EPEC [17], *Hafnia alvei* [2], and *Citrobacter freundii* biotype 4280 [38, 39]). Whether B2F1 or other O91:H21 EHEC isolates encode all or part of the LEE locus is currently under investigation.

The shorter fusion, RVHdHisEae, also complemented 86-24*eae*Δ*10*, a finding which implies that the region(s) of intimin that binds the bacterium to the epithelial cell is not in the amino-terminal one-third of the molecule. The reported homology between EPEC intimin and the invasin protein of *Yersinia pseudotuberculosis* extends to EHEC intimin and invasin (17, 51). Invasin is the major protein involved in the penetration of intestinal epithelial cells by *Y. pseudotuberculosis* (12, 13, 15) and has a pivotal role in the initiation of infection by *Y. enterocolitica* (34). Overall, the EHEC intimin and invasin predicted amino acid sequences are 31% identical and 51% similar (51). The carboxyl termini of intimin and invasin are the most divergent regions between the two proteins. The carboxyl-terminal 192 amino acids of invasin comprise the receptor binding domain (21–23). Isberg and Leong (14) identified members of the β_1 family of integrins as the cellular receptors for invasin. The aspartate residue at position 911, critical for integrin binding by invasin (23), is absent in intimin, but two cysteine residues essential for binding of invasin to its integrin receptor (22) are shared by the intimins from all four of the A/E-promoting bacteria (8). Frankel et al. (8) demonstrated by use of MBP fusions that the C-terminal 280 amino acids of intimin from EPEC, EHEC, *H. alvei*, or *C. freundii* 4280, although somewhat different from one another at the amino acid level, were capable of binding and mediating the attachment of staphylococci to HEp-2 cells. The purified MBP-invasin fusion proteins were also able to bind HEp-2 cells. However, the patterns of attachment of the isolated MBP-intimins were different from the pattern demonstrated for the MBP-invasin fusion protein (8). These investigators did not explore the potential nucleation of F-actin as a result of the binding of the intimin carboxyl domain to the HEp-2 cells.

The rabbit and mouse antisera raised against the longer intimin fusion protein recognized EHEC intimin as well as EPEC intimin. These data confirm the findings of Louie et al. (25), who found that antisera raised against an intimin–glutathione-*S*-transferase fusion protein recognized an OMP expressed by EHEC of various serogroups and EPEC. However, the capacity of the antibodies against the intimin–glutathione-*S*-transferase fusion to block adherence to HEp-2 cells was not demonstrated in that analysis. In the present study, the anti-intimin antibodies generated against the longer fusion protein were capable of blocking the adherence of wild-type EHEC O157:H7 to HEp-2 cells. Our anti-intimin antisera together with the histidine fusions created for purification of intimin will be used as tools for further structure-function analysis of this EHEC adhesin.

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