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Characterization of the C-Terminal Domains of Intimin-Like Proteins of Enteropathogenic and Enterohemorrhagic *Escherichia coli*, *Citrobacter freundii*, and *Hafnia alvei*

GAD FRANKEL,[1]* DAVID C. A. CANDY,[2] PAUL EVEREST,[1] AND GORDON DOUGAN[1]

Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AZ,[1] and Department of Child Health, King's College School of Medicine and Dentistry, London SE5 9PJ,[2] United Kingdom

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Surface proteins called intimins (Int), which are homologous to the invasin protein (Inv) of *Yersinia* spp., play a role in inducing brush border damage, termed attachment and effacement, which follows infection by enteropathogenic and enterohemorrhagic *Escherichia coli*, *Citrobacter freundii* biotype 4280, and *Hafnia alvei*. Maltose-binding protein (MBP) fusions containing the C-terminal 280 amino acids of Int-like proteins of strains of enteropathogenic *E. coli*, enterohemorrhagic *E. coli*, *H. alvei*, and *C. freundii* biotype 4280 and of *Yersinia pseudotuberculosis* Inv were constructed and purified. The 3′ end of the gene for the *H. alvei* Int-like protein was sequenced and showed homology to corresponding regions of other Int-encoding genes. Binding of MBP–Int-like and MBP-Inv fusion proteins to HEp-2 cells was demonstrated by immunofluorescence microscopy and by fluorescence-activated cell sorting. MBP-Inv induced attachment and spreading of HEp-2 cells to plastic-coated wells, but MBP–Int-like fusion proteins did not. Preincubation of HEp-2 cells with MBP-Inv, but not with MBP–Int-like fusion proteins, inhibited MBP-Inv-induced cell attachment. Fixed staphylococci and fluorescent polymer microspheres coated with both MBP–Int-like and MBP-Inv fusion proteins showed enhanced adhesion to HEp-2 cells. These fusion proteins will facilitate studies of the role of intimin in the pathogenesis of diarrhea associated with members of the family *Enterobacteriaeceae* that induce attachment and effacement.

An increasing number of species of enterobacteria, including enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) (15, 29), *Hafnia alvei* (1), and *Citrobacter freundii* biotype 4280 (13, 26), have been shown to induce a specific lesion of the intestinal brush border, termed attachment and effacement (A/E) (19). Attachment of these enterobacteria to brush borders induces elongation and then effacement of microvilli by the formation of microvesicles of brush border membrane. The bacteria then adhere to bare plasma membrane of the enterocytes by close apposition. The bacteria are partly invested by plasma membrane, forming structures called pedestals (19). The cytoskeleton immediately subjacent to this lesion becomes reorganized by polymerization of actin (6, 14, 22).

The formation of the A/E lesion in the intestinal brush border is determined by a family of outer membrane proteins with molecular masses of ca. 100 kDa encoded by chromosomal genes. The first of these proteins to be characterized, that of EPEC, was initially termed *E. coli* attaching/effacing protein (EAE) and is now called intimin (Int) (12). Loss of the *eae* gene (now called *eaeA*) (3, 10–12) from EPEC resulted in loss of the ability to cause the A/E lesion and also loss of the ability to invade tissue culture cells. However, transfer of the gene to an avirulent *E. coli* strain did not confer the ability to induce the A/E lesion (10). An additional, as yet unidentified, chromosomal virulence gene(s) (class IV mutants [*cfm*]) was required to induce tyrosine phosphorylation of cellular proteins, while the intimin was required for pedestal formation (22).

* Corresponding author. Mailing address: Department of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AZ, UK. Phone: 44-71-225-8287. Fax: 44-71-225-8288.

An *eaeA* gene probe hybridized with DNAs from *H. alvei* and the 4280 biotype of *C. freundii* (1, 26). The *eaeA* genes of EPEC, EHEC, and *C. freundii* biotype 4280 have been cloned and sequenced (2, 12, 26, 34). The N-terminal ends of the *eaeA*-like gene products (designated Int_{EPEC}, Int_{EHEC}, and Int_{CF}, respectively) had a high degree of homology with each other and with the N-terminal ends of the *inv* gene products of *Yersinia pseudotuberculosis* (9) and *Yersinia enterocolitica* (33). The C-terminal 280 amino acids of the three sequenced intimins had less homology with each other (Int_{EPEC} and Int_{EHEC}, 50% identity; Int_{EPEC} and Int_{CF}, 50% identity) and showed very little similarity with the analogous domain of the *Y. pseudotuberculosis inv* gene product (Int_{EPEC} and Inv, 15% identity). The *Y. pseudotuberculosis* and *Y. enterocolitica* gene products, termed invasin (Inv), are outer membrane proteins of 103 and 94 kDa, respectively, which induce eukaryocytic cells that are not normally phagocytic to ingest coated bacteria and inert particles (21, 31). The binding activity of *Y. pseudotuberculosis* Inv resides in the 192 C-terminal amino acids of a total of 986 amino acids (17, 21). A disulfide loop in this region is necessary for this biological activity (18). The cell membrane receptors for Inv are the $\alpha_{3-6}\beta_1$ integrins, which are known to bind extracellular matrix glycoproteins such as fibronectin and vitronectin and to be associated with intracellular proteins such as actin, α-actinin, talin, tropomyosin, and vinculin (7, 8) that are implicated in either pedestal formation or invasion of cells by enterobacteria (6, 14, 22, 23).

Recently, Schauer and Falkow (27) showed that the *eaeA* gene product of *C. freundii* biotype 4280 is necessary for colonic colonization in mice. This, together with an earlier observation by Jerse et al. (12) that in the absence of EAF plasmid, *eaeA* mutants of EPEC do not adhere to cultured cells in vitro, suggested that the *eaeA* gene product is in fact an

TABLE 1. Bacterial strains and plasmid used in this study and names of the C-terminal domains of the intimin or invasin proteins isolated from them

Strain or plasmid	Name of Inv or Int-like C-terminal domain	Source[a]
EPEC O127ab:H6	Int_{EPEC}	A. D. Phillips
EHEC O157:H7 3787	Int_{EHEC}	B. Rowe
H. alvei	Int_{HA}	R. Robins-Browne
C. freundii biotype 4280	Int_{CF}	S. W. Barthold
pRI203	Inv	R. R. Isberg

[a] The addresses of the donors of strains or plasmid are detailed in the Acknowledgments.

adhesin. In this study we have cloned and sequenced the 3′ end of the *eaeA* gene homolog of *H. alvei*, and we report a high degree of homology of the gene product with the corresponding region of Int_{EPEC}. The differences in the C-terminal ends of Int-like proteins from EPEC, EHEC, *H. alvei*, and *C. freundii* compared with those of Inv from *Yersinia* spp. could reflect differences in properties of binding to cell surface receptors or different receptor specificities. This in turn could explain the different phenotypic properties of these organisms compared with those of *Yersinia* spp. We therefore cloned the C-terminal ends of Inv and Int-like proteins as maltose-binding protein (MBP) fusions to characterize their binding properties and biological activities.

MATERIALS AND METHODS

Bacterial strains and plasmid. The bacterial strains and plasmid used in this study and the names of the C-terminal domains of the Inv and Int-like proteins isolated from them are detailed in Table 1.

Construction of MBP-Inv and MBP–Int-like fusion proteins and DNA sequencing of the Int_{HA} C-terminal domain. PCR (20) was employed to amplify the DNA sequences encoding Int-like and Inv C-terminal regions, using whole bacterial DNA and plasmid pRI203, respectively, as templates. Two oligonucleotide primer pairs were synthesized on the basis of the published sequences of the *inv* gene of *Y. pseudotuberculosis* (9) and the *eaeA* family of genes of EPEC, EHEC (2, 34), and *C. freundii* (26). The forward primers, including an *Eco*RI restriction site, were derived from the 3′ ends of the conserved N-terminal domains (Table 2). The reverse primers were derived from the 3′ ends of the genes, including the stop codons and an *Xba*I restriction site (Table 2). One primer pair was used to amplify *eaeA*-like C-terminal domains, and the other primer pair was used to amplify the *inv* C-terminal domain. Bacteria, picked from a single colony with a toothpick, were transferred to a PCR mixture (which excluded the *Taq* DNA polymerase) and boiled for 10 min. Two units of *Taq* DNA polymerase (Boehringer Mannheim) was added, and DNA amplification was obtained after 30 cycles of 95°C for 20 s, 50°C for 45 s, and 72°C for 1 min. The ca. 840-bp PCR products (encoding ca. 280 amino acids) were purified by the Magic PCR cleanup system (Promega), digested with *Eco*RI and *Xba*I restriction endonucleases, and subcloned downstream from the *malE* gene (encoding MBP) of *E. coli* by using the pMAL C2 plasmid (New England Biolabs). The recombinant plasmids were used to transform *E. coli* TG1 by electroporation. Successful cloning was confirmed after purification of recombinant plasmids by the Magic Minipreps Kit (Promega) and DNA sequencing of the fusion junctions by the dideoxy chain termination procedure (25). The recombinant plasmid encoding the Int-like protein of *H. alvei* (Int_{HA}) was used as a template for determining its DNA sequence. The sequencing was started with a forward primer derived from pMAL C2, and additional (walking) primers were synthesized in the forward and backward orientations (for sequencing of both DNA strands) on the basis of the emerging DNA sequence.

Purification of MBP fusion proteins. The MBP fusion proteins were purified by amylose chromatography. Luria broth (500 ml) containing 0.2% glucose and 100 mg of ampicillin per liter was inoculated with 5 ml of an overnight culture of TG1 harboring a recombinant plasmid and incubated at 37°C with shaking for 2 h. Isopropyl-β-D-thiogalactoside (IPTG) (Bethesda Research Laboratories) was added to a final concentration of 0.3 mM, and incubation was continued for an additional 2 h. Subsequent purification was conducted at 4°C. The cultures were then centrifuged at 3,500 × *g* for 30 min. The supernatants were discarded, and the pellets were resuspended in 30 ml of column buffer (50 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM NaN_3) and then frozen overnight at −20°C. After thawing and sonication at maximum intensity in 10-s bursts for a total of 2 min, the sonicates were centrifuged at 6,300 × *g* for 30 min. The supernatants (crude extracts) were diluted with 100 ml of column buffer. The amylose resin (New England Biolabs) was poured into 15-cm columns (bed volume, 20 ml). The columns were washed with 200 ml of column buffer. The crude extracts were loaded at a rate of 1 ml/min and then washed with 160 ml of column buffer. The bound fusion proteins were eluted with column buffer containing 10 mM maltose (elution buffer). Twenty 3-ml fractions were collected, and the optical densities of the fractions were measured at 280 nm. Protein concentrations were determined with Bradford reagent (Bio-Rad). The purity of fractions eluted was tested by polyacrylamide gel electrophoresis and immunoblotting with anti-MBP antiserum (New England Biolabs).

Detection of binding of MBP fusion proteins to HEp-2 cells by immunofluorescence microscopy. HEp-2 cells were grown overnight on glass coverslips in 24-well tissue culture plates (1.5×10^5 cells per well) in 1 ml of Dulbecco's modified Eagle's medium with 10% fetal calf serum, 2 mM L-glutamine, and 50,000 U of penicillin and 50 mg of streptomycin per liter (complete medium). Dulbecco's modified Eagle's medium was replaced with 200 μl of RPMI–0.4% bovine serum albumin

TABLE 2. Sequences of the primers used for DNA amplification of Inv and Int-like C-terminal domains

Primer sequence[a]	Orientation	Protein(s) PCR obtained from products	Reference(s)
5′GTGAATTCACCGTCTCCACACCGGATATC	Forward	Inv	9
5′CTTCTAGATTATATTGACAGCGCACAGAG	Reverse	Inv	9
5′CTGAATTCGCCAGCATTACTGAGATTAAG	Forward	Int_{EPEC}, Int_{EHEC}, Int_{HA}, Int_{CF}	2, 26, 34
5′CTTCTAGAGACTATTTTACACAAACTGC	Reverse	Int_{EPEC}, Int_{EHEC}, Int_{HA}, Int_{CF}	2, 26, 34

[a] Underlined sequences represent the added nucleotides specifying the restriction enzyme sites.

(BSA)–20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.0]–2.5 mM maltose (binding solution), and cells were incubated for 45 min at 37°C in the presence of 5 μg of MBP fusion protein or of MBP as a negative control. The cells were washed five times with phosphate-buffered saline (PBS). Rabbit anti-MBP antiserum was diluted 1:1,000 in binding solution, and 250 μl was added to each well. The cells were incubated for 30 min at 37°C and then washed five times with PBS. Fluorescence-labelled porcine antirabbit antibody (Dakopatts) was diluted 1:1,000 with binding solution, and 250 μl was added to each well. After 30 min of incubation at 37°C, the cells were washed four times with PBS and fixed in 95% ethanol–5% acetic acid at −20°C for 15 min. The coverslips were removed from the wells, washed with deionized water, blotted, mounted on glass slides, and examined by UV microscopy.

Detection of binding of MBP fusion proteins to HEp-2 cells by FACS. The same methodology was used as for the immunofluorescence study, except that cells were grown directly in 24-well tissue culture plates and were not fixed. For each measurement, 5-μg portions of MBP fusion proteins were tested in triplicate. The cells were harvested after incubation at 37°C for 5 min with 0.3 ml of cell separation solution (Sigma) per well. Fluorescent cells were detected with a fluorescence-activated cell sorter (FACS) (Becton Dickinson).

Adhesion of HEp-2 cells to wells coated with the different MBP fusion proteins. Costar 96-well enzyme immunoassay/radioimmunoassay (EIA/RIA) plates were coated overnight at 37°C with 50-μl aliquots (per well) of fivefold dilutions of 0.2-mg/ml MBP and MBP fusion protein solutions in PBS. The wells were washed twice with PBS and blocked with 1% BSA in PBS for 2 h at 37°C. Successful coating with MBP-Int$_{EPEC}$ was assessed by using rabbit anti-MBP antiserum in an enzyme-linked immunosorbent assay (ELISA). HEp-2 cells, cultured in complete medium, were added to the wells (50 μl of a suspension of 10^6 cells per ml) and incubated for 40 to 120 min at 37°C in 5% CO_2. The wells were washed gently with PBS three times. The adherent cells were fixed with 70% methanol for 10 min at room temperature, stained with 0.2% crystal violet for 5 min, and washed with deionized water until no further stain was eluted. The cells were lysed with 1% sodium dodecyl sulfate (SDS) at 37°C, and the optical density at 562 nm was measured after 10 min.

Inhibition of HEp-2 cell adhesion to MBP-Inv-coated wells. HEp-2 cells at a concentration of 5×10^4 cells per ml in complete medium were incubated for 20 min at room temperature with column buffer or 50 μg of MBP, MBP-Inv, or MBP-Int$_{EPEC}$ per ml. Fifty-microliter aliquots of cells were added to wells of EIA/RIA plates coated with 80 ng of MBP-Inv per well. The plates were treated as described above for cell adherence, except that after crystal violet staining and washing with deionized water, the number of cells adhering to the wells was determined by examination with an inverted microscope.

Coating of staphylococci with MBP fusion proteins and HEp-2 adherence assay. Fixed, washed staphylococci (Sigma) expressing protein A were coated with anti-MBP antiserum, washed, coated with MBP fusion proteins or MBP, washed, and incubated with HEp-2 cell monolayers for 90 min at 37°C and 5% CO_2 by the method of Rankin et al. (21). The preparations were washed, fixed in 3% glutaraldehyde in PBS at 4°C, and stained with Giemsa stain for examination by light microscopy.

Coating of Covaspheres MX with MBP fusion proteins and HEp-2 adherence assay. Fluorescent polymer microspheres (Covaspheres MX; Duke Scientific) were coated with the different MBP fusion proteins according to the manufacturer's instructions. Twenty-five microliters of the coated Covaspheres suspension was added to subconfluent HEp-2 cells in 1 ml of complete Dulbecco's modified Eagle's medium, incubated for 30 to 60 min at 37°C, washed three times with PBS, fixed with 70% methanol, and examined by fluorescence microscopy.

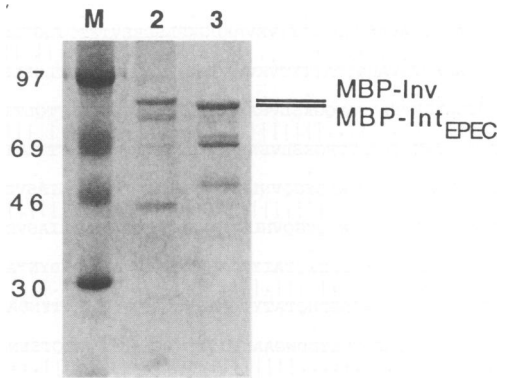

FIG. 1. Polyacrylamide gel electrophoresis of MBP-Inv (lane 2) and MBP-Int$_{EPEC}$ (lane 3) after amylose chromatography. Lane M, molecular weight markers (numbers are molecular weights in thousands).

Nucleotide sequence accession number. The nucleotide sequence encoding Int$_{HA}$ has been deposited with GenBank under accession no. L29509.

RESULTS

Construction and purification of MBP-Inv and MBP–Int-like fusion proteins and comparison of the amino acid sequences of Int$_{HA}$ and Int$_{EPEC}$. PCR products of ca. 840 bp, encoding ca. 280 amino acids of the C-terminal ends of Inv and the various Int-like proteins, were cloned in frame downstream from the *malE* gene of *E. coli*. Because of the relative high degree of similarity between the genes, one set of primers (Table 2) could be used to amplify the EPEC, EHEC, *Hafnia*, and *Citrobacter eaeA* gene family. A second primer pair was used to amplify the *Yersinia inv* gene (Table 2). The different recombinant plasmids were transformed into *E. coli* TG1, and the junction between the *malE* and the *eaeA/inv* domains was sequenced to confirm accurate cloning (not shown). Ten to 30 mg of fusion proteins, purified from 500-ml Luria broth cultures, was obtained for each of the constructs. Polyacrylamide gel electrophoresis (Fig. 1) and immunoblotting (not shown) showed high degrees of purification of the different preparations. The amino acid sequence of the C-terminal domain of Int$_{HA}$ was deduced from the DNA sequence of the cloned domain. Alignment of the Int$_{HA}$ amino acid sequence with the published sequence of Int$_{EPEC}$ (12, 34) revealed 77.5% similarity and 67% identity (Fig. 2).

Detection of binding of MBP fusion proteins to HEp-2 cells by fluorescence microscopy and FACS. The ability of the four MBP–Int-like fusion proteins and MBP-Inv to bind to HEp-2 cells was demonstrated by immunofluorescence microscopy. Cells exposed to MBP did not show any fluorescent signal. Cells exposed to the MBP–Int-like fusion proteins showed a fluorescence pattern that appeared to originate from minute, discrete surface structures on the cells, rather than general fluorescence of the cell surface. Cells that were exposed to MBP-Inv generated a punctate fluorescence pattern with localized areas of intense staining, which was not seen on cells

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  1 ITEIKADKTTAVANGKDAVTYTVKVMKDGKPLSGEEVTFTTTLGTLSKST 50
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660 ITEIKADKTTAVANGQDAITYTVKVMKGDKPVSNQEVTFTTTLGKLSNST 709

 51 EKTNTNGYRKVSLTSANQGKSLVSASVTMPQLMLKLLEVEFFTQLTIDNG 100
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710 EKTDTNGYAKVTLTSTTPGKSLVSARVSDVAVDVKAPEVEFFTTLTIDDG 759

101 NVEIVGTGAKGKLPNVWLQYGQVNLKANGGNGKTYWYSANPAIASVDPSS 150
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760 NIEIVGTGVKGKLPTVWLQYGQVNLKASGGNGKYTWRSANPAIASVDASS 809

151 GQVTLKDKGETTITVVSGDKQTAIYTIAMPNSIVSVNSSGRVDYNTANNI 200
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810 GQVTLKEKGTTTISVISSDNQTATYTIATPNSLIVPNMSKRVTYNDAVNT 859

201 CKNIKGSLPSSIKELKDLYDDWGAANKYQHY.SQESITAWTLQTSENKVQ 249
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860 CKNFGGKLPSSQNELENVFKAWGAANKYEYYKSSQTIISWVQQTAQDAKS 909

250 GVASTYDLVRKNPLIDKVDIAGNYAYAVCVK 280
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910 GVASTYDLVKQNPL.NNIKASESNAYATCVK 939
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FIG. 2. Alignment of the amino acid sequences of the Int_{EPEC} (EPEC E2348/69) (12, 34) (lower row) and Int_{HA} (upper row) domains. The amino acid sequence of Int_{EPEC} starts at position 660, and the amino acid sequence of Int_{HA} starts at the N-terminal end defined by the primers used for the PCR. Underlining indicates amino acid sequences encoded by the PCR primers. Vertical lines represent identical residues, colons represent highly conserved substitutions, and periods represent conserved substitutions.

exposed to MBP–Int-like fusion proteins (Fig. 3). FACS was also used to demonstrate MBP fusion protein binding to the eukaryotic cells. The four MBP–Int-like fusion proteins had higher signals, as indicated by higher median channels of fluorescence intensity, than the MBP-Inv fusion protein (Fig. 4), which is in agreement with the fluorescence microscopy images showing brighter staining with Int-like fusion proteins (Fig. 3). Both the Inv and Int-like fusion proteins had higher fluorescence intensities than the negative control (Fig. 4). The results summarized are the averages for six separate assays each performed in triplicate.

Adhesion of HEp-2 cells to wells coated with MBP fusion proteins. Spreading and attachment of HEp-2 cells to plastic wells coated with MBP-Inv occurred within 40 min of incubation. The degree of attachment, measured by release of crystal violet by 1% SDS from fixed, stained HEp-2 cells, was maximum when 2 μg of MBP-Inv was used to coat the wells (Fig. 5). By contrast, MBP and MBP–Int-like fusion proteins did not induce HEp-2 cell attachment at coating concentrations of up to 10 μg per well (Fig. 5). MBP–Int-like fusion proteins did not promote cell adherence even after 2 h of incubation. Successful coating of EIA/RIA plate wells by MBP-Int_{EPEC} was confirmed by ELISA (not shown). The ability of MBP–Int-like fusion proteins to inhibit attachment of cells to EIA/RIA plate wells coated with MBP-Inv was investigated. As demonstrated in Fig. 6, preincubation of the cells with MBP-Inv inhibited cell attachment. However, neither MBP nor MBP-Int_{EPEC} fusion protein had any inhibitory effect (Fig. 6).

Adherence of staphylococci and fluorescent Covaspheres coated with MBP fusion proteins to HEp-2 cells. Staphylococci and fluorescent Covaspheres coated with MBP could be seen only occasionally adhering to HEp-2 cells, whereas staphylococci and beads coated with either MBP-Inv (Fig. 7 and 8), MBP-Int_{EPEC} (Fig. 7 and 8), or MBP-Int_{CF} (not shown) could be seen covering large areas of the cell membranes. After examination of many independent monolayers exposed to the coated particles, it appeared that the numbers of attached staphylococci were similar for the particles coated with MBP-

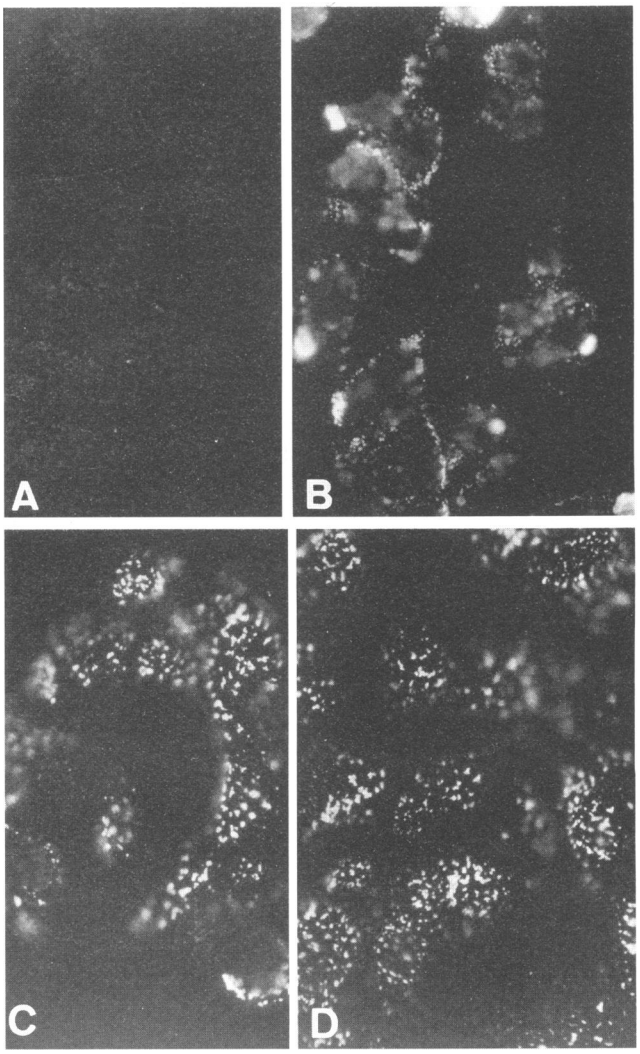

FIG. 3. Binding of MBP (A), MBP-Inv (B), MBP-Int_{EPEC} (C), and MBP-Int_{EHEC} (D) to HEp-2 cells. Anti-MBP and secondary fluorescein isothiocyanate-conjugated antibodies were used to detect bound fusion proteins. Cells were incubated with 5 μg of fusion proteins for 45 min. Patterns similar to that for MBP-Int_{EPEC} were also obtained following incubation of HEp-2 cells with MBP-Int_{HA} and MBP-Int_{CF} fusion proteins.

Int_{EPEC} (Fig. 7) and those coated with MBP-Inv or MBP-Int_{CF} (not shown), while MBP-Int_{EPEC} (Fig. 8) and MBP-Int_{CF} (not shown) coated Covaspheres were seen at higher frequencies than beads coated with MBP-Inv (Fig. 8).

DISCUSSION

Interaction with epithelial cells is among the first events in the pathogenesis of many infectious diseases. As a result, the attached bacteria may invade eukaryotic cells. *Yersinia* spp. are one of several genera of invasive bacteria whose mechanisms of cell invasiveness are well characterized (8, 9, 17, 21, 30, 33). A chromosomal *inv* gene of *Y. pseudotuberculosis* was identified as mediating surface attachment and entry into cultured HEp-2 cells. Invasin of *Y. enterocolitica*, which has 77% identity with the *Y. pseudotuberculosis* homolog, was also found to have

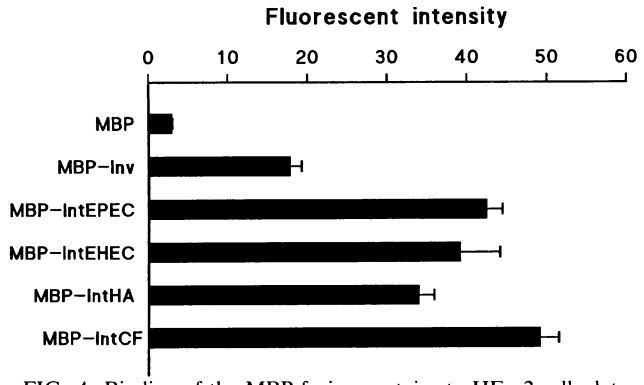

FIG. 4. Binding of the MBP fusion proteins to HEp-2 cells determined by FACS. Incubation of cells with the fusion proteins was performed at 37°C for 45 min. Bars represent the means of six measurements of fluorescence intensity determined for MBP and the Int-like and Inv fusion proteins; thin lines represent standard errors of the means.

similar biological properties, i.e., the ability to convert an *E. coli* K-12 strain to an attaching and invading organism (33).

Yersinia spp. invade the intestinal epithelium and proliferate in the lamina propria and regional lymph nodes. In contrast, EPEC, EHEC, *H. alvei*, and *C. freundii* biotype 4280 colonize the intestinal lumen, by adhering to and effacing enterocyte brush border (10, 13, 19, 32). EPEC strains of human origin colonize the entire intestinal tract of humans (24) and can induce the A/E lesion in human, rabbit, and pig intestinal brush borders (15, 19). EHEC strains have been shown to colonize the distal intestine preferentially, in vivo, in infant rabbits (29) and gnotobiotic pigs (32). They are distinguished from EPEC by hyperproduction of Shiga-like cytotoxin (29). *H. alvei* resembles EPEC in its ability to efface rabbit small intestine (1). *C. freundii* biotype 4280 appears to efface only the colonic mucosae of mice (13). The ability of all four of these pathogens to efface brush border and to intimately interact with the epithelial cell membranes involves the *eaeA* gene family located on the bacterial chromosome.

An alignment of the sequences of the EPEC, EHEC, and *C. freundii* biotype 4280 proteins encoded by *eaeA*-like genes (2, 12, 26, 34) and the *eaeA* C-terminal domain of *H. alvei* (this study) shows striking sequence similarities of around 80%. The

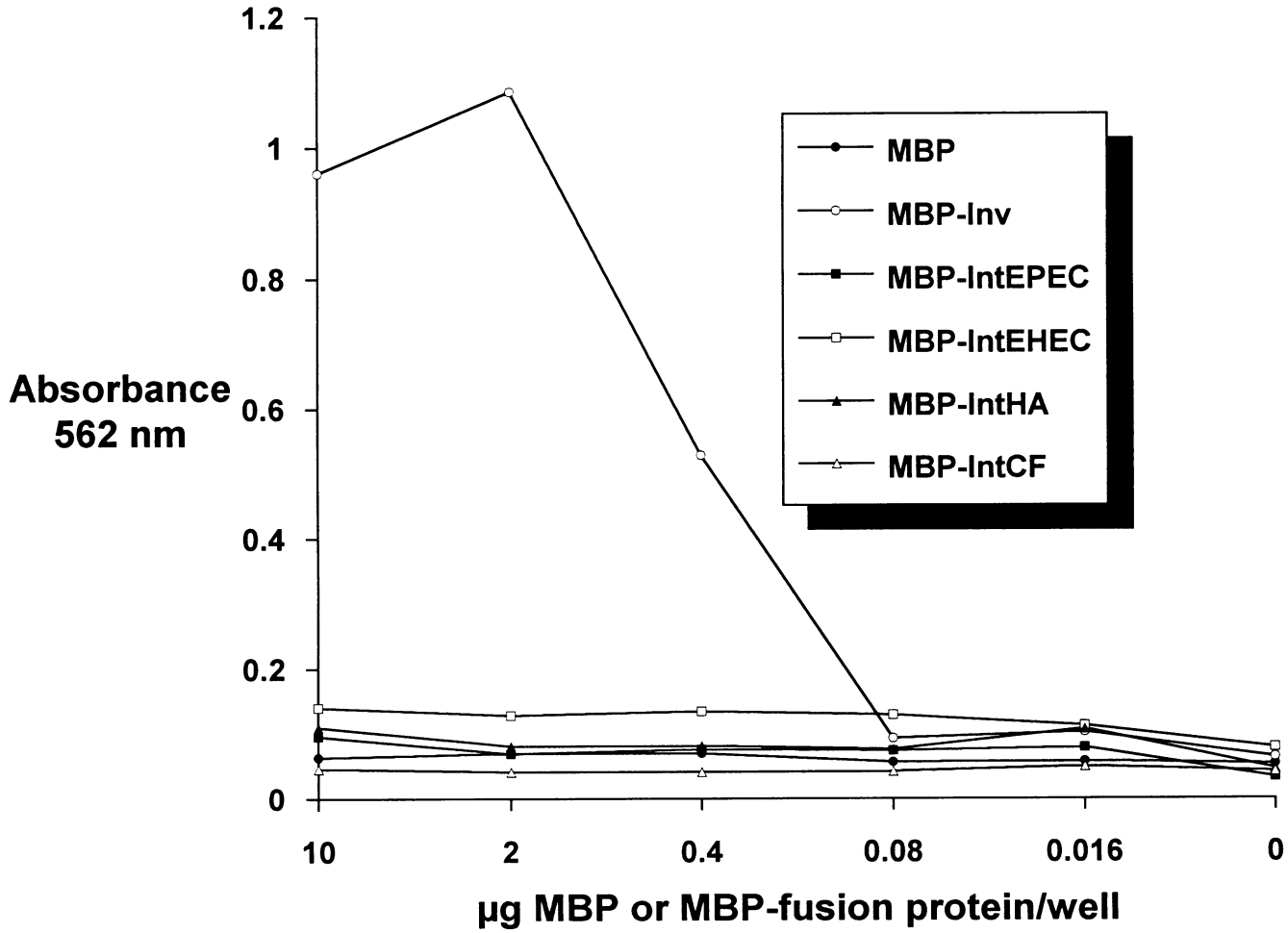

FIG. 5. Adherence of HEp-2 cells to tissue culture wells coated with various amounts of MBP fusion proteins. Fifty microliters of cells (10^6) was incubated in the coated wells for 40 min at 37°C. Unbound cells were washed, and cell adherence was recorded at 562 nm after release of the crystal violet stain by SDS. A single representative experiment of three independent repeats is displayed.

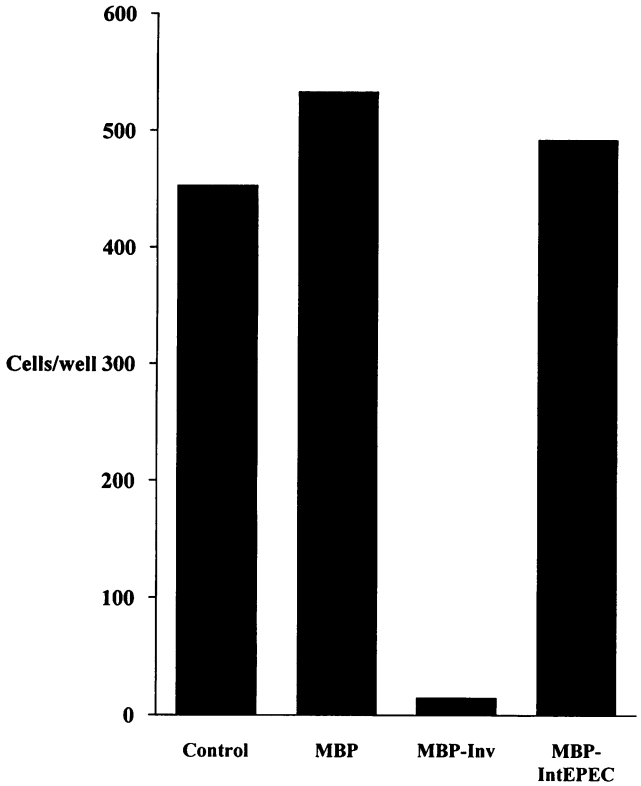

FIG. 6. Inhibition of HEp-2 adherence to MBP-Inv-coated tissue culture wells. Cells (5×10^4 per well) were preincubated with either column buffer, MBP, MBP-Inv, or MBP-Int_{EPEC} before addition to the wells. Nonadhering cells were removed by gentle washing with PBS. The cells were fixed with 70% methanol and stained with crystal violet. The number of adherent cells was determined by direct counting. The height of each bar represents the mean for three independent experiments.

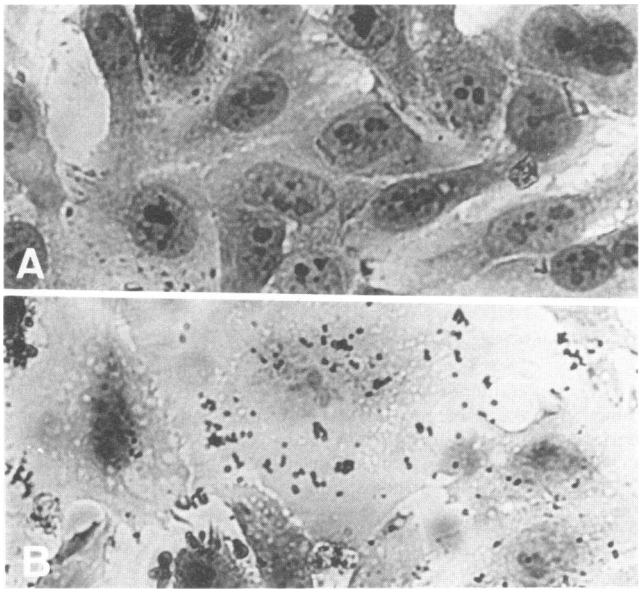

FIG. 7. Fixed staphylococci coated with either MBP or MBP fusion proteins were incubated with HEp-2 monolayers. Unbound bacteria were removed by washing; the culture was fixed, stained, and examined by light microscopy. Similar distributions of coated bacteria on cell surfaces were seen with MBP-Int_{EPEC} (B) and MBP-Inv fusion proteins, while MBP-coated staphylococci (A) were seen attached to the HEp-2 cells only occasionally.

eaeA-encoded proteins also show significant similarly to the *Y. pseudotuberculosis* and *Y. enterocolitica inv* gene products, especially among the first 700 amino acids of the N-terminal end (34). The integrin-binding site of Inv resides in the 192 amino acids of its C-terminal end (17), and on the basis of similarities with the Int_{EPEC} family of proteins, it has been suggested that Int_{EPEC} is the adhesin that mediates the intimate interaction between EPEC and the epithelial cell, through the analog domain. Despite the striking differences in the amino acid sequences between Inv and Int C-terminal domains, two cystine residues that were previously shown to be essential for Inv binding to its receptor (18) are also present, at the same relative locations, in all four intimins (2, 12, 26, 34; this study). However, unlike Inv, which by itself can induce the invasive process of nonphagocytic cells, Int_{EPEC} requires the cooperation of other EPEC proteins (3, 10, 11, 22; see reference 4 for a review), and the expression of the *eaeA* gene in an *E. coli* K-12 strain did not confer the A/E property (10). Nevertheless, Int_{EPEC} is a virulence factor that induces seroconversion in human volunteers (5).

In this study we investigated the ability of the C-terminal domain of the *eaeA* gene product to attach to and induce attachment of HEp-2 cells. We have done so by generating *malE* fusions (obtained by cloning of amplified segments) with the DNA sequences of the C-terminal domains of the four *eaeA* genes listed in Table 1. We have used, in the PCR, a forward primer which was derived from the end of the N-terminal conserved region that is located at about 840 bp upstream from the stop codon (encoding amino acids SITEIK). The reverse primer was derived from the end of the *eaeA* gene itself (encoding amino acids A[V/T]CV[K/E]Z). Since both of these sites are conserved among the three sequenced *eaeA* genes, with only minor differences in DNA sequences, we were able to use one set of primers to amplify the three genes. This set of primers was also suitable for amplifying the *H. alvei eaeA*-like gene. Since we intended to use the Inv C-terminal domain as a positive control and to compare the properties of Inv with those of the *eaeA* gene products, we have amplified the same region of *inv* by using pRI203, which encodes the whole *Y. pseudotuberculosis inv* gene, as the template.

Using both fluorescence microscopy and FACS, we were able to demonstrate that the four MBP–Int-like fusion proteins bound to HEp-2 cells. MBP by itself did not bind. The binding properties of MBP-Int_{EPEC}, MBP-Int_{EHEC}, MBP-Int_{HA}, and MBP-Int_{CF} were very similar to each other but differed from those of MBP-Inv in the following ways: (i) fluorescence microscopy examination revealed that MBP-Inv generated localized, intense staining which resembled the pattern obtained by antibody-mediated clustering of integrins (16), while MBP-Int fusion proteins bound to discrete structures on the cell surface; (ii) the FACS analysis showed greater fluorescence (binding) with MBP-Int fusion proteins than with MBP-Inv; (iii) fluorescent beads coated with an MBP-Int fusion protein showed better binding to HEp-2 cells than beads coated with MBP-Inv; (iv) MBP-Inv was the only construct that facilitated HEp-2 binding to coated tissue culture wells; and (v) MBP-Inv was the only fusion protein that inhibited HEp-2 spreading in wells previously coated with Inv.

Rankin et al. (21) have previously shown that *Staphylococcus aureus* coated with an MBP-Inv fusion protein (through staphylococcal protein A and anti-MBP antibody) will invade HEp-2

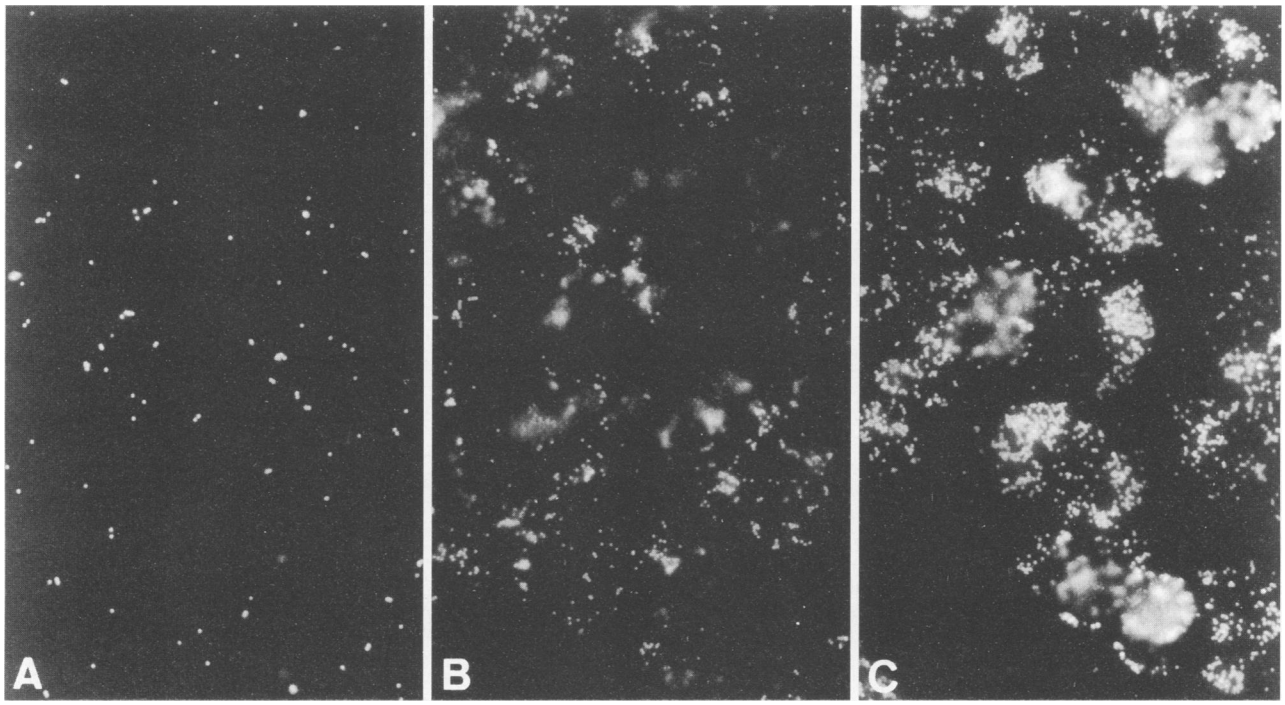

FIG. 8. Covaspheres MX coated with either MBP or MBP fusion proteins were incubated with HEp-2 monolayers. Greater numbers were seen on cell surfaces when Covaspheres coated with MBP-Int_{EPEC} were used (C) than when those coated with MBP-Inv were used (B). Covaspheres coated with MBP were seen attached to HEp-2 cells only occasionally (A).

cells, while same bacteria coated with fibronectin will only adhere to the cell membrane. Since both of these molecules will bind integrin, the difference in their biological activity is because Inv has higher affinity for the receptor than does fibronectin (31). Staphylococci and polystyrene beads coated with MBP-Int_{EPEC} fusion protein mediated attachment to HEp-2 cells. However, although coated staphylococci showed a similar attachment capability, MBP-Int-coated beads showed enhanced binding compared with that of Inv-coated beads. This might be the result of the covalent binding of the proteins to the beads, which may modify the flexibility of the 76-amino-acid loop necessary for Inv binding (18). Site-directed mutagenesis to substitute the cystine residues of intimins will be needed to determine their necessity for binding of intimins. The induction of cell spreading and adhesion is a property of integrin ligands such as fibronectin (30), laminin (28), and Inv. Therefore, the facts that the intimins did not facilitate cell spreading, did not inhibit spreading mediated by Inv, and did not seem to induce receptor clustering may suggest that Int-like proteins bind to different entities than Inv does. However, at this stage we can not entirely exclude the possibility of binding to same receptor with different biological outcomes.

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