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An aspartate residue of the *Yersinia pseudotuberculosis* invasin protein that is critical for integrin binding

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The *Yersinia pseudotuberculosis* invasin protein mediates bacterial entry into mammalian cells by binding multiple β_1-chain integrins. Invasin binding to purified $\alpha_5\beta_1$ integrin is inhibited by Arg–Gly–Asp (RGD)-containing peptides, although invasin contains no RGD sequence. Fifteen mutations that diminished binding and bacterial entry were isolated after mutagenesis of the entire *inv* gene. All of the mutations altered residues within the C-terminal 192 amino acids of invasin, previously delineated as the integrin binding domain, and 10 of the mutations fell within an 11 residue region. This small region was subjected to site-directed mutagenesis and almost half of the 35 mutations generated decreased invasin-mediated entry. D911 within this region was the most critical residue, as even a conservative glutamate substitution abolished bacterial penetration. Purified invasin derivatives altered at this residue were defective in promoting cell attachment and this defect was reflected in a 10-fold or greater increase in IC_{50} for integrin binding. D911 may have a function similar to that of the aspartate residue in RGD-containing sequences.

Key words: bacterial entry/cell adhesion/integrin/invasin/ *Yersinia pseudotuberculosis*

Introduction

The interaction of a pathogenic microorganism with host cells is a critical determinant of infection. In many instances, attachment to host cell surfaces is required for the establishment of an infectious niche (Beachey, 1981; Finlay and Falkow, 1989). Most bacterial pathogens encode surface factors that either directly bind host cells (Relman *et al.*, 1990; Hoepelman and Tuomanen, 1992) or bind host adhesion factors, such as fibronectin, that act as 'bridging' molecules (Courtney *et al.*, 1983; Van de Water *et al.*, 1983; Flock *et al.*, 1987). Penetration into host cells follows binding for many bacterial pathogens (Moulder, 1985; Isberg, 1991; Falkow *et al.*, 1992; Bliska *et al.*, 1993b). Disease caused by the enteropathogenic *Yersinia pseudotuberculosis* and *Y.enterocolitica* is initiated by the ingestion of contaminated foodstuffs. The bacteria apparently penetrate M cells to cross the intestinal epithelial barrier (Hanski *et al.*, 1989; Grutzkau *et al.*, 1990; A.Marra, unpublished observation). Upon gaining access to the mesenteric lymphatic system, and subsequently the liver and spleen, the bacteria replicate predominantly outside of host cells (Simonet *et al.*, 1990).

The enteropathogenic *Yersiniae* possess multiple mechanisms to enter cultured mammalian cells (Miller and Falkow, 1988; Isberg, 1990; Bliska *et al.*, 1993a; Yang and Isberg, 1993). The pathway mediated by the invasin protein has been the most studied (Isberg, 1990) and is involved in translocation from the intestinal lumen into regional lymph nodes (Pepe and Miller, 1993; A.Marra, unpublished observation). Invasin is a 986 amino acid outer membrane protein that tightly binds at least five different β_1-chain integrins prior to uptake into cultured cells (Isberg *et al.*, 1987; Isberg and Leong, 1988; E.Krukonis and R.R.Isberg, unpublished observation). The C-terminal 192 amino acids of the protein contains the integrin binding activity of invasin (Leong *et al.*, 1990) and this region is also sufficient to promote entry when used to coat non-invasive bacteria, such as *S.aureus* (Rankin *et al.*, 1992). The N-terminal portion of invasin is required for secretion to and proper localization within the bacterial outer membrane (Leong *et al.*, 1990).

Integrins are a large family of heterodimeric receptors that mediate a variety of cell–cell and cell–extracellular matrix interactions (Hynes, 1992). The cytoplasmic domains of integrins interact with factors associated with the cytoskeleton and integrin binding can trigger signaling pathways that result in changes in tyrosine phosphorylation (Kornberg *et al.*, 1991; Guan and Shalloway, 1992), intracellular pH (Schwartz *et al.*, 1991) and the adhesive properties of other receptors (Springer, 1990). Several integrin ligands, such as fibronectin, contain the sequence Arg–Gly–Asp (RGD) and small peptides that contain RGD sequences can inhibit the binding of these ligands to some integrins (Ruoslahti and Pierschbacher, 1987; D'Souza *et al.*, 1991). In spite of this common motif, RGD-containing ligands bind distinct sets of integrins (Hynes, 1992), indicating that other features of ligands determine receptor specificity. The particular conformation of the RGD sequence in a given ligand may contribute to integrin binding (Pierschbacher and Ruoslahti, 1987; Samanen *et al.*, 1991; Barker *et al.*, 1992; O'Neil *et al.*, 1992) and, in addition, regions of a ligand distant from the RGD sequence may also be involved in receptor recognition (Aota *et al.*, 1991; Bowditch *et al.*, 1994).

A 76 amino acid disulfide loop in the C-terminus of invasin is required for activity (Leong *et al.*, 1993), but it remains unclear what specific sequences in the 192 amino acid integrin binding domain are responsible for high affinity binding. Invasin and fibronectin bind integrin $\alpha_5\beta_1$ at mutually exclusive sites and binding by both ligands is inhibited by RGD peptides (Argraves *et al.*,

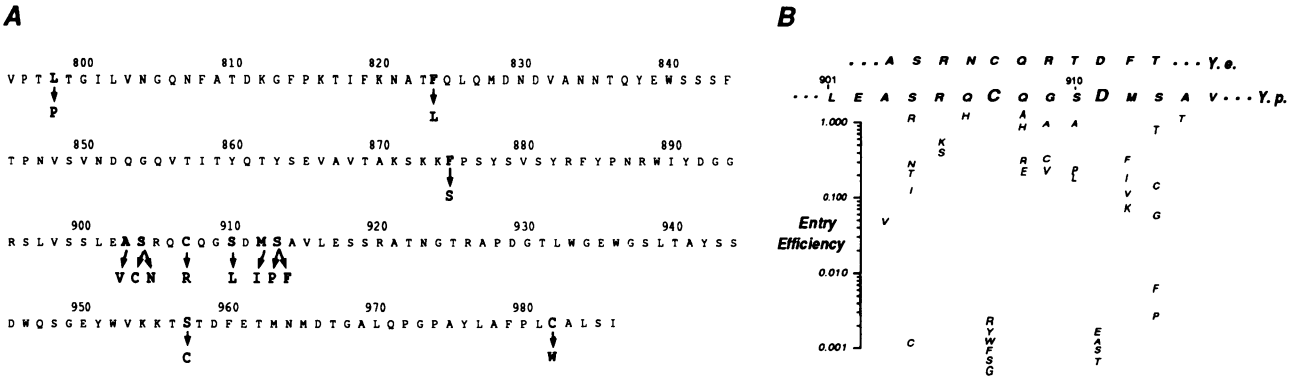

Fig. 1. Invasin mutations. (**A**) All of the Inv$^-$ mutations isolated after random mutagenesis alter residues in the integrin binding domain. The cell attachment domain of invasin is represented by single letter amino acid code; numbers above residues denote the position in the protein sequence. A plasmid (pJL272) that carries the entire *inv* gene was mutagenized and mutants that decreased HEp-2 cell binding and penetration were isolated (Materials and methods). All of the mutations affected residues in the cell attachment domain and the amino acid changes are represented by letters below the wild-type sequence.(**B**) D911 is critical for invasin-mediated cellular entry. Denoted is the entry efficiency of all of the single mutants of invasin in the region from residues 903–913 (Tables I and II). The amino acid sequence of residues 901–915 are given in single letter code. Approximate entry efficiency for each mutant is denoted by location relative to the scale on the left. Entry efficiency mediated by wild-type invasin is defined as 1.0. The sequence of *Y.enterocolitica* (*Y.e.*) invasin that corresponds to residues 903–913 is given above the *Y.pseudotuberculosis* (*Y.p.*) sequence.

1986; Tran Van Nhieu and Isberg, 1991). However, invasin contains no RGD sequence nor any sequence that closely resembles RGD (Isberg *et al.*, 1987). By generating point mutants of invasin that bind integrins with lower affinity, we have identified a sequence containing a critical aspartate in the integrin binding domain that is required for receptor recognition.

Results

Invasin mutants that decrease penetration of bacteria into mammalian cells fall within the previously defined integrin binding domain

To identify residues of invasin that are critical for cellular penetration, a plasmid carrying the entire *inv* gene was randomly mutagenized and *Escherichia coli* strains that expressed full-length invasin on the bacterial surface, but did not avidly bind HEp-2 cell monolayers, were enriched from independent pools of transformants (see Materials and methods). Individual clones were tested for the ability to enter cultured mammalian cells and 15 independent mutants that decreased the entry efficiency by 5- to 1000-fold were isolated (Figure 1 and Table I). Each of the mutants expressed apparently full-length invasin and the levels of invasin expression were approximately equivalent to wild-type (Figure 2, Immunoblot). By exchanging restriction fragments between mutant and wild-type *inv* genes, the site of each mutation was mapped and the exact mutation in each case was determined by DNA sequencing (Materials and methods).

In spite of the fact that the entire *inv* gene was randomly mutagenized, all 15 mutations altered residues in the previously defined integrin binding domain (Figure 1 and Table I; Leong *et al.*, 1990). Of the 15 mutants, the two that were most defective for penetration altered the Cys907 (C907) or C982 residues that participate in a 76 amino acid disulfide loop and were described previously (Leong *et al.*, 1993). Nine of the remaining 13 mutations mapped within an 11 amino acid region that encompasses C907 (Figure 1 and Table I), suggesting that this small region of the 986 amino acid invasin protein may be particularly important for integrin recognition. Two mutations in this region, *invS913P* and *invS910L*, were each isolated independently from two separate pools, so that a total of seven different amino acid changes in this region were isolated.

Expression of invasin mutant proteins

The method of enrichment was predicted to select for bacterial mutants that expressed full-length invasin on their surface, so immunoblotting and indirect immunofluorescence was used to examine the invasin produced. Bacterial lysates were fractionated by SDS−PAGE and probed with the monoclonal antibody (mAb) 2A9-1, an antibody that recognizes the central portion of invasin (Leong *et al.*, 1991). All of the mutants appeared to express approximately wild-type levels of full-length invasin (Figure 2A). (Note multiple species of invasin, a property previously described; Isberg and Leong, 1988.)

Surface exposure of invasin was assessed for the mutants by subjecting live bacteria to quantitative indirect immunofluorescence using mAb 3A2-1, an antibody that recognizes a surface-exposed epitope in the integrin binding domain of invasin. Most of the mutants were found to efficiently express invasin on the bacterial surface. InvA903V was not evaluated, because it was not efficiently recognized by mAb 3A2-1 in immunoblots (data not shown). Three of the mutants that affected residues distant from C907 expressed the epitope 10- to 15-fold less efficiently than wild-type (Table I, InvL798P, InvF875S and InvF824L). One other mutant, InvS913P, expressed the epitope ~2-fold less efficiently. The C-terminal domain of invasin of these mutants may not be fully exported or may be present in a conformation that is not efficiently recognized by mAb 3A2-1. Given that the density of invasin molecules on the bacterial surface can affect the level of cellular penetration (Rankin *et al.*, 1992), these mutants may be deficient in entry because of inefficient invasin export. In addition, if surface recognition by mAb 3A2-1 is diminished in these mutants because of altered

Table I. Inv$^-$ mutants selected for inefficient cell attachment

Plasmid	Protein produced[a]	Entry efficiency[b]	Surface expression[c]	Cell binding[d]
pJL272	wild-type invasin	1.00	1.00	+++
pT7-12	none	0.001	<0.005	−
pJL272-3	InvL798P	0.005 ± 0.004	0.08 ± 0.01	+
pJL272-17–2	InvF824L	0.131 ± 0.024	0.09 ± 0.08	+
pJL272-26-3	InvF875S	0.013 ± 0.002	0.07 ± 0.01	++
pJL272-18-1	InvA903V	0.044 ± 0.011	ND[e]	++
pJL272-5	InvS904C	0.001 ± 0.003	1.16 ± 0.06	+
pJL272-34a	InvS904N	0.270 ± 0.065	1.10 ± 0.06	+++
pJL272-C6b[f]	InvC907R	0.002 ± 0.002	0.17 ± 0.06	−
pJL272-13-2	InvS910L	0.148 ± 0.036	0.76 ± 0.08	+++
pJL272-21a-1	InvS910L	ND	ND	ND
pJL272-20	InvM912I	0.148 ± 0.030	0.82 ± 0.05	+++
pJL272-19-3	InvS913F	0.005 ± 0.002	0.67 ± 0.06	++
pJL272-23-3	InvS913P	0.002 ± 0.002	0.41 ± 0.02	+
pJL272-C6d	InvS913P	ND	ND	+
pJL272-19-3	InvS957C	0.089 ± 0.004	0.72 ± 0.01	+++
pJL272-21a-2$^+$	InvC982Y	0.009 ± 0.005	0.33 ± 0.05	−

[a]Protein was expressed in *E.coli* MC4100 harboring the denoted plasmid. Invasin point mutants are indicated by single letter amino acid designation and number indicates invasin residue. For example, InvS904C carries a Ser→Cys mutation at invasin residue 904.
[b]Percent entry was calculated as the percentage of bacteria that survive gentamicin treatment relative to wild-type control and is the mean of three determinations ± SD (Leong *et al.*, 1990).
[c]Surface expression was determined using quantitative indirect immunofluorescence probing with mAb 3A2-1, which recognizes an epitope between amino acids 795 and 986 of invasin (Leong *et al.*, 1990). Values shown are expressed relative to the fluorescence obtained with *E.coli* MC4100/pJL272, defined as 1.00, and are the mean of three determinations ± SD (see Materials and methods).
[d]Cell binding activity when filter replicas of SDS−PAGE fractionated bacterial extracts were probed with dispersed HEp-2 cells (see Materials and methods): +++, activity indistinguishable or nearly indistinguishable from wild-type invasin; ++, activity significantly less than for wild-type invasin; +, activity detectable only when filter was probed with a high concentration (>2×10^6/ml) of HEp-2 cells; −, undetectable cell binding activity.
[e]Not determined.
[f]Previously described (Leong *et al.*, 1993).

invasin folding, this conformational change may similarly diminish integrin recognition. All of the other nine mutants expressed the 3A2-1 epitope at roughly wild-type levels (i.e. at least 67% of wild-type; Table I).

Cell attachment mediated by the invasin mutant proteins

Quantitation of the invasin epitope on the bacterial surface indicated that for most of the mutants, diminished surface expression was insufficient to explain the penetration defect. In order to assess the ability of each mutant protein to mediate cell binding, bacterial lysates containing these proteins were fractionated by SDS−PAGE, transferred to membrane filters and probed with live mammalian cells (Figure 2, lower panel and Table I, cell binding; Isberg and Leong, 1988). In these filter assays, all of the mutants that were at least 40-fold less efficient in cellular penetration than wild-type were clearly diminished in cell binding activity (Figure 2, relative entry efficiency of 0).

In the 903–913 region, Asp911 is most critical for invasin activity

As described above, most of the mutations that diminished invasin-mediated cellular entry affected residues in the region 903–913. To clarify which residues in this region were most critical for invasin function, this region was altered using mutagenic oligonucleotides (Material and methods). In addition, because of the importance of aspartate residues in other integrin ligands (e.g. the aspartate in RGD sequences), the aspartate codon at position 911 (D911) was changed to several different codons. A collection of 35 different mutations was

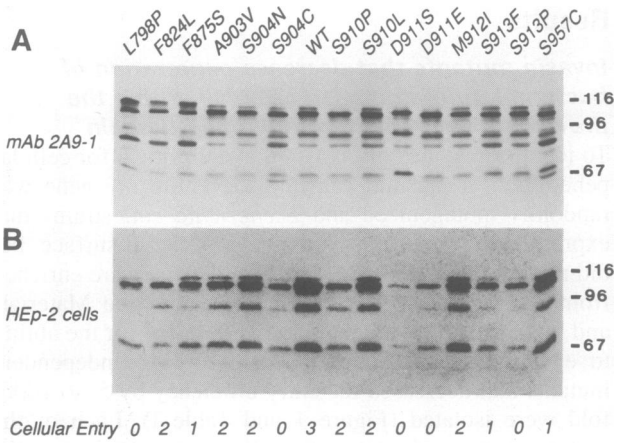

Fig. 2. Invasin mutants that are defective for mediating bacterial entry are defective for mediating mammalian cell attachment. Lysates were prepared from 3×10^6 *E.coli* harboring pJL272 derivatives and then subjected to SDS−PAGE fractionation and (**A**) immunoblotting with anti-invasin mAb 2A9-1 (Leong *et al.*, 1991) or (**B**) cell probing with HEp-2 cells, as previously described (Isberg and Leong, 1988). The invasin derivative produced from each bacterial strain is indicated above each lane in (A) (see Tables I and II). WT, wild-type invasin. Cellular entry efficiency: 0, entry reduced 40- to 500-fold; 1, entry reduced 10- to 40-fold; 2, entry reduced 5- to 10-fold (from Tables I and II); 3, wild-type levels of entry. Note that multiple species of invasin, observed here after expression of *inv* in *E.coli*, are also generated after expression in wild-type *Y.pseudotuberculosis*, as described previously (Isberg and Leong, 1988). The electrophoretic migration of molecular weight standards are given at right.

Table II. Site-directed invasin mutants, residues 903–914

Plasmid	Protein produced[a]	Entry efficiency[a]	Surface expression[a]	Cell binding[b]
pJL272	wild-type invasin	1.00	1.00	+++
pT7-12	none	0.001	<0.005	–
Single mutations				
pJL272-100	S904I	0.102 ± 0.013	0.54 ± 0.05	++
pJL272-117	S904T	0.206 ± 0.007	0.55 ± 0.18	++
pJL272-43	S904R	0.990 ± 0.105	ND	+++
pJL272-139	R905S	0.337 ± 0.060	1.00 ± 0.04	+++
pJL272-123	R905K	0.438 ± 0.104	1.02 ± 0.14	+++
pJL272-114	Q906H	0.996 ± 0.145	ND	+++
pJL272-61	Q908E	0.195 ± 0.014	0.52 ± 0.10	+++
pJL272-130	Q908R	0.210 ± 0.018	ND	+++
pJL272-25	Q908H	0.843 ± 0.081	ND	+++
pJL272-140	Q908A	0.982 ± 0.224	ND	+++
pJL272-G1	G909V	0.194 ± 0.038	0.95 ± 0.17	++
pJL272-G7	G909C	0.321 ± 0.034	0.47 ± 0.05	++
pJL272-81	G909A	0.866 ± 0.187	0.66 ± 0.07	+++
pJL272-S910P	S910P	0.159 ± 0.002	ND	++
pJL272-G14	S910A	0.780 ± 0.102	ND	+++
pJL316-D911A	D911A	0.005 ± 0.005	1.17 ± 0.15	±
pJL316-D911T	D911T	<0.001 ± <0.001	1.31 ± 0.14	±
pJL316-D911S	D911S	0.001 ± <0.001	1.12 ± 0.05	±
pJL272-D911E	D911E	0.001 ± 0.001	1.03 ± 0.20	+
pJL272-97	M912K	0.066 ± 0.010	0.87 ± 0.09	++
pJL272-M912V	M912V	0.118 ± 0.015	0.80 ± 0.01	++
pJL272-M912F	M912F	0.258 ± 0.046	0.94 ± 0.08	+++
pJL272-S913G	S913G	0.044 ± 0.005	1.81 ± 0.12	++
pJL272-G3	S913C	0.143 ± 0.023	0.56 ± 0.12	++
pJL272-S913T	S913T	0.535 ± 0.066	ND	+++
pJL272-148	A914T	1.16 ± 0.129	ND	+++
Multiple mutations				
pJL272-200	A903G/R905K/Q906H	1.56 ± 0.184	ND	ND
pJL272-157	A903G/S904R/S913A	0.471 ± 0.092	0.92 ± 0.15	+++
pJL272-118	S904R/G909V	0.496 ± 0.041	0.72 ± 0.12	+++
pJL272-8	S904N/Q906H	0.833 ± 0.082	ND	+++
pJL272-161	S904R/S913T	0.746 ± 0.036	ND	ND
pJL272-7	R905T/Q908H	1.03 ± 0.226	ND	+++
pJL272-30	R905S/S913Y	0.001 ± 0.001	1.11 ± 0.14	±
pJL272-143	R905S/G909D	0.108 ± 0.029	1.72 ± 0.42	+++
pJL272-45	Q908H/A914T	1.01 ± 0.136	ND	+++

[a]Data are expressed as defined in Table I.
[b]Cell binding by filter assay are expressed as defined in Table I. ±, activity detectable only when filters were probed with high concentrations of mammalian cells (i.e. 2×10^6 cells/ml).

generated, of which 26 were single mutations. Every residue in the region from codon 903 to 914 was changed to at least one different amino acid and most residues were altered to more than one different amino acid (Figure 1B). Each of these mutants were characterized for: (i) expression of full-length protein by immunoblotting, (ii) expression of the 3A2-1 epitope on the surface of the bacterium, (iii) cell binding activity when immobilized on filter membranes and (iv) the ability to mediate cellular entry; (Table II, Figure 2 and data not shown).

Apparently full-length invasin was produced for all of the mutants at approximately wild-type levels of expression and stability, as judged by immunoblotting (Figure 2 and data not shown). All of the mutants that affected cellular entry (see below) were also tested for expression of invasin on the bacterial surface by indirect immunofluorescence with mAb 3A2-1 (Leong *et al.*, 1990). None of the mutations affected the expression of the 3A2-1 epitope by more than 2-fold and most of the mutants expressed wild-type levels (Table II). These results indicate that full-length invasin is produced and exported to the outer membrane in these mutants.

In spite of the fact that these mutants express full-length invasin on the bacterial surface, many of the mutations resulted in significantly decreased cell binding and penetration. Fourteen of the 35 mutations promoted entry at levels <20% of wild-type; collectively, these mutations affected seven of the residues (904 and 908–913) in this 11 amino acid region (Table II). When combined with the mutations that were isolated by enrichment on HEp-2 cells described above (Table I), nine of the 11 residues in the region 903–913, i.e. all except Arg905 and Gln906, were implicated in invasin function (Figure 1B).

Of all the residues in the 903–913 interval, outside of the C907 residue involved in the disulfide bond (Leong *et al.*, 1993), D911 was found to be the most critical residue for cell attachment and entry. Changing this aspartate to alanine, threonine, serine or glutamate lowered bacterial penetration to background levels, yet had no

Table III. Entry-defective bacterial *inv* mutants are defective for mammalian cell binding

Plasmid	Invasin produced[a]	10^7 bacteria added/well	Bacteria bound/mammalian cell[b]
pT7-12	none	3.4	0.18 ± 0.57
pJL272	wild-type invasin	3.0	13.1 ± 10.11
pJL272-D911A	InvD911A	3.2	0.37 ± 0.87
pJL272-D911E	InvD911E	3.3	0.41 ± 1.14
pJL272-S913G	InvS913G	3.0	1.51 ± 2.11
pJL272-M912I	InvM912I	3.0	1.55 ± 2.05

[a]Invasin was expressed in *E.coli* MC4100 harboring the denoted *inv*-encoding plasmid. Invasin point mutants affecting residues Asp911, Ser913 or Met912 are indicated by single letter amino acid designation.
[b]Approximately 10^5 HEp-2 cells were incubated with the denoted number of bacteria as described in Materials and methods. Results shown are the mean of four monolayer infections treated identically for a total of 200 mammalian cells observed. $P < 0.0001$ by the Whitney test, comparing pJL272 with pJL272-M912I and comparing pJL272-S913G with pT7-12.

effect on export of invasin to the bacterial surface (Table II and Figure 1B). Three of these four mutations almost entirely abrogated cell attachment; only the most conservative mutation, D911E, retained detectable cell attachment activity (Table II and Figure 2, D911S and D911E). Other than D911 and C907, all of the residues in this region could be changed to at least one other amino acid without a >5-fold effect on entry. Additionally, no single amino acid change other than the D911 or C907 mutations completely abolished either cellular penetration or cell attachment activity in the filter assay (Table II). The double mutant R905S/S913Y resulted in an almost complete loss of invasin function, but neither R905 nor S913 are absolutely required for function, because single amino acid changes affecting these residues retained significant invasin activity (e.g. Table II, R905S and S913T).

D911 of invasin is required for bacterial attachment to HEp-2 cell monolayers

To quantitatively evaluate the ability of mutant invasin proteins to promote bacterial attachment to HEp-2 cells, monolayers were infected with bacteria expressing mutant invasin and the number of bacteria stably associated with the mammalian cells was counted by microscopic observation of stained samples (Table III). Four invasin mutants were analyzed: strains expressing InvD911A and D911E were unable to penetrate cells, while InvM912I and S913G were 7- and 20-fold defective for entry. The level of cellular association for each of the four mutants roughly paralleled the level of cellular penetration (Table III). For bacteria expressing wild-type invasin, an average of 13.1 bacteria were associated with each mammalian cell, in contrast to control *E.coli*, which bound at <0.2 bacteria/cell. Bacteria expressing InvD911E or InvD911A bound at levels barely above background (~0.4 bacteria/cell). It is notable that InvD911E displayed significant cell binding activity (compared with InvD911A or a no protein control) when tested in the filter assay after SDS–PAGE (Figure 2). Apparently, when this mutant invasin is expressed in the outer membrane of *E.coli* K12, this residual binding activity is not sufficient to promote stable bacterial attachment. Bacteria expressing InvM912I or InvS913G, mutations that cause less defective entry phenotypes than the D911 lesions, bound cells at ~10-fold less than wild-type (~1.5 bacteria/cell; Table III).

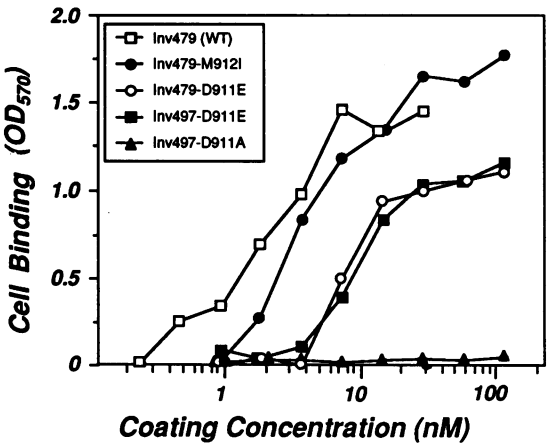

Fig. 3. Purified MBP–invasin derivatives altered at D911 are deficient in mediating cell attachment to microtiter wells. MBP–Inv fusion proteins were purified and analyzed for promoting cell attachment. Microtiter wells were coated with serial dilutions of purified protein, washed, blocked with BSA and incubated with dispersed HEp-2 cells for 1 h at 37°C (Materials and methods). After washing away unbound cells with PBS, bound cells were fixed with methanol, stained with crystal violet and quantitated by absorbance at 570 nm. Each point represents the mean of three determinations. A key to symbols is given in the inset. MBP alone does not mediate cell attachment at the concentrations tested here (Leong *et al.*, 1990, 1993).

Purified invasin derivatives altered at D911 and M912 have diminished cell binding activity

Maltose binding protein–invasin (MBP–Inv) fusion proteins that carry the integrin binding domain of invasin retain cell binding activity (Leong *et al.*, 1990; Rankin *et al.*, 1992). In order to closely examine the effect of specific mutations on invasin, MBP–Inv fusion proteins that carry mutations at residues D911 or M912 were purified. The mutants span a range of binding and penetration activity: InvD911A has lost invasin activity entirely, InvD911E has retained residual binding activity in the filter assay but has lost all penetration activity and InvM912I has retained binding activity but mediates entry at levels 7-fold lower than wild-type (Tables I and II and Figure 2). Two different MBP–InvD911E fusion proteins were generated, MBP–Inv479-D911E and MBP–Inv497-D911E, which differ by 19 in the number of invasin residues that are contained in the hybrid protein.

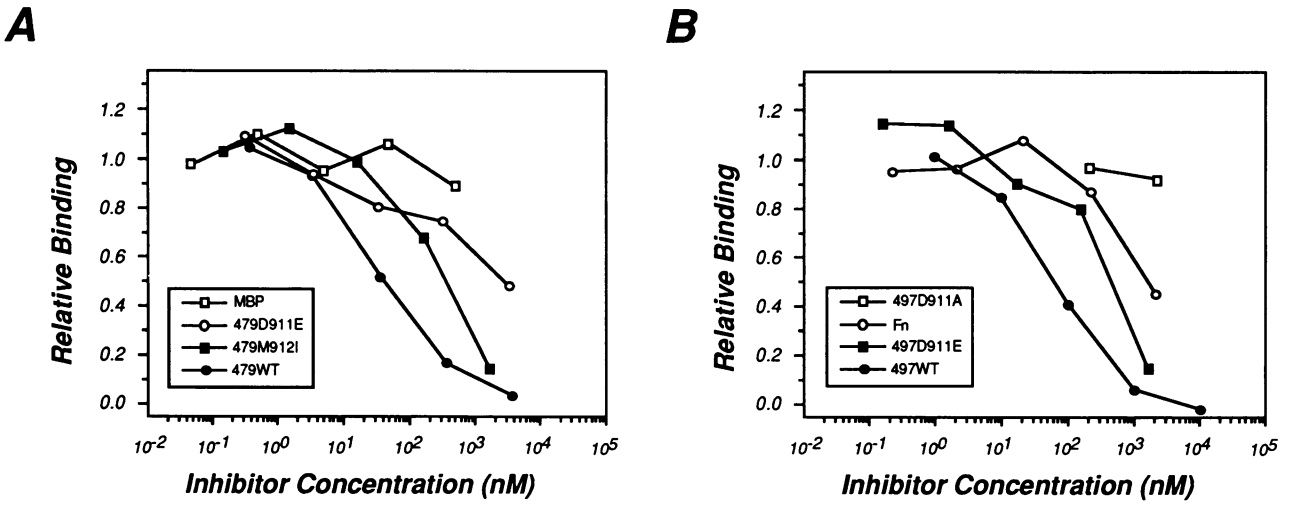

Fig. 4. Binding of integrin $\alpha_5\beta_1$ to immobilized invasin is inhibited by purified invasin derivatives. Microtiter wells coated with the Inv497 fragment were challenged with 7×10^{-9} M integrin $\alpha_5\beta_1$ in the presence of increasing concentrations of MBP–invasin or control proteins (Materials and methods). Bound receptor was detected by ELISA and relative binding is the ELISA signal in the presence of inhibitor divided by the signal in the absence of inhibitor. The key to symbols is given in the lower left inset; Fn, human fibronectin. Each point represents the mean of four replicates. All of the proteins were tested in parallel, but results are shown in two panels for simplicity of presentation.

Microtiter wells were coated with serial dilutions of protein to quantitate the minimal coating concentration required for cell attachment by each invasin derivative (Figure 3). As expected, MBP–Inv479, which carries a wild-type integrin binding domain, promoted cell attachment more efficiently than any of the mutants. The D911A mutant protein was completely devoid of cell binding activity (Figure 3). MBP–Inv479-D911E and the slightly longer MBP–Inv497-D911E mediated cell attachment much less efficiently than wild-type and even at the highest coating concentrations tested, the level of cell binding to these proteins was less than the maximal wild-type level. The M912I mutant protein required slightly higher coating concentrations than the wild-type protein to promote equivalent cell adhesion.

Invasin mutants are defective for binding purified $\alpha_5\beta_1$

To obtain a quantitative measure of binding by the invasin mutants, binding of the purified MBP–Inv hybrids was analyzed using purified $\alpha_5\beta_1$ integrin. Microtiter wells were coated with a C-terminal invasin fragment, probed with purified $\alpha_5\beta_1$ (Hautanen *et al.*, 1989) and binding was detected by ELISA (see Materials and methods). Addition of MBP–Inv proteins to $\alpha_5\beta_1$ in solution phase resulted in an inhibition of receptor binding to the invasin immobilized in microtiter wells (Figure 4). Wild-type MBP–invasin fusion proteins inhibited binding most efficiently, giving 50% inhibition (IC_{50}) at 39 or 48 nM, depending upon the length of invasin present in the particular hybrid protein (479 versus 497 amino acids; Figure 4). Fibronectin and invasin bind to $\alpha_5\beta_1$ in a mutually exclusive fashion, but the K_d for fibronectin is ~100-fold higher than that for invasin (Tran Van Nhieu and Isberg, 1991). Consistent with this, fibronectin inhibited invasin binding by $\alpha_5\beta_1$ in this assay, with an IC_{50} of ~1.6 μM (Figure 4B). MBP (Figure 4A) and MBP–InvD911A (Figure 4B) were unable to inhibit wild-type MBP–Inv, even at the highest concentrations of inhibitors tested. The M912I mutation resulted in an ~10-fold increase in the IC_{50} compared with wild-type (340 nM; Figure 4A). The magnitude of the integrin binding defect of the D911E substitution depended upon the site of the fusion protein joint, as the IC_{50} for MBP–Inv497-D911E was 470 nM, while that for MBP–Inv479-D911E was 2.5 μM. Although the reason for the difference in IC_{50} for the two D911E mutants is not clear, these experiments provide direct evidence that the D911 residue is critical for avid binding of integrin $\alpha_5\beta_1$ by invasin and that even a highly conservative change (D→E) caused disruption of binding.

Residues D911 and M912 are not required for disulfide loop formation in purified invasin derivatives

Our previous analysis of mutations affecting C907 or C982 revealed that a disulfide bond between these residues was required for integrin recognition (Leong *et al.*, 1993). To test whether the mutations at residues 911 or 912 diminished activity by affecting the formation of this disulfide bond, partial proteolysis products of the purified MBP–invasin hybrid proteins were analyzed by two dimensional gel electrophoresis. Purified derivatives of mutant invasin were subjected to partial proteolysis with V8 protease, which cleaves at several sites within the disulfide loop of invasin (Leong *et al.*, 1993). After SDS–PAGE of these cleavage products in the absence of reduction, partial proteolysis products that contain disulfide bonds can be detected by reduction with β-mercaptoethanol followed by SDS–PAGE in a second dimension; novel species of smaller molecular weight that migrate below the diagonal are generated only from protease products that contained a disulfide bond (Figure 5; Leong *et al.*, 1993). No such species are generated when the protease products are not reduced prior to the second dimension of SDS–PAGE. Because the MBP portion of each hybrid contains no cysteine residues, C907 and C982 of invasin are the only residues that can participate in a disulfide loop that generates the faster migrating species. All of the mutant invasin derivatives gave rise to clearly

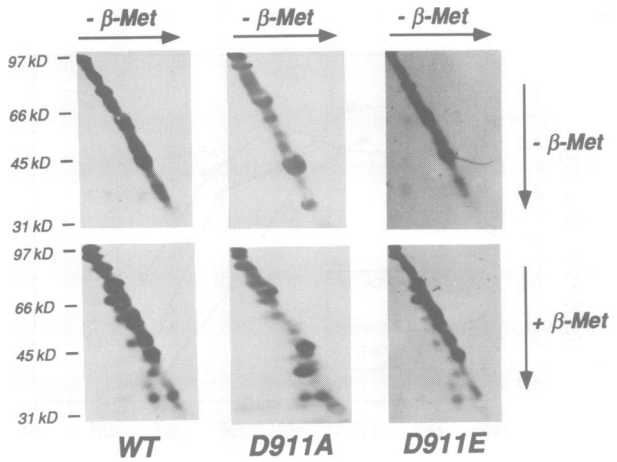

Fig. 5. D911 is not required for disulfide loop formation. MBP–Inv479 (WT), MBP–Inv497-D911A (D911A) and MBP–Inv479-D911E (D911E) were subjected to partial proteolysis with V8 protease and fractionated by non-reducing (– β-Met) 10% SDS–PAGE. Identical lanes were cut from the gel and overlaid on a second gel, either in the absence (top panels, – β-Met) or presence (bottom panels, + β-Met) of reductant (see Materials and methods). Invasin-related products were detected by transferring the second gel to an Immobilon filter and immunoprobing with the anti-invasin mAb 2A9-1. Molecular weight standards are given at left. Partial proteolysis products that are linked by the disulfide bond between C907 and C982 before reduction, are dissociated into smaller species by reduction. These novel, smaller species migrate below the diagonal. Note that because V8 protease cleaves after Asp and Glu residues, the D911A and D911E mutations may directly affect cleavage products.

discernible novel smaller molecular weight species, indicating the presence of a disulfide bond (Figure 5). We cannot rule out that the efficiency of formation of the critical 76 amino acid disulfide loop is decreased by the mutations at residues 911 or 912, but these results suggest that disruption of the disulfide loop cannot explain their integrin binding defect.

Discussion

To identify invasin residues critical for binding integrins, mutations that diminish invasin-mediated cell attachment and penetration were isolated. All 15 mutations isolated after mutagenesis of the entire gene altered residues within the C-terminal 192 amino acid integrin binding domain. Of these, *invC907R* and *invC982Y*, the two mutations that disrupted the 76 amino acid disulfide loop required for invasin function, have been previously described (Leong *et al.*, 1993). Two mutations, *invS904C* and *invS957C*, resulted in the generation of new cysteine residues and it is possible that the altered proteins form alternative, aberrant disulfide loops. Three mutations, *invL798P*, *invF824L* and *invF875S*, resulted in poor expression of the C-terminus of invasin on the surface of the bacterium. Each of the other eight mutations affected amino acids in a small region of invasin flanking C907, residues 903–913. All of these mutants efficiently expressed invasin on the surface of the bacterium, indicating that aberrant localization of the mutants could not explain the cell penetration defect. Almost half of the mutations generated by site-directed mutagenesis of this region resulted in a greater than 5-fold defect in invasin-mediated entry, in spite of the fact that the mutant proteins were surface localized. A total of nine of the 11 residues in this region were implicated in the cell attachment function of invasin by this mutational analysis.

Although a large fraction of mutations affecting the 903–913 interval diminished invasin activity, only one amino acid in this region, D911, appeared to be absolutely essential. All of the other residues in the region could be changed to at least one other amino acid without a substantial loss of entry efficiency and no single mutants except those affecting D911 resulted in a complete absence of invasin activity. In contrast, even the conservative mutation *invD911E* resulted in a total loss of invasin-mediated penetration and a significant decrease in integrin binding activity. Bacteria that expressed either the D911A or the D911E invasin mutants could not stably bind to HEp-2 cell monolayers. Compared with wild-type MBP–Inv, purified D911E invasin derivatives required 5-fold higher coating concentrations to promote half-maximal cell attachment in microtiter wells and a D911A derivative was devoid of all activity. Finally, these proteins were also defective in binding purified integrin $\alpha_5\beta_1$, as measured by IC_{50}s in an invasin binding assay. The IC_{50}s for two D911E derivatives were at least 10-fold higher than wild-type and the D911A mutant had no detectable inhibitory activity. The loss of binding activities could not be attributed to disruption of the disulfide loop involving C907 and C982.

The identification of an aspartate as a critical residue for integrin recognition by invasin raises the question of the relationship, if any, between D911 and the aspartate residue in the RGD sequences found in many integrin ligands. The aspartate residue in RGD peptides appears to be the most critical amino acid for the inhibitory activity of these peptides (D'Souza *et al.*, 1991), an observation that parallels the importance of D911 of invasin. Additionally, although *Y.pseudotuberculosis* invasin contains no RGD homology, the related *Y.enterocolitica* invasin contains the sequence RTD at the residues corresponding to 909–911 in the *Y.pseudotuberculosis* protein (Figure 1B; Young *et al.*, 1990). It has been postulated that the aspartate in RGD sequences provides an oxygenated residue needed to complex divalent cation in conjunction with putative Ca^{2+} binding sequences (EF-hand motifs) found in the α chain (Corbi *et al.*, 1987; Lawler *et al.*, 1988; Tuckwell *et al.*, 1992) or β chain (Loftus *et al.*, 1990) of integrins, so perhaps D911 of invasin plays a similar role. Aspartate residues are also found in many of the peptide sequences that are critical for integrin binding (Hynes, 1992) and an aspartate residue in the endothelial cell adhesion molecule VCAM has recently been shown to be required for $\alpha_4\beta_1$ recognition (Osborn *et al.*, 1994; Vonderheide *et al.*, 1994).

The conformation of the 903–913 region is presumably also important for the ability of invasin to recognize integrins. One possibility is that the difference in integrin binding by the two D911E MBP–invasin fusion proteins reflects differences in their conformation; consistent with this model, only one of them (MBP–Inv479-D911E) retains maltose binding activity (R.R.Isberg, unpublished observation). Therefore, the amino acids flanking D911, e.g. M912, could themselves be recognized by integrins directly or could influence recognition of D911 by maintaining this region in the proper conformation. Only three residues separate D911 from C907 and the disulfide bond

may place conformational constraints on a recognition site. Synthetic peptides based on the invasin sequence in this region, including cyclized peptides that could mimic the effect of the disulfide bond, do not inhibit invasin binding or mediate cell attachment themselves, indicating the importance of conformation (R.R.Isberg and J.M.Leong, unpublished observation).

Although this analysis clearly establishes the importance of the 903–913 region of invasin in cell attachment, it does not preclude the direct involvement of other regions of invasin. Indeed, the inability to detect cell adhesion by any invasin fragment smaller than the last 192 amino acids indicates that more than the 903–913 region is required for activity (Leong *et al.*, 1990) and several mutations that were isolated by enrichment on HEp-2 cells altered residues outside of this region (Figure 1). Several of these mutants diminished both invasin export and its avidity for integrin binding, as judged by the filter assay (Figure 2). Interestingly, recent work with MBP–invasin fusion proteins indicates that mutations affecting D811 alter the invasin cell binding phenotype (L.Saltman and R.Isberg, unpublished observation). Mutations at D811 destabilize native invasin and thus they would not be predicted to be isolated using the enrichment procedure described here. The importance of D811 may parallel results from analysis of integrin recognition of fibronectin, as, in addition to the fibronectin RGD-containing sequence, a site ~100 residues N-terminal to this sequence (the 'synergistic site') is involved in binding (Aota *et al.*, 1991; Bowditch *et al.*, 1994). Thus, although invasin and other β_1-chain integrin ligands lack discernible sequence homology, they may share similar recognition elements. Future analysis of integrin binding by invasin may provide further insight into important features of such elements.

Materials and methods

Media

Escherichia coli were grown in L broth, supplemented with 100 μg/ml ampicillin or 25 μg/ml kanamycin when appropriate. *Staphyloccus aureus*, Cowan 1, was grown in Penassay broth. HEp-2 cells (ATCC #CCl23) were maintained in RPMI 1640 supplemented with 5% newborn calf serum (Gibco-BRL, Bethesda, MD) at 37°C in 5% CO_2.

Internalization of bacteria into cultured mammalian cells

Penetration of bacteria into mammalian cells was quantitated by the gentamicin protection assay, as described previously (Leong *et al.*, 1990). Approximately 5×10^5 bacteria from a fresh overnight culture were added to nearly confluent monolayers of HEp-2 cells in 24-well culture dishes and allowed to penetrate cells for 2–3 h at 37°C. Survival after a 90 min treatment with 50 μg/ml gentamicin was determined by plating for viable counts on LB agar after lysing the HEp-2 cell monolayer in 0.5 ml of 0.5% Triton X-100.

Isolation, mapping and sequencing of inv mutations that decrease cell attachment and penetration

pJL272, which carries the *inv* gene, was randomly mutagenized and point mutations of invasin that decrease binding and penetration of HEp-2 cells were isolated as previously described (Leong *et al.*, 1993). Briefly, pJL272, which carries the *Y.pseudotuberculosis inv* gene was transformed into a mutator strain, KD1087 (*mutD5*; Fowler *et al.*, 1974) for random mutagenesis. *Escherichia coli* MC4100 Inv$^-$ (Kumamoto and Beckwith, 1983) harboring pJL272 were isolated from independently mutagenized pools by infecting HEp-2 cell monolayers and enriching for bacteria that could be recovered in the supernatant after gentle washing of the monolayer. In order to monitor the enrichments, a bacterial internalization assay was performed on the population of bacteria after every 10–20 cycles of enrichment, using the gentamicin protection assay as described above. When these pools of enriched bacteria gave internalization frequencies ~3- to 10-fold less than that for MC4100/pJL272 (which produces wild-type invasin), the HEp-2 cell monolayer enrichment was terminated. Most bacteria that survived this enrichment protocol no longer expressed full-length invasin on their surface. Therefore, magnetic beads coated with two different anti-invasin mAbs that recognize the C-terminus of invasin were used to enrich for bacteria that expressed full-length invasin (Leong *et al.*, 1993). The resulting population of bacteria were subjected to a second series of enrichments on HEp-2 cell monolayers. Single colonies were isolated and tested for the ability to penetrate HEp-2 cells by the gentamicin protection assay and for the production of full-length invasin by immunoblotting. Cell binding blots of gel-fractionated bacterial lysates were performed to assess adhesion promoted by each invasin mutant (Isberg and Leong, 1988).

Each mutation was mapped by exchanging restriction fragments between *inv*$^-$ and *inv*$^+$ plasmids and testing recombinants for the ability to mediate bacterial entry into HEp-2 cells, as described (Leong *et al.*, 1993). Convenient restriction sites allowed for mapping of mutations into seven intervals: 5′ to codon 206, codons 207–632, 633–711, 712–730, 731–902, 903–926 and 3′ to codon 927. After mapping, the DNA sequence of the relevant region of each mutant was determined by dideoxynucleotide chain termination (Sequenase kit, US Biochemical Corp.) on double-stranded plasmid by alkaline minilysis of 3.0 ml of fresh overnight culture of *E.coli* JM109 followed by purification on Magic Miniprep resin (Promega).

Site-directed mutagenesis of codons 903–914 of the inv gene

Two methods were used to make mutations in the region of *inv* surrounding C907. First, codons 903–915 of *inv* were mutagenized using gapped circle oligonucleotide-directed mutagenesis (Inouye and Inouye, 1987). A 56 base oligonucleotide corresponding to codons 898–915 was synthesized using doped reagents for codons 903–915, such that the mixture of nucleotides used to synthesize codons 903–915 consisted of 98.5% of the wild-type nucleotide and 0.5% each of the other three nucleotides. This oligonucleotide was used as a primer to fill in a gapped version of pJL272, derived as described (Inouye and Inouye, 1987). To specifically mutagenize codon D911, an analogous 56 base oligonucleotide primer was synthesized with random nucleotides at the three positions corresponding to codon 911.

The second method of site-directed mutagenesis has been previously described (Leong *et al.*, 1993). The region of *inv* located between the *Xho*I (codons 901–902) and *Mlu*I (codons 926–927) sites was replaced by a mutagenic double-stranded *Xho*I–*Mlu*I cassette generated from synthetic oligonucleotides by mutually primed synthesis (Hill *et al.*, 1986). RIJL-3, an oligonucleotide corresponding to the coding strand of codons 901–920 of the *inv* gene, was synthesized using doped reagents for codons 903–914, such that the mixture of nucleotides used to synthesize this region of the oligonucleotide consisted of 94% of the wild-type nucleotide and 2% each of the other three nucleotides. RIJL-4 is a 41 base oligonucleotide that corresponds to the wild-type sequence of the non-coding strand from codons 915 to 926. RIJL-3 and RIJL-4 were annealed and the single-stranded regions of the resultant partial duplex were converted to double-stranded DNA using Klenow fragment of DNA polymerase (New England Biolabs, Beverly, MA) according to the manufacturer's instructions. This mutagenic cassette was then cleaved with *Xho*I and *Mlu*I and used to replace the homologous fragment in pJL272. After transformation of the mutagenized plasmid into *E.coli* MC4100, 225 colonies were picked at random and the DNA sequence of the *inv* gene between the two restriction sites was determined. For several of these mutations, restriction fragment exchange experiments as described above confirmed that the mutation identified in the codon 903–913 interval was responsible for the Inv$^-$ phenotype.

Immunoblotting and cell binding filter assays of Inv$^-$ mutants

Standard immunoblotting (Towbin *et al.*, 1979) of bacterial extracts using anti-invasin mAbs was performed in order to confirm that full-length invasin was synthesized and to estimate the relative amount of invasin protein stably expressed. Bacteria from fresh overnight 3.0 ml L broth cultures were pelleted and lysed in 300 μl of SDS–PAGE sample buffer (Laemmli, 1970) at 37°C. Aliquots (0.5 μl) of each of these lysates was subjected to electrophoresis on a 7.5% polyacrylamide–SDS gel under non-reducing conditions, transferred to Immobilon filters and probed with the anti-invasin mAb 2A9-1, as described (Leong *et al.*,

1990). To assess cell binding activity, 1.5 µl was subjected to SDS–PAGE under non-reducing conditions, transferred to Immobilon filters and probed with live HEp-2 cells (Isberg and Leong, 1988; Leong *et al.*, 1990).

Quantitation of invasin epitope on the surface of E.coli

Surface expression of invasin was determined by mAb probing, using a modification of a method described previously (Leong *et al.*, 1990, 1991). Fresh overnight cultures of *E.coli* MC4100 harboring invasin-producing plasmids were diluted 1:100 into Luria broth and grown to mid-logarithmic phase (OD_{600} = 0.4–0.6) at 37°C. Cultures were chilled in ice water and the bacteria were washed twice in cold 150 mM NaCl, 2 mM $MgCl_2$, 20 mM HEPES, pH 7.0 (IF buffer) and resuspended in 1/20th of the original culture volume in IF buffer containing 1% bovine serum albumin (BSA), 3% normal goat serum and 0.5–1.0 µg/ml mAb. Bacteria and mAbs were incubated together for 2 h at room temperature with rocking. Bacteria were washed three times in cold IF buffer and resuspended in 1/20th of the original culture volume in IF buffer containing 1% BSA, 3% normal goat serum and TRITC-conjugated rabbit anti-mouse IgG (Zymed) diluted to 5 µg/ml. The secondary antibody incubation was allowed to proceed in the dark for 16 h at 4°C, with rocking. Bacteria were washed twice in 0.75 ml IF buffer and the OD_{600} of a 1:10 dilution was determined. The bacteria were pelleted and resuspended in a volume to give a theoretical OD_{600} of 3.5. The fluorescence associated with 0.1 ml of bacterial suspension was determined in a microtiter fluorimeter at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The fluorescence associated with *E.coli* MC4100/pJL272*inv*$^+$ was determined as a positive control in each experiment. Background fluorescence was determined by omitting primary anti-invasin mAb from this procedure and was subtracted from all experimental values to yield net fluorescence. The net fluorescence associated with MC4100/pJL272*inv*$^+$ was assigned a value of 1.0 and the net fluorescence associated with all other strains was expressed relative to this value.

Purification of MBP fusion proteins

pJL309 produces MBP–Inv479, a protein that contains the C-terminal 479 amino acids of invasin fused to the C-terminus of MBP (Leong *et al.*, 1990). pJL309-D911E and pJL309-M912I express MBP–Inv479-D911E and MBP–Inv479-M912I (MBP–invasin fusion proteins carrying the mutations D911E and M912I respectively) and were derived from pJL309 and the parental pJL272 plasmids containing the corresponding point mutations using standard techniques (Sambrook *et al.*, 1989). MBP–Inv479, MBP–Inv479-D911E and MBP–Inv479-M912I fusion proteins were over-expressed in *E.coli* SR2 (Kumamoto and Beckwith, 1983; Rankin *et al.*, 1992) and purified by affinity chromatography on cross-linked amylose as previously described (Maina *et al.*, 1988; Leong *et al.*, 1990).

pRI285 is derived from the MBP fusion vector pMAL-cRI (New England Biolabs, Beverly, MA) and contains an *inv* gene fragment encoding the C-terminal 497 amino acids fused to the *malE* gene. The expression of MBP–Inv497 from this plasmid is under the P_{tac} promoter control. pAM201 and pAM202 are derived from pRI285 and produce MBP–Inv497-D911A and MBP–Inv497-D911E respectively. Lysates of 500 ml cultures of *E.coli* SR2 strains harboring these plasmids were prepared as described previously (Leong *et al.*, 1990). The proteins were precipitated in 50% ammonium sulfate and resuspended in 50 mM NaCl, 150 µg/ml phenylmethylsulfonyl fluoride (PMSF), 10 mM Tris–HCl, pH 8.0, and dialyzed twice against 100 volumes of the same buffer. After centrifugation at 15 000 *g* for 10 min, the supernatants were loaded onto a DE52 ion exchange column in the above buffer and eluted with a linear gradient of NaCl (50–250 mM) corresponding to 5 column volumes. Fractions containing purified fusion proteins were identified by SDS–PAGE, pooled and stored as aliquots at −70°C. The concentration of purified proteins was determined by the bicinchoninic acid microassay (Pierce Biochemicals).

For some experiments, MBP–Inv497 was cleaved with Factor Xa (New England Biolabs, Beverly, MA) to yield MBP and the C-terminal fragment Inv497. MBP–Inv497 (350 µg) was cleaved with 10 µg of Factor Xa for 48 h at 15°C in 400 µl of buffer supplied by the manufacturer. The cleavage products were passed over a cross-linked amylose column and homogeneous Inv497 fragment was collected in the flow-through fractions.

Quantitation of mammalian cell binding by E.coli strains expressing invasin

Approximately 4×10^4 HEp-2 cells were cultured overnight at 37°C in a 5% CO_2 atmosphere on 22 mm circular glass coverslips in 24-well microtiter dishes. The monolayers were washed twice in phosphate-buffered saline (PBS) and covered with 1 ml of binding buffer (RPMI 1640 supplemented with 0.4% BSA and 20 mM HEPES, pH 7.0). Saturated bacterial cultures were diluted 1:10 in M9 salts and 40 µl of this dilution were added to each of four wells. Microtiter plates were rotated for 10 min then incubated for 60–90 min at 37°C in a 5% CO_2 atmosphere. The monolayers were washed 5–7 times with PBS to remove unbound bacteria, fixed in methanol for 10 min at room temperature, dried and stained in 0.4% modified Giemsa (Sigma Chemical Co., St Louis, MO) diluted 1:40 in H_2O for 60 min at room temperature. The coverslips were mounted and individual bacteria associated with the cells were counted under light microscopy. The binding observed was expressed as the number of associated bacteria per mammalian cell after observation of 50–150 mammalian cells per cover slip for each of 2–4 cover slips (total of at least 200 mammalian cells observed for each strain).

HEp-2 cell binding to microtiter wells coated with purified invasin derivatives

The coating concentrations of invasin required to give half-maximal HEp-2 cell binding was determined as previously described (Leong *et al.*, 1990). To confirm that the microtiter well coating efficiency was equivalent for each of the proteins tested, parallel wells coated in an identical fashion were probed with the anti-invasin mAb 3A2-1, which recognizes the C-terminal 192 amino acids of invasin, and bound antibody was quantitated using standard ELISA (Leong *et al.*, 1991).

Purification of integrin $\alpha_5\beta_1$

Integrin $\alpha_5\beta_1$ was purified from human placenta by invasin affinity chromatography using a modification of a previously described procedure (Tran Van Nhieu and Isberg, 1994). MBP–Inv497 was linked to Affi-Gel 10 agarose beads (Bio-Rad Laboratories, Hercules, CA) in 80 mM $CaCl_2$, 30 mM HEPES, pH 8.0, for 16 h at 4°C with rocking, to give a final concentration of 1.8 mg MBP–Inv/ml resin. Fresh placental tissue was stored in ~50 g aliquots at −70°C. Placenta (100 g) was thawed on ice, added to 275 ml of homogenization buffer (HB; 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.25 mM $CaCl_2$, 10^{-2} TIU/ml aprotinin, 25 mM HEPES, pH 7.8) and homogenized in a Waring blender. PMSF was immediately added to a final concentration of 1 mM. The homogenate was centrifuged at 15 000 *g* for 30 min and the pellet (~50 g) was resuspended in 50 ml of HB. An equal volume (100 ml) of extraction buffer (HB + 100 mM octyl-β-glucopyranoside) was added and the extraction proceeded for 80 min at 4°C with rocking. After centrifugation at 15 000 *g* for 30 min, the pellet was discarded and the supernatant was subjected to a second centrifugation at 350 000 *g* for 30 min. The supernatant from this second centrifugation was loaded onto a 10 ml MBP–Inv497–agarose column, prepared as described above. The column was washed with 100 ml of wash buffer (HB + 0.1% NP-40) and invasin receptor was eluted in wash buffer + 10 mM EDTA. $MgCl_2$ was immediately added to each elution fraction to a final concentration of 20 mM and 1 M Tris, pH 9.3, was added to a final concentration of 8 mM. Fractions were analyzed by SDS–PAGE and those that contained receptor were pooled and concentrated by affinity chromatography on a 1 ml wheatgerm agglutinin (WGA)–agarose column (Vector Labs; Pytela *et al.*, 1987). The WGA–agarose column was washed in HB + 0.1% NP-40 and receptor was eluted in HB + 0.1% NP-40 containing 5% (w/v) *N*-acetylglucosamine. Fractions containing receptor were stored as aliquots at −80°C.

Determination of IC_{50}s for purified invasin derivatives

Invasin binding of purified integrin $\alpha_5\beta_1$ was measured using a modification of an enzyme-linked immunosorbent assay previously developed for fibronectin (Hautanen *et al.*, 1989). Linbro ELISA plates (96-well; Flow Laboratories) were coated with 3 µg/ml Inv497 (cleaved from MBP–Inv497) in 125 mM NaCl, 25 mM HEPES, pH 7.0, at 4°C for 16 h. Wells were washed three times in PBS, then blocked with 3.5% BSA in PBS for 2 h at 20°C with rocking. Wells were probed with 40 µl of 2 µg/ml integrin $\alpha_5\beta_1$ in 125 mM NaCl, 1 mM $MnCl_2$, 20 mM octyl-β-glucopyranoside, 25 mM HEPES, pH 7.0, in the presence of increasing amounts of competitor protein in the identical buffer for 3 h at 20°C with rocking. (In pilot experiments, 2 µg/ml of $\alpha_5\beta_1$ was found to give approximately half-maximal binding.) Wells were washed three times with 125 mM NaCl, 1 mM $MnCl_2$, 25 mM HEPES, pH 7.0, 0.1% NP-40 to remove unbound receptor and then fixed in 3% para-formalde-hyde, 2 mM $MnCl_2$, 0.1% NP-40 in PBS for 30 min–16 h at 20°C. Wells were washed three times in PBS, 0.2% Tween-20 and bound integrin $\alpha_5\beta_1$ was revealed by standard ELISA using anti-fibronectin receptor antiserum (Telios Pharmaceuticals, San Diego, CA) and alkaline

phosphatase-conjugated goat anti-rabbit IgG antibody (Promega; Leong *et al.*, 1990).

Two dimensional gel electrophoresis of reduced partial protease products

A disulfide bond between Cys907 and Cys982 of invasin was demonstrated by two dimensional gel electrophoresis using a modification of a previously described technique (Leong *et al.*, 1993). MBP–Inv fusion protein (25 μg) in 25 μl of 10 mM Tris (pH 8.0), 50 mM NaCl, 0.2% SDS was boiled for 2 min and equilibrated at 20°C for 10 min. Two microliters of 50 μg/ml V8 protease (Boehringer Mannheim) was then added and the digestion was allowed to proceed for 30 min at 37°C. Eleven microliters of 4× SDS-PAGE sample buffer (Laemmli, 1970) supplemented with 5 μg/ml 3,4-dichloro-isocoumarin but lacking reducing agent was added and the samples were boiled for 10 min before being loaded on three lanes of a 10% non-reducing SDS gel. After electrophoresis, one of the lanes was stained with Coommassie blue, to determine the extent of proteolysis, while the other two lanes were processed as separate samples for analysis on second dimension gels. For the non-reduced control sample, one of these lanes was immersed in a tube containing 4% SDS, 5 μg/ml 3,4-dichloro-isocoumarin and 125 mM Tris, pH 6.8. To analyze the effects of disulfide bond reduction, the other lane was immersed in the same buffer containing 2% mercaptoethanol. After soaking for 2 h at room temperature, the two samples were boiled for 10 min and laid lengthwise over separate 10% SDS–polyacrylamide gels and fractionated by electrophoresis. The reduced sample was fractionated in the presence of 0.02% mercaptoethanol in the top reservoir buffer. After electrophoresis, the gels were transferred to Immobilon filters (Towbin *et al.*, 1979) and probed with 0.5 μg/ml mAb 2A9-1, which recognizes fragments containing residues 711–803 of invasin (Leong *et al.*, 1991). Immunoprobed products were detected as previously described (Leong *et al.*, 1991).

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References

Aota,S.I., Nagai,T. and Yamada,K. (1991) *J. Biol. Chem.*, **266**, 15938–15943.

Argraves,W.S., Pytela,R., Suzuki,S., Millan,J.L., Pierschbacher,M.D. and Ruoslahti,E. (1986) *J. Biol. Chem.*, **261**, 12922–12924.

Barker,P. *et al.* (1992) *J. Med. Chem.*, **35**, 2040–2048.

Beachey,E.H. (1981) *J. Infect. Dis.*, **143**, 325–345.

Bliska,J., Copass,M. and Falkow,S. (1993a) *Infect. Immun.*, **61**, 3914–3921.

Bliska,J., Galan,J. and Falkow,S. (1993b) *Cell*, **73**, 903–920.

Bowditch,R., Hariharan,M., Tominna,E., Smith,J., Yamada,K., Getzoff,E. and MH,G. (1994) *J. Biol. Chem.*, **269**, 10856–10863.

Corbi,A.L., Miller,L.J., O'Connor,K., Larson,R.S. and Springer,T.A. (1987) *EMBO J.*, **6**, 4023–4028.

Courtney,H.S., Simpson,W.A. and Beachey,E.H. (1983) *J. Bacteriol.*, **153**, 763–770.

D'Souza,S.E., Ginsberg,M.H. and Plow,E.F. (1991) *Trends Biochem. Sci.*, **16**, 246–250.

Falkow,S., Isberg,R. and Portnoy,D. (1992) *Annu. Rev. Cell Biol.*, **8**, 333–363.

Finlay,B.B. and Falkow,S. (1989) *Microbiol. Rev.*, **53**, 210–230.

Flock,J.I. *et al.* (1987) *EMBO J.*, **6**, 2351–2357.

Fowler,R., Degnen,G. and Cox,E. (1974) *Mol. Gen. Genet.*, **133**, 179–191.

Grutzkau,A., Hanski,C., Hahan,H. and Riecken,E. (1990) *Gut*, **31**, 1011–1015.

Guan,J.-L. and Shalloway,D. (1992) *Nature*, **358**, 690–693.

Hanski,C., Kutschka,U., Schmoranzer,H., Naumann,M., Stallmach,A., Hahn,H., Menge,H. and Riecken,E. (1989) *Infect. Immun.*, **57**, 673–678.

Hautanen,A., Gailit,J., Mann,D.M. and Ruoslahti,E. (1989) *J. Biol. Chem.*, **264**, 1437–1442.

Hill,D.E., Hope,I.A., Macke,J.P. and Struhl,K. (1986) *Science*, **234**, 451–457.

Hoepelman,A.I.M. and Tuomanen,E.I. (1992) *Infect. Immun.*, **60**, 1729–1733.

Hynes,R.O. (1992) *Cell*, **69**, 11–27.

Inouye,F. and Inouye,M. (1987) *DNA and RNA Synthesis*. Academic Press, New York, NY, pp. 181–206.

Isberg,R.R. (1990) *Mol. Biol. Med.*, **7**, 73–82.

Isberg,R.R. (1991) *Science*, **252**, 934–938.

Isberg,R.R. and Leong,J.M. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 6682–6686.

Isberg,R.R., Voorhis,D.L. and Falkow,S. (1987) *Cell*, **50**, 769–778.

Kornberg,L., Earp,H.S., Turner,C.E., Prockop,C. and Juliano,R.L. (1991) *Proc. Natl Acad Sci. USA*, **88**, 8392–8396.

Kumamoto,C.A. and Beckwith,J. (1983) *J. Bacteriol.*, **154**, 253–260.

Laemmli,U.K. (1970) *Nature*, **227**, 680–685.

Lawler,J., Weinstein,R. and Hynes,R.O. (1988) *J. Cell. Biol.*, **107**, 2351–2361.

Leong,J.M., Fournier,R. and Isberg,R.R. (1990) *EMBO J.*, **9**, 1979–1989.

Leong,J.M., Fournier,R.S. and Isberg,R.R. (1991) *Infect. Immun.*, **59**, 3424–3433.

Leong,J., Morrissey,P. and Isberg,R. (1993) *J. Biol. Chem.*, **268**, 20524–20532.

Loftus,J.C., O'Toole,T.E., Plow,E.F., Glass,A., Frelinger,A.L.I. and Ginsberg,M.H. (1990) *Science*, **249**, 915–918.

Maina,C.V., Riggs,P.D., Grandea,A.G.I., Slatko,B.E., Moran,L.S., Tagliamonte,J.A., McReynolds,L.A. and Guan,C. (1988) *Gene*, **74**, 365–373.

Miller,V.L. and Falkow,S. (1988) *Infect. Immun.*, **56**, 1242–1248.

Moulder,J.W. (1985) *Microbiol. Rev.*, **49**, 298–337.

O'Neil,K.T., Hoess,R.H., Jackson,S.A., Ramachandran,N., Mousa, S.A. and DeGrado,W.F. (1992) *Proteins*, **14**, 509–515.

Osborn,L., Vassallo,C. Browning,B., Tizard,R., Haskard,D., Benjamin,C., Dougas,I. and Kirchhausen,T. (1994) *J. Cell. Biol.*, **124**, 601–608.

Pepe,J.C. and Miller,V.L. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 6473–6477.

Pierschbacher,M.D. and Ruoslahti,E. (1987) *J. Biol. Chem.*, **262**, 17294–17298.

Pytela,R., Pierschbacher,M.D., Argraves,S., Suzuki,S. and Ruoslahti,E. (1987) *Methods Enzymol.*, **144**, 475–89.

Rankin,S., Isberg,R. and Leong,J. (1992) *Infect. Immun.*, **60**, 683–686.

Relman,D., Tuomanen,E., Falkow,S., Golenbock,D.T., Saukkonen,K. and Wright,S.D. (1990) *Cell*, **61**,1375–1382.

Ruoslahti,E. and Pierschbacher,M.D. (1987) *Science*, **238**, 491–497.

Samanen,J. *et al.* (1991) *J. Med. Chem.*, **34**, 3114–3124.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (eds) (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schwartz,M., Lechene,C. and Ingber,D. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 7849–7853.

Simonet,M., Richard,S. and Berche,P. (1990) *Infect. Immun.*, **58**, 841–845.

Springer,T.A. (1990) *Nature*, **346**, 425–34.

Towbin,H., Staeheli,T. and Gordon,J. (1979) *Proc. Natl Acad. Sci. USA*, **76**, 4350–4354.

Tran Van Nhieu,G. and Isberg,R.R. (1991) *J. Biol. Chem.*, **266**, 24367–24375.

Tran Van Nhieu,G. and Isberg,R. (1994) *Methods Enzymol.*, **236**, 307–318.

Tuckwell,D., Brass,A. and Humphries,M. (1992) *Biochem. J.*, **285**, 325–331.

Van de Water,L., Destree,A.T. and Hynes,R.O. (1983) *Science*, **220**, 201–204.

Vonderheide,R., Tedder,T., Springer,T. and Staunton,D. (1994) *J. Cell. Biol.*, **125**, 215–222.

Yang,Y. and Isberg,R.R. (1993) *Infect. Immun.*, **61**, 3907–3913.

Young,V., Miller,V., Falkow,S. and Schoolnik,G.K. (1990) *Mol. Microbiol.*, **4**, 1119–1128.

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