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Identification of the integrin binding domain of the *Yersinia pseudotuberculosis* invasin protein

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The invasin protein of the pathogenic *Yersinia pseudotuberculosis* mediates entry of the bacterium into cultured mammalian cells by binding several β_1 chain integrins. In this study, we identified the region of invasin responsible for cell recognition. Thirty-two monoclonal antibodies directed against invasin were isolated, and of those, six blocked cell attachment to invasin. These six antibodies recognized epitopes within the last 192 amino acids of invasin. Deletion mutants of invasin and maltose-binding protein (MBP) – invasin fusion proteins were generated and tested for cell attachment. All of the invasin derivatives that carried the carboxyl-terminal 192 amino acids retained cell binding activity. One carboxyl-terminal invasin fragment and seven MBP – invasin fusion proteins were purified. The purified derivatives that retained binding activity inhibited bacterial entry into cultured mammalian cells. These results indicated that the carboxyl-terminal 192 amino acids of invasin contains the integrin-binding domain, even though this region does not contain the tripeptide sequence Arg – Gly – Asp.

Key words: bacterial invasiveness/integrin receptors/intracellular pathogen/invasin/*Yersinia*

Introduction

Entry into the host mammalian cell is an important step in the course of infection for many microbial pathogens (Devenish and Shiemann, 1981; Formal *et al.*, 1983; Sansonetti *et al.*, 1986). Obligate intracellular pathogens, such as chlamydia and rickettsia, must invade the host cell in order to replicate (for review, see Moulder, 1985). Facultative intracellular pathogens may also find the intracellular environment favorable for growth, or use their ability to enter into host cells in order to spread from one body compartment to another (Takeuchi, 1967). One such example is the enteric bacterium *Yersinia pseudotuberculosis*. Like most other enteric pathogens, it is characteristically acquired by ingestion of contaminated foodstuff (Brubaker, 1972; Gemski *et al.*, 1980). The bacteria gain access to the lamina propria from the intestinal lumen by entering into and passing through intestinal epithelial cells (Lian *et al.*, 1987; Une, 1977). They can then spread via the lymphatic system to the liver and spleen, where multiplication occurs (Richardson and Harkness, 1971).

Y.pseudotuberculosis is particularly useful for studying cellular entry because it efficiently enters mammalian cells in culture (Bovallius and Nilsson, 1975; Carter, 1975). This tissue culture model has been used to identify three independent pathways for cellular penetration that enteropathogenic *Yersinia* utilize (for reviews, see Miller *et al.*, 1988; Isberg, 1989). First, the *Y.pseudotuberculosis inv* gene was identified by its ability to convert the normally non-invasive *Escherichia coli* K12 into an efficient invader of mammalian cells in culture (Isberg and Falkow, 1985). Using a similar approach, Miller and Falkow (1988) isolated a second chromosomal gene, termed *ail*, from the related species *Y.enterocolitica* that mediates surface attachment to many cell lines, as well as entry into a subset of these lines. Finally, studies of an *inv*$^-$ mutant of *Y.pseudotuberculosis* has revealed yet another pathway for cellular entry. In contrast to the chromosomally-encoded *inv* and *ail* genes, this pathway is mediated by the large virulence plasmid found in all clinical isolates of *Yersinia* (Portnoy and Falkow, 1981; Bolin *et al.*, 1982; Perry *et al.*, 1986; Isberg, 1989). These multiple pathways for entry could explain why a *Y.pseudotuberculosis inv*$^-$ mutant remains virulent in the mouse model, although it has a delayed time course for mouse fatality when the animals are infected orally (Rosqvist *et al.*, 1988).

The *inv*-mediated pathway is a well-characterized system for analyzing bacterial entry into mammalian cells. *Inv* encodes a 103 kd protein, called invasin, that has been shown to be localized in the outer membrane (Isberg *et al.*, 1987). Invasin has the property of tightly binding cultured mammalian cells (Isberg and Leong, 1988) and recognizes multiple β_1 chain integrins (Isberg and Leong, 1990). This integrin receptor family is a group of related heterodimeric proteins that promote cell attachment to extracellular matrix proteins (for review, see Hynes, 1987; Ruoslahti, 1988). For many integrin receptors, a tripeptide sequence Arg – Gly – Asp present in the ligand molecule has been demonstrated to be critical in receptor recognition (Pytela *et al.*, 1987; Ruoslahti and Pierschbacher, 1987). For example, fibronectin and collagen, which are ligands for β_1 receptors, contain Arg – Gly – Asp sequences, and synthetic peptides containing this sequence can inhibit cell binding to these proteins (Pierschbacher *et al.*, 1983; Pierschbacher and Ruoslahti, 1984; Dedhar *et al.*, 1987).

The fibronectin receptor has been shown to also bind cytoplasmic proteins that may interact with actin filaments (Horwitz *et al.*, 1986; Argraves *et al.*, 1989) and it has been postulated that a general function of integrin receptors is to link the extracellular environment to the cytoskeleton. By mimicking host β_1 chain ligands, *Y.pseudotuberculosis* may be utilizing the cellular machinery that is capable of initiating the cytoskeletal events necessary for bacterial internalization. It is unclear, however, exactly how invasin mimics a natural host ligand: there is no Arg – Gly – Asp sequence present in the protein and no obvious candidate for an integrin binding sequence. Therefore, to better understand how invasin recognizes β_1 chain integrins and to begin to define

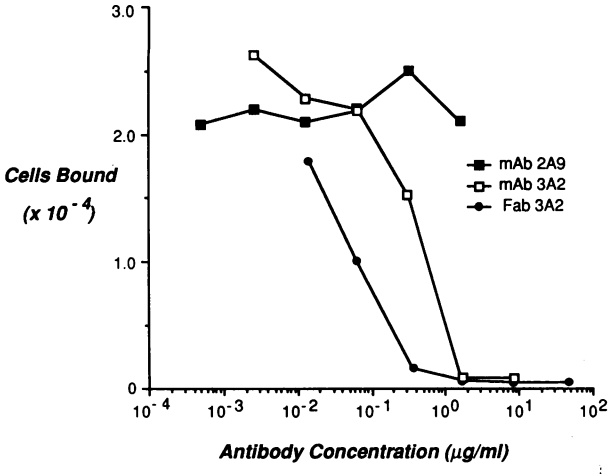

Fig. 1. Inhibition of HEp-2 cell binding by anti-invasin mAb. Wells coated with 0.4 μg/ml invasin fragment InvΔ53C, a carboxyl-terminal fragment of invasin that retains cell-binding activity (see Figure 6). All blocking mAbs also blocked cell attachment to full-length invasin (data not shown). mAbs were then incubated for 30 min prior to the addition of HEp-2 cells. After allowing for attachment at 37°C, wells were washed with PBS and bound cells were quantitated using PNP-NAG, the substrate for the lysosomal enzyme hexosaminidase (see Materials and methods). Each point represents the average of at least two determinations. mAb 3A2 is a blocking mAb; Fab 3A2 is the Fab fragment derived from mAb 3A2; mAb 2A9 is an anti-invasin mAb that does not block binding by HEp-2 cells and recognizes an epitope in the central portion of the protein.

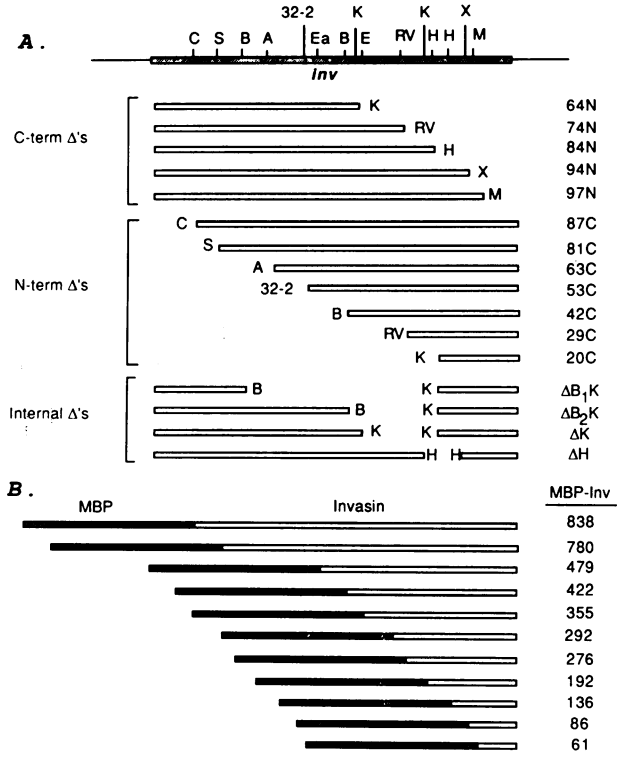

Fig. 2. Invasin derivatives generated. The *inv* gene is shown schematically with the restriction sites that were used to generate the deletions and fusion proteins: C, *Cla*I; S, *Sna*BI; B, *Bss*HII; A, *Acc*I; 32-2, the site of a *Hin*dIII linker in an N-terminal deletion derivative of *inv* generated by Bal 31 digestion (Isberg *et al.*, 1987); Ea, *Eag*I; K, *Kpn*I; B, *Bst*YI; E, *Eco*0109; RV, *Eco*RV; H, *Hpa*I; X, *Xho*I; M, *Mlu*I. (**A**) Set of *inv* deletions analyzed. The restriction site used for each deletion is designated by the letter at the deletion endpoint. Our designation of each protein fragment is given at right. The number in each designation corresponds to the approximate molecular weight (in kd) for each fragment; the N or C preceding the number denotes the intact end of the invasin fragment produced. The four internal deletions are in-frame. (**B**) Set of MBP–invasin fusion proteins analyzed. The MBP portion of each hybrid is symbolized by the shaded bar; the invasin portion is symbolized by the open bar. The number of amino acids in the invasin-derived portion of each hybrid is given at right.

which peptide sequences are required for cellular entry we have identified the region of invasin that interacts with its receptors on HEp-2 cells.

Results

Identification of monoclonal antibodies (mAbs) that block cell attachment

Two approaches were used to identify the region of invasin protein responsible for cell attachment. First, a bank of mAbs was generated against invasin and those mAbs that blocked cell attachment were identified. The epitopes recognized by these mAbs were mapped using deletion derivatives of invasin and maltose-binding protein (MBP)–invasin fusion proteins. Second, the invasin fragments and fusion proteins were directly tested for their ability to bind mammalian cells.

We showed previously that mammalian cells bound to microtiter wells coated with invasin (Isberg and Leong, 1988). We generated a set of 32 mAbs directed against invasin and screened for those that could inhibit cell attachment in this assay. Those mAbs that inhibit binding may recognize epitopes at or near the site of invasin that interacts with mammalian cells. Six of the 32 hybridomas secreted mAbs that efficiently blocked attachment to invasin in the microtiter well assay. An example of one blocking mAb, mAb 3A2, is shown in Figure 1. Blocking was independent of the Fc portion of the antibody and of the bivalent nature of these IgG molecules, since the Fab fragment purified from mAb 3A2 blocked attachment at least as efficiently as intact mAb (Figure 1).

Blocking mAbs recognize epitopes in the carboxyl-terminal 192 amino acids of invasin

To map the epitopes recognized by the blocking mAbs, invasin and various deletion derivatives were overproduced using the phage T7 promoter as described (Tabor and Richardson, 1985). One such plasmid derivative, pRI253, contained the entire *inv* gene and results in synthesis of full-length invasin as well as many smaller species of the protein (Isberg and Leong, 1988; see Figure 4A). Multiple species were also observed in *Y.pseudotuberculosis* and in *E.coli* when the *inv* gene was not overexpressed (Isberg and Leong, 1988). The relationship of the smaller species to full-length invasin is complex and their biological significance is unknown.

In order to produce invasin fragments lacking internal or carboxyl-terminal segments of the protein, internal deletions and 4 bp insertion mutations were generated in the *inv* gene in pRI253 using standard techniques (Materials and methods). Derivatives containing only the carboxyl-terminal portion of invasin were constructed in a similar fashion in T7 promoter vectors that ensure high level expression (Materials and methods). Each plasmid constructed was tested for the production of a protein of the predicted molecular weight by pulse-labeling with [^{35}S]Met after thermal induction or by using immunoblot analysis probing with a mixture of mAbs directed against invasin. Proteins

Table I. The carboxyl-terminus of invasin binds mammalian cells

Plasmid	Fragment produced[a]	Deleted amino acids	Cell-binding activity		Relative surface exposure[d]	Percent invasion[e]
			filter assay[b]	microtiter assay[c]		
pRI253	WT(103 kd)	none	+++	+++	1.0	1.5
pJL207	InvΔ97N	928–986	–	–	1.1	7.6×10^{-4}
pJL201	InvΔ94N	902–986	–	–	0.9	1.0×10^{-3}
pJL258	InvΔ84N	804–986	ND[f]	ND	1.0	6.3×10^{-4}
pJL257	InvΔ74N	711–986	ND	ND	1.1	3.3×10^{-4}
pJL256	InvΔ64N	608–986	ND	ND	1.4	4.3×10^{-4}
pJL213	InvΔ87C	1–148	+++	+++	0.0	1.5×10^{-3}
pJL217	InvΔ81C	1–206	+++	+++	0.0	1.0×10^{-4}
pJL216	InvΔ63C	1–370	+++	+++	0.0	4.3×10^{-4}
pJL225	InvΔ53C	1–464[g]	+++	+++	0.0	3.0×10^{-4}
pJL229	InvΔ42C	1–564	+++	+++	0.0	9.3×10^{-4}
pJL228	InvΔ29C	1–710	+++	+++	0.0	4.6×10^{-4}
pJL222	InvΔ20C	1–794	–	–	ND	1.3×10^{-3}
pJL205	ΔB_1K	276–794	–	–	0.0	1.6×10^{-3}
pJL204	ΔB_2K	565–794	+	–	0.0	2.5×10^{-4}
pJL202	ΔK	608–794	++	+	0.2	2.4×10^{-3}
pJL206	ΔH	803–849	–	–	1.2	1.2×10^{-3}
pT7-12	none		–	–	0.0	2.8×10^{-4}

[a]For amino-terminal and carboxyl-terminal deletions the number in each designation corresponds to the approximate molecular weight of the invasin fragment. The letter in each designation indicates which terminus of invasin is present. For example, 'InvΔ97N' is a 97 kd fragment containing the amino-terminus of invasin, but missing the carboxyl-terminus (see Figure 2). Deletions designated by 'Δ' are internal deletions.
[b]Cell-binding activity after SDS–PAGE and transfer to a filter membrane: '+++' = activity indistinguishable from full-length invasin; '++' = activity easily detectable, but less than full-length invasion; '+' = activity detectable only with large amounts of extract (10- to 50-fold more than full-length invasin); '–' = no detectable activity.
[c]Cell-binding activity when extracts were linked to microtiter wells: '+++' = activity indistinguishable from full-length invasin; '+' = activity detectable, with ~10- to 20-fold fewer HEp-2 cells bound at maximum concentration; '–' = no detectable activity.
[d]Surface exposure was determined using indirect immunofluorescence using mAb directed against the carboxyl-terminus of invasin (see Materials and methods). *E.coli* MC1000 harboring the plasmid pGP1 encoding T7 DNA polymerase and *inv* deletion plasmids were grown in the absence of induction. Fluorescence was quantitated using a fluorescence spectrophotometer, correcting for cell number determined by OD_{600}. The corrected values shown in table are expressed relative to the fluorescence of bacteria harboring pRI253, defined as 1.0 (Materials and methods).
[e]HEp-2 cells (2×10^5) were infected with 2×10^7 *E.coli* 71/18 harboring the *inv* deletion plasmid (Materials and methods). Percent invasion was calculated as the percentage of bacteria that survive gentamicin treatment and is the average of two values (Isberg *et al.*, 1987). Amino-terminal deletions presumably abolish invasiveness by preventing surface localization; carboxy-terminal deletions disrupt cell-binding activity.
[f]Not determined.
[g]Approximate deletion end-point (Materials and methods).

cross-reactive with invasin of the predicted molecular weight were produced for all of the plasmids used (Figure 4A). As is true for full-length invasin, multiple invasin-related species were observed for each overproduced fragment. The set of amino-terminal, carboxyl-terminal and internal deletions used in this study is shown in Figure 2A and listed in Table I.

Small carboxyl-terminal fragments of invasin were unstable, even in a protease-deficient strain. In order to map epitopes in this region, as well as to evaluate the cell-binding properties of carboxyl-terminal peptides, restriction fragments of the *inv* gene encoding the carboxyl-terminus of invasin were fused to the *E.coli malE* gene. The product of the *malE* gene, MBP, is a periplasmic protein that is involved in the utilization of maltose and can bind to a cross-linked amylose matrix, allowing easy purification after elution with maltose (Ferenci and Klotz, 1978; Kellerman and Ferenci, 1982; Guan *et al.*, 1988). Riggs and co-workers have constructed vectors to easily generate gene fusions to the 3′ end of *malE* under the control of an inducible promoter (Maina *et al.*, 1988). Plasmids were constructed that encoded virtually full-length MBP fused to varying amounts (61–880 amino acids) of the carboxyl-terminus of invasin (Figure 2B). Immunoblotting techniques using anti-invasin mAb or anti-MBP antiserum were used to confirm that the appropriate fusion protein was generated in each case (see Figure 7A). In spite of evidence of some protease degradation, even those hybrids that contain only small portions of invasin were easily detected.

We tested each mAb for its ability to recognize invasin fragments and MBP–invasin fusion proteins using immunoblotting. An immunoblot for the mAb 3A2 is shown in Figure 3. The epitopes for all of the blocking antibodies were contained within the last 192 amino acids of invasin. None of the blocking mAbs recognized MBP–Inv136 or InvΔ97N, the 59 amino acid carboxyl-terminal deletion of invasin (Figure 3). These results implicated the carboxyl-terminal region of invasin in cell attachment.

The carboxyl-terminus of invasin is required for cell binding activity

Sonicated cell extracts containing deletion derivatives of invasin were tested for cell attachment activity using a membrane filter assay as previously described (Isberg and Leong, 1988). Proteins were subjected to SDS–PAGE, transferred to a filter membrane and the filter probed with live dispersed HEp-2 cells. Staining of bound cells revealed bands on the filter due to the presence of cell binding proteins. Deletions of as few as 59 amino acids from the carboxyl-terminus of invasin completely abolished binding activity (Figure 4B, 97N; Table I, pJL207). In contrast, all of the amino-terminal deletions that could be stably overproduced retained binding activity. Most notably, the 29 kd

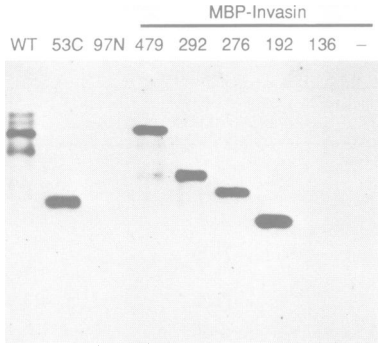

Fig. 3. Blocking mAbs recognize epitopes in the carboxyl-terminal 192 amino acids of invasin. Deletion derivatives of invasin and MBP–invasin fusion proteins were subjected to SDS–PAGE, transferred to Immobilon filters and probed using the blocking mAb 3A2. Lanes: 'WT', invasin-containing cell extract prepared from SW5AA2Q(pRI253*inv*$^+$); '53C', purified InvΔ53C; '97N', InvΔ97N-containing cell extract from SW5AA2Q(pJL207); 'MBP–invasin', purified MBP–invasin hybrids–the number at the top of each lane indicates the number of residues derived from the carboxyl terminus of invasin that are present in the hybrid and '–' denotes purified MBP alone.

carboxyl-terminal fragment InvΔ29C, which is deleted for the first 710 residues of invasin, still efficiently bound HEp-2 cells (Figure 4, 29C; Table I, pJL228). Two internal deletions implicated a smaller region of invasin that could bind animal cells. The internally deleted invasin protein, InvΔK, missing residues 608–794, bound HEp-2 cells, albeit much less efficiently than full-length invasin (Figure 4B, ΔK; Table I, pJL202). The deletion InvΔB_2K, lacking residues 565–794, also bound animal cells, but even less efficiently than InvΔK (Figure 4B, ΔB_2K; Table I, pJL204). The fact that these deletions retained some activity, combined with the result that InvΔ29C bound mammalian cells, indicated that only the last 192 amino acids (residues 795–986) were absolutely required for binding β_1 chain integrins. This is also consistent with analysis of fusion proteins carrying this sequence (see below). No invasin derivatives that were deleted in this 192 amino acid region, such as InvΔH, which is missing residues 803–849, had detectable cell binding activity (Figure 4, ΔH; Table I, pJL206). Not all invasin derivatives that contain the last 192 amino acids were able to mediate binding, since InvΔB_1K and InvΔ20C are binding-defective (Table I, pJL205 and pJL222). This result and the observation that the binding activities of InvΔK and InvΔB_2K are lost upon cycles of freezing and thawing (data not shown) suggest that the conformation of the binding domain may be critical for activity.

The cell attachment activity of overproduced fragments of invasin was also assessed using the cell attachment assay in microtiter wells (Isberg and Leong, 1988). Microtiter wells were coated with cell extracts containing invasin fragments, and dispersed HEp-2 cells were incubated in these wells (Materials and methods). Analysis of invasin fragments using this assay gave results entirely consistent with those obtained using the filter assay (Table I). Fragments containing the carboxyl-terminal 29 kd region retained binding activity, whereas deletion of the extreme carboxyl-terminus eliminated cell attachment. Consistent with the filter assay, the internal deletion fragment InvΔK retained ~10% binding activity compared to full-length invasin (data not shown). Cell binding by the internally deleted invasin fragment

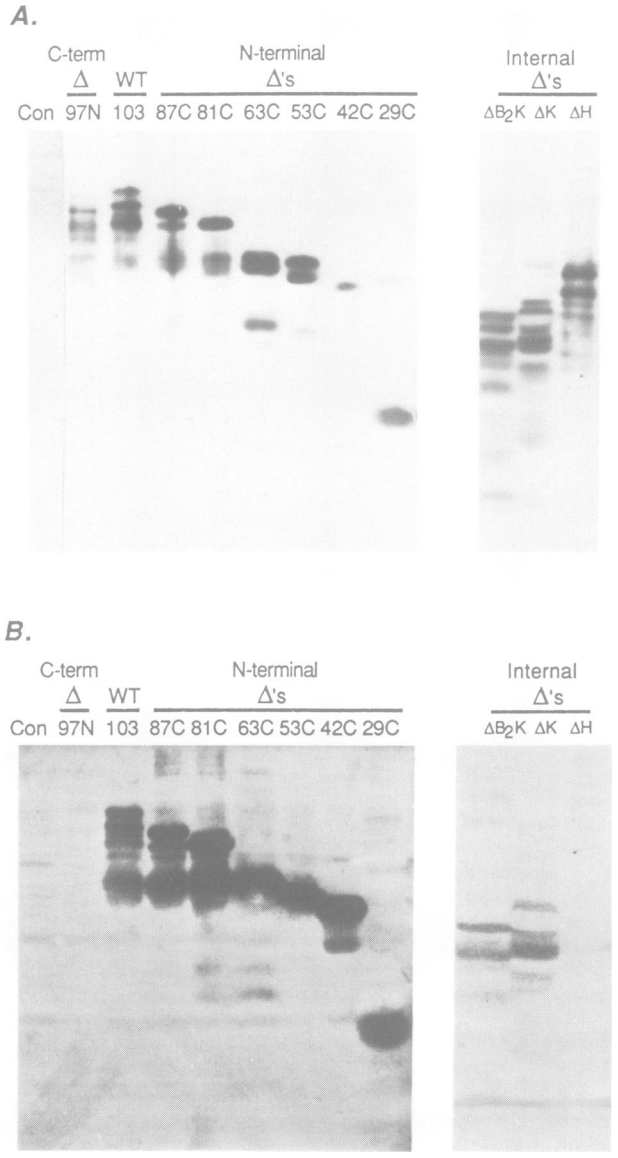

Fig. 4. Fragments containing the carboxyl-terminus of invasin bind animal cells. Sonicated cell extracts were subjected to electrophoresis on an 8% polyacrylamide SDS gel and transferred to Immobilon filters. Numbers above each lane give the approximate molecular weight (in kd) of the invasin species for full-length invasin (WT), the carboxyl-terminal and amino-terminal deletions. The internal deletions InvΔK, InvΔB_2K and InvΔH are in-frame deletions shown schematically in Figure 1A. Control extract, from identically-treated bacteria that carry pGP1 and a pT7-12, a promoter plasmid with no insert, was used in lanes marked Con. **Panel A**: immunoblot. The filter was probed with a mixture of mAbs directed against invasin in order to detect all invasin species. **Panel B**: cell-binding assay. The filter was probed with live HEp-2 cells and bound cells were stained with amido black. Ten-fold more extract was used in cell-binding experiments than was used for immunoblotting, except in the case of internal deletions InvΔK and InvΔB_2K: 50-fold and 100-fold more extract was needed to detect cell-binding by InvΔK and InvΔB_2K, respectively.

InvΔB_2K could not be demonstrated in the microtiter well assay, reflecting the lower sensitivity of this assay compared to the filter assay.

To confirm that the cell binding activity in the microtiter well assay was due to invasin derivatives and to obtain a quantitative estimate of that activity, the 53 kd carboxyl-terminal fragment of invasin, termed InvΔ53C, was purified

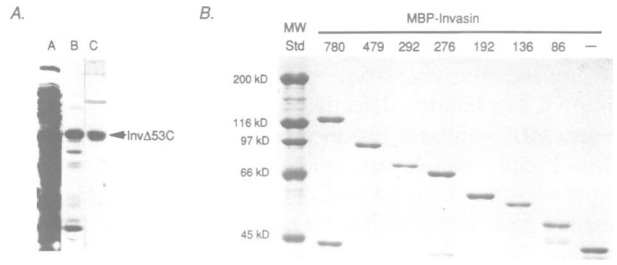

Fig. 5. Purification of invasin fragment InvΔ53C and MBP−invasin fusion proteins. **Panel A**: purification of InvΔ53C. InvΔ53C was overproduced in *E.coli* SW5AA2Q (*htpR*$^-$ *lon*$^-$) and purified as described (Materials and methods). Displayed is a Coomassie blue-stained 10% polyacrylamide SDS gel of purification steps. Lane A: total cell extract. Lane B: precipitate from 30% ammonium sulfate cut. Lane C: fraction from DEAE−cellulose column containing purified InvΔ53C. Note that incongruities above protein band in lane C are due to the cracking of the gel during drying. **Panel B**: purified MBP−invasin fusion proteins. Proteins were overproduced in *E.coli* and purified on cross-linked amylose columns (Materials and methods). A Coomassie blue-stained 7.5% polyacrylamide SDS gel of the set of purified proteins is shown. The first lane contains molecular weight standards, with the molecular weights indicated at left. For each of the other lanes, the number above each lane indicates the number of amino acids in the hybrid protein that are derived from invasin. The species with an apparent molecular weight of 40 kd, which appears in several MBP−invasin lanes, is MBP-related. This species co-migrates with MBP, reacts with anti-MBP antiserum and is less prominent when the proteins are overproduced in a *degP*$^-$ *E.coli* host.

(Figure 5A; Materials and methods). In contrast to full-length invasin, which is membrane associated and therefore difficult to purify, InvΔ53C was found to be water soluble (data not shown). A corresponding carboxyl-terminal fragment defective for cell attachment, InvΔ254, was also purified. This mutant has the same amino-terminus as InvΔ53C but contains two additional amino acids at its carboxyl-terminus that almost completely eliminate cell binding activity (R.Isberg and D.Voorhis, unpublished observations). Serial dilutions of the proteins were used to coat microtiter wells and the coating concentration required to give half-maximal cell attachment was determined. As shown in Figure 6, InvΔ53C mediates cell attachment to the wells, while InvΔ254, even at the highest concentration tested, at best promotes inefficient cell attachment. InvΔ53C at a coating concentration of 0.25 μg/ml gave half-maximal binding; by comparison, the half-maximal coating concentration for the extracellular matrix protein fibronectin is ~2 μg/ml for HEp-2 cells, illustrating the high avidity of this cell line for invasin.

The carboxyl-terminus of invasin retains binding activity when fused to MBP

The results of the deletion analysis suggested that only the carboxyl-terminal 192 residues were absolutely required for cell attachment. However, this was difficult to test directly using deletion derivatives of invasin because of the instability of the small invasin fragment containing only these residues. Therefore, the MBP−invasin proteins that carry varying amounts of the carboxyl-terminus of invasin were tested for cell attachment activity using the filter assay. When 136 or fewer amino acids were present, no cell binding activity was detectable (Figure 7b). In contrast, all of the fusion proteins that contained at least the carboxyl-terminal 192 amino acids

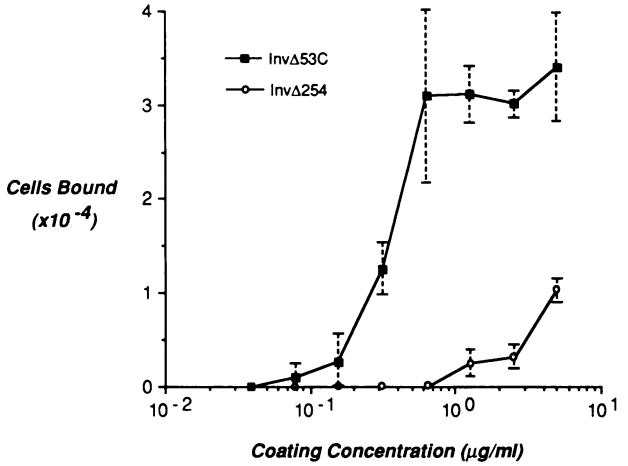

Fig. 6. Purified carboxyl-terminus of invasin binds mammalian cells. Purified InvΔ53C and its binding-defective counterpart InvΔ254 were tested for their ability to mediate HEp-2 cell binding in 96-well microtiter plates. Wells were coated at various coating concentrations of protein, washed and incubated with 5 × 10^4 HEp-2 cells. After washing 3 times in PBS, bound cells were quantitated using crystal violet staining (Materials and methods). Each point represents the average of three determinations and error bars indicate ±SD.

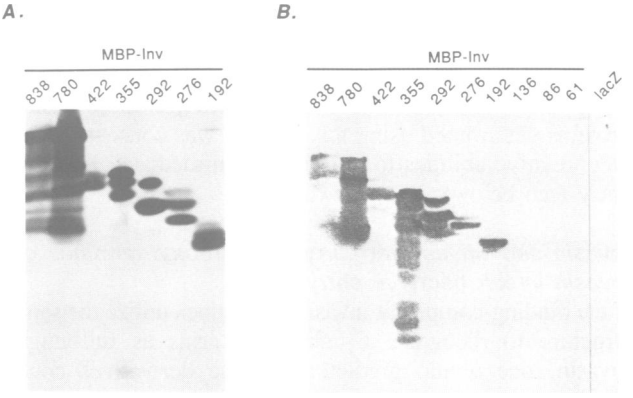

Fig. 7. MBP−invasin fusion proteins that carry the carboxyl-terminal 192 amino acids of invasin bind HEp-2 cells. Approximately 10^9 bacteria producing MPB−invasin fusion proteins were lysed in 0.1% SDS, subjected to electrophoresis on a 7.5% polyacrylamide SDS gel and transferred to Immobilon filters (see Materials and methods). Numbers above each lane give the number of amino acids of the invasin derived portion of each fusion protein. Control extract containing MBP fused to a portion of the *lacZ* gene (Guan *et al.*, 1988) was used in lane marked *lacZ*. **Panel A**: immunoblot. mAb 1B10, directed against the carboxyl terminal region of invasin was used to detect MBP-invasin fusion proteins. **Panel B**: cell-binding assay. The filter was probed with live HEp-2 cells and bound cells were stained with XP, the substrate for alkaline phosphatase. Five-fold more extract was used in the cell-binding assay than was used for immunoblotting.

of invasin bound Hep-2 cells in this assay. Seven of the fusion proteins were purified on cross-linked amylose columns and tested for their ability to mediate mammalian cell binding in microtiter wells (Figure 5B). Consistent with the results using the filter assay, fusion proteins carrying at least 192 amino acids from the carboxyl-terminus of invasin efficiently bound HEp-2 cells and fusion proteins with 136 or fewer amino acids showed no attachment activity (Figure 8).

To obtain an estimate of the relative cell binding efficiency

of the different fusion proteins, microtiter wells were coated with different concentrations of purified fusion proteins and the concentration required to give half-maximal binding was determined (Figure 8; Table II). The binding-proficient fusion proteins could be grouped roughly into three categories on the basis of their half-maximal coating concentration. MBP−Inv780 bound the most efficiently, with a half-maximal coating concentration of 3.4 nM. MBP−Inv479, MBP−Inv292 and MBP−Inv192 showed intermediate binding efficiencies, with half-maximal coating concentrations of between 5.5 and 7.3 nM. Finally, MBP−Inv276 bound the least efficiently, requiring a coating concentration of 14.7 nM in order to promote half-maximal HEp-2 cell binding. The fact that MBP−Inv780 had the highest binding efficiency suggests either that there are specific sequences located in the amino-terminal region of the protein that are recognized by HEp-2 cells or that this portion of the protein enhanced cell binding by promoting the proper conformation of the carboxyl-terminal cell attachment regions. The fact that MBP-Inv276 contains all the sequence information of MBP-Inv192 (and more) and yet bound HEp-2 cells apparently less efficiently indicated that regions of invasin not directly involved in receptor interaction can affect binding. Obviously, the half-maximal coating concentration is not a direct measure of binding avidity, since factors such as the efficiency of coating the well may vary with each hybrid. However, the hierarchy of apparent cell attachment efficiencies for these fusion proteins determined using this method was consistent with their relative abilities to inhibit invasin-mediated bacterial entry (see below).

Invasin derivatives that carry the carboxyl-terminus of invasin inhibit bacterial entry

If the binding-competent invasin derivatives utilize the same structure to recognize β_1 chain integrins as full-length invasin, one would predict that these derivatives could competitively inhibit bacterial penetration. We therefore tested the ability of invasin derivatives to inhibit the entry of *inv*-encoding bacteria into cultured epithelial cells. InvΔ53C, its binding-defective counterpart InvΔ254 and the seven MBP−invasin fusion proteins were incubated with HEp-2 cell monolayers prior to infection with *E.coli* expressing the *inv* gene (MC4100/pRI203*inv*$^+$). All of the binding-proficient proteins and none of the binding-defective proteins inhibited bacterial entry (Figure 9). In these experiments, none of the purified invasin derivatives caused detachment of the HEp-2 cells from the microtiter wells, consistent with the observation that invasin does not bind all β_1 chain integrins present on these cells (Isberg and Leong, 1990). InvΔ53C inhibited entry the most efficiently−700-fold inhibition at a concentration of 50 μg/ml. None of the MBP−invasin fusion proteins inhibited bacterial entry as efficiently as InvΔ53C−apparently the presence of MBP sequences were antagonistic in this assay. The relative abilities of MBP−invasin fusion proteins to inhibit invasion generally paralleled their binding efficiencies, as measured by the coating concentrations that gave half-maximal cell binding in microtiter wells. MBP−Inv780, which bound cells more efficiently than the other fusion proteins, also inhibited entry most efficiently: 60-fold inhibition at 200 μg/ml. MBP−Inv479, MBP−Inv292 and MBP−Inv192 bound with intermediate efficiency, and inhibited entry 6- to 9-fold. Finally, MBP−Inv276 bound least efficiently and inhibited entry 4-fold (Figure 9).

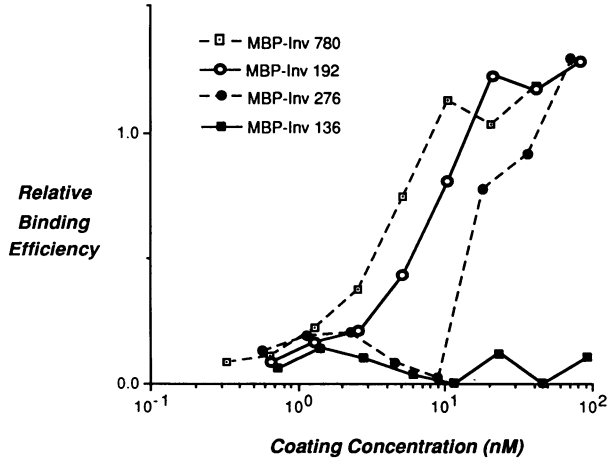

Fig. 8. Quantitation of cell-binding activity of MBP−invasin fusion proteins. Purified fusion proteins were tested for their ability to mediate HEp-2 cell binding in 96-well microtiter plates. Wells were coated with serial dilutions of protein, washed and then 5×10^4 HEp-2 cells were added in 0.1 ml of RMPI 1640, 0.4% BSA, 20 mM HEPES (pH 7.0). After a 1 h incubation at 37°C, wells were washed 3 times in PBS and bound cells were quantitated using crystal violet staining (Materials and methods). On each plate the number of HEp-2 cells bound for each concentration of fusion protein was divided by the number of cells bound to wells coated with InvΔ53C at 5 μg/ml. Each point represents the mean of six determinations.

Table II. MBP−invasin fusion proteins retain cell-binding activity

Plasmid	MBP−invasin protein produced[a]	Cell-binding activity: filter assay[b]	Cell-binding activity: ½ max coating concentration[c]	Percent invasion[d]
pJL269	MBP−Inv838	+++	ND[e]	0.80
pJL270	MBP−Inv780	+++	3.4 nM	2.2×10^{-3}
pJL309	MBP−Inv479	+++	5.5 nM	7.5×10^{-3}
pJL302	MBP−Inv422	+++	ND	1.0×10^{-2}
pJL310	MBP−Inv355	+++	ND	4.8×10^{-3}
pJL311	MBP−Inv292	+++	7.3 nM	5.1×10^{-3}
pJL301	MBP−Inv276	+++	14.7 nM	9.3×10^{-3}
pJL300	MBP−Inv192	+++	6.2 nM	1.6×10^{-3}
pJL307	MBP−Inv136	−	−	1.7×10^{-3}
pJL306	MBP−Inv86	−	−	1.9×10^{-3}
pJL305	MBP−Inv61	−	ND	6.2×10^{-3}
pCG806	MBP−*lacZ*	−	−	4.2×10^{-3}

[a]All fusion proteins have the carboxyl-terminus of invasin fused to MBP−each designation indicates the number of residues from the carboxyl-terminus of invasin present in the hybrid protein. For example, MBP-Inv192 carries the carboxyl-terminal 192 amino acids fused to the carboxyl-terminus of MPB (see Figure 2).
[b]Cell-binding activity after SDS−PAGE and transfer to a filter membrane: '+++' = activity indistinguishable from full-length invasin; '−' = no detectable activity.
[c]Microtiter well coating concentration of MBP-invasin hybrid protein that gave half-maximal HEp-2 cell binding. '−' indicates that the protein did not promote cell-binding.
[d]HEp-2 cells (2×10^5) were infected with 7.5×10^6 *E.coli* 71/18 harboring a *malE*−*inv* plasmid. The bacteria were induced at mid-logarithmic phase with 1 mM IPTG for 2 h prior to infection in order to induce synthesis of the fusion protein. Percent invasion was calculated as the percentage of bacteria that survive gentamicin treatment (Materials and methods).
[e]Not determined.

Carboxyl-terminal fragments of invasin are not properly localized in the outer membrane

By analogy to other outer membrane proteins, we predicted that amino-terminal sequences of invasin would be required for the proper export of the protein to the outer membrane. To test which deletion derivatives were exposed on the surface of the bacteria, we used mAbs directed against invasin to probe the surface of bacteria that expressed them using quantitative immunofluorescence (Table I; Materials and methods). As predicted, none of the plasmids that were deleted for the amino-terminal portion of the *inv* gene resulted in the detectable exposure of invasin on the surface of the bacteria. Deletion derivatives of invasin that were missing as much as 379 amino acids from the carboxyl-terminus, however, were exported to the surface of bacteria as efficiently as full-length invasin (Table I, pJL256). Therefore, the amino-terminal 608 amino acids of invasin are sufficient for proper localization.

Binding-deficient invasin derivatives do not mediate bacterial entry into HEp-2 cells

All of the plasmids generated were tested for their ability to promote bacterial entry into HEp-2 cell monolayers (Table I). Plasmids that produced deletions of the amino-terminal of invasin did not promote entry, presumably due to improper localization of the protein. Deletions of the carboxyl-terminus, which have lost their ability to recognize HEp-2 cells, also did not confer the invasive property. pJL202, which encodes the internal deletion InvΔK, did not promote detectable invasiveness when harbored in *E.coli* 71−18, the strain background used in Table I; however, in *E.coli* MC4100 it promoted entry ~50-fold less efficiently than pRI253(inv^+) (data not shown). The variable efficiency at which this derivative promoted entry may be a reflection of its relative instability.

Plasmids that produced MBP−invasin fusion proteins were also tested for their ability to confer the invasive phenotype on *E.coli* harboring them (Table II). Interestingly, one of the plasmids, pJL269, promoted entry even though the hybrid protein it produced, MBP−Inv838, is missing 148 amino acids from the amino terminus of invasin. The level of invasion was ~2-fold less than the level conferred by pRI253(inv^+) (Table I). The MBP sequences in MBP−Inv838 apparently direct translocation of the protein across the inner membrane. Since MBP is a periplasmic protein and does not normally cross the outer membrane, presumably the carboxyl-terminal 838 residues directs localization to the outer membrane. Combined with the analysis of the carboxyl-terminal deletions of invasin described above, this result suggested that the information required for outer membrane localization resides between invasin residues 149 and 608.

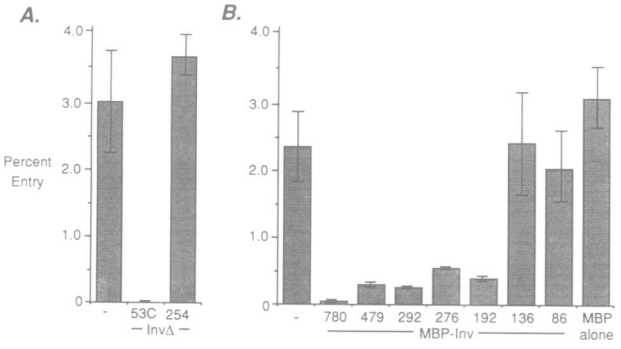

Fig. 9. Inhibition of bacterial invasion by purified derivatives of invasin. Purified invasin derivatives were tested for their ability to inhibit *inv*-encoding *E.coli* entry into monolayers of HEp-2 cells. Monolayers were incubated with protein for 60 min at room temperature prior to infection with 2.5×10^7 MC4100 (pRI203inv^+). The number of intracellular bacteria after a 2 h infection was determined by gentamicin protection (Materials and methods). Each bar represents the average of four determinations, and the superimposed error bars indicate ±SD. '−' indicates levels of invasion in the absence of protein. **Panel A**: inhibition by InvΔ53C. The concentration of InvΔ53C, and InvΔ254 was 50 μg/ml. **Panel B**: inhibition by MBP−invasin fusion proteins. All proteins were used at 200 μg/ml.

Discussion

In this report, several lines of evidence were presented that the carboxyl-terminal 192 amino acids of invasin, residues 795−986 (shown in Figure 10), are responsible for attachment to HEp-2 cells. First, all six of the anti-invasin mAbs that inhibited attachment of HEp-2 cells to invasin recognized epitopes in the last 192 amino acids. This inhibition was not dependent on the Fc portion of the antibodies since the Fab fragment also blocked binding. Second, carboxyl-terminal fragments of invasin as small as 276 amino acids and MBP−invasin fusion proteins containing as few as 192 amino acids of invasin retained cell attachment activity. The attachment activity of these invasin derivatives reflected that of full-length invasin. Purified carboxyl-terminal fragment and MBP−invasin fusion proteins could inhibit entry by *inv*-encoding bacteria, and the cell binding activity of these derivatives, like that of full-length invasin, was abolished by heating to 95°C, inhibited by the same blocking mAbs and required divalent cations (data not shown). None of the derivatives deleted in the carboxyl-terminal 192 amino acids

```
        800                 810                 820                 830
V P T L T G I L V N G Q N F A T D K G F P K T I F K N A T F Q L Q M D N D V A N

        840                 850                 860                 870
N T Q Y E W S S S F T P N V S V N D Q G Q V T I T Y Q T Y S E V A V T A K S K K

        880                 890                 900                 910
F P S Y S V S Y R F Y P N R W I Y D G G R S L V S S L E A S R Q C Q G S D M S A

        920                 930                 940                 950
V L E S S R A T N G T R A P D G T L W G E W G S L T A Y S S D W Q S G E Y W V K

        960                 970                 980
K T S T D F E T M N M D T G A L Q P G P A Y L A F P L C A L S I
```

Fig. 10. Amino acid sequence of the cell attachment domain of invasin. Numbers above one letter amino acid code indicate residue position in protein sequence.

of invasin retained integrin-binding activity: InvΔ97N, which is missing residues 928–986 and MBP–Inv136, which is missing residues 1–849, have no cell attachment activity.

Because we have been unable to demonstrate a binding-proficient region of less than 192 residues, it seems unlikely that the receptors for invasin recognize a simple linear sequence of amino acids in the protein. One possibility is that the relatively large region is required to hold the recognition element(s) in the proper conformation. Conformation of the Arg–Gly–Asp sequence has been shown to be important for recognition by integrin receptors. For example, cyclization of a peptide containing the Arg–Gly–Asp sequence results in an increase in its ability to inhibit vitronectin-dependent cell attachment and a decrease in its ability to inhibit fibronectin-dependent attachment (Pierschbacher and Ruoslahti, 1987). Collagen receptor recognizes the Arg–Gly–Asp sequence in native triple helical collagen, but not when the triple helix is destroyed by heat denaturation (Dedhar *et al.*, 1987). The cell binding activity of invasin shares this heat-sensitive characteristic. The observation that MBP–Inv192, InvΔK, InvΔB_2K, InvΔB_1K and InvΔ20C each carry the carboxyl-terminal 192 amino acids, but varied widely in their ability to bind HEp-2 cells, may also be related to the conformation of integrin recognition sequences.

Another possible explanation for the inability to detect cell binding activity with a smaller region of invasin is that there may be distinct recognition elements in different regions of the integrin-binding domain, and interaction with all of the elements may be required for efficient cell attachment. Fibronectin, which also binds to members of the β_1 integrin family, contains multiple recognition sequences for cell attachment (McCarthy *et al.*, 1986). Efficient fibronectin binding by BHK fibroblasts required not only the Arg–Gly–Asp-containing sequence, but also another sequence located ~170 amino acids towards the amino-terminus (Obara *et al.*, 1988). The two sites act synergistically not only for promoting cell adhesion but also for promoting actin microfilament bundle organization and cell spreading. Invasin and fibronectin share at least two of the same receptors ($\alpha_3\beta_1$ and $\alpha_5\beta_1$) and binding of HEp-2 cells to invasin is also accompanied by marked cell spreading (Isberg and Leong, 1988). In spite of these similarities we have been unable to detect amino acid homology between the cell attachment region of invasin and the recognition sequences in fibronectin. In particular, invasin contains no sequence that closely resembles Arg–Gly–Asp, the tripeptide sequence that is present in many ligands for β_1 chain integrins. Recent evidence suggests that a region of fibronectin which does not contain Arg–Gly–Asp can also bind a member of the β_1 family, $\alpha_4\beta_1$ (Wayner *et al.*, 1989; Guan and Hynes, 1990). The ease of genetic analysis in this bacterial system should allow for the identification of the exact residues within the binding domain that are critical for integrin recognition.

The fact that only the carboxyl-terminal 192 amino acids are required for receptor recognition raises the question of what role is played by the amino-terminal 794 amino acids. These residues are not required for entry, since bacteria coated with MBP–invasin fusion protein containing only the carboxyl-terminal 192 amino acids of invasin can efficiently enter mammalian cells (J.Leong, S.Rankin and R.Isberg, unpublished observations). Certainly, one function of this region is to promote the proper localization of invasin in the outer membrane. Analysis of deletion derivatives of invasin and MBP–invasin fusion proteins suggests that the information required for outer membrane localization is contained between residues 149 and 608. The carboxyl-terminal 379 amino acids of invasin, which includes the integrin binding domain, is entirely dispensible for localization to the outer membrane (Table I, pJL256). This is in contrast to several other bacterial outer membrane proteins, in which large carboxyl-terminal deletion mutants cannot be detected immunochemically on the surface of the bacteria (Bosch *et al.*, 1986; Boulain *et al.*, 1986). The size of invasin is reminiscent of large excreted proteins such as hemolysin and IgA protease which require accessory functions for excretion (Mackman *et al.*, 1985; Pohlner *et al.*, 1987). Analogous to the 500 amino acid 'helper' domain of IgA protease that is postulated to form a pore for excretion of the protease domain, the large central region of invasin, residues 149–608, might form an amphipathic channel to translocate the carboxyl-terminal integrin binding domain of invasin to the outer membrane.

Finally, the experiments described here define a small region of invasin protein that is sufficient for binding a particular cell line, the HEp-2 epithelial line. This cell line expresses at least two of the four β_1 chain integrins known to bind invasin and it is possible that the 192 amino acid region is able to mediate binding to only one of these receptors. It will be important to determine if all of the invasin receptors recognize the same structure(s) in invasin. The availability of different cell lines that express different members of the β_1 chain integrin family should make this analysis straightforward.

Materials and methods

Construction of plasmids that overproduce fragments of invasin

The two-plasmid phage T7 promoter system developed by Tabor and Richardson (1985) was used to overproduce invasin derivatives. One plasmid, pGP1, carries the gene for T7 RNA polymerase distal to the lambda P_L promoter. A second plasmid, pRI253, carries the *inv* gene downstream of a T7 promoter sequence (Isberg and Leong, 1988). Therefore, heat induction results in the expression of T7 RNA polymerase and high-level expression of the *inv* gene. Plasmids that produce internally-deleted invasin protein were derived from pRI253 simply by deleting restriction fragments internal to the gene, while retaining the correct reading frame at the deletion endpoints. pRI253 was also modified to generate plasmids that overproduce amino-terminal invasin fragments, either by deleting the carboxyl-terminal portion of the *inv* gene or by making 4 bp insertion mutations within the *inv* gene. These insertions were made by cleaving the *inv* gene with restriction enzymes that leave 4 bp 5′ protruding ends, then blunting the ends using Klenow fragment of DNA polymerase or T4 DNA polymerase and recircularizing the plasmid. The change in reading frame results in the premature termination of invasin, and the addition of a small number of amino acids at the carboxyl-terminus (encoded by the new reading frame before a stop codon is encountered).

T7 promoter plasmids that produce carboxyl-terminal fragments of invasin were constructed by inserting restriction fragments of the 3′ end of the *inv* gene downstream of a Shine–Dalgarno sequence and an ATG start codon. The two starting plasmids were pT7-7 (a gift of S.Tabor and C.Richardson) and pJL230. In pT7-7, just distal to the ATG, is a polylinker into which *inv* gene fragments were inserted. pJL230 is derived from pT7-12 (Tabor and Richardson, 1985) by the insertion of a 20 bp synthetic DNA fragment carrying a Shine–Dalgarno sequence and a ATG codon just proximal to the *SalI*/*AccI*/*Hin*cII site. *Inv* gene fragments were inserted using this site: the six base sequence recognized by these three enzymes is cleaved at a

different position by each of the three enzymes, so that the translation start signal can be adapted to any of the three possible reading frames. Restriction sites within the *inv* gene coding sequence were used to construct all except two of the plasmids that produce carboxyl-terminal fragments. One of these, pJL225, which produces the 53 kd carboxyl-terminal fragment of invasin, was constructed using an *inv* deletion derivative, *inv*32-2. *Inv*32-2 was constructed in the vector pEMBL18 by the exonuclease III plus S1 nuclease unidirectional deletion procedure previously described (Henikoff, 1985; Isberg *et al.*, 1987) and places a unique *Hin*dIII site approximately in the middle of the *inv* gene. The *Hin*dIII−*Bam*HI fragment containing the 3′ end of the *inv* locus from this construction was then ligated into pT7-7. The other, pRI254, is identical to pJL225 except that it encodes the two additional amino acids, Ile and Leu, at the 3′ end of the *inv* gene. The additional codons are the result of oligonucleotide-directed mutagenesis as previously described (Boyd *et al.*, 1988), and result in an invasin derivative defective for cellular attachment (Figure 6). The *malE* gene fusion plasmids pCG806 and pCG807fX (Maina *et al.*, 1988) were used to generate plasmids that produce MBP−invasin hybrid proteins. Restriction fragments carrying the 3′ end of the *inv* gene were inserted at the 3′ end of the *malE* gene using standard techniques.

Preparation of bacterial cell extracts containing fragments of invasin

E.coli MC1000 F$^-$ Δ(*ara-leu*) Δ(*lac*)X74 *rpsL galE galK* (Kumamoto and Beckwith, 1983) or SW5AA2Q F$^-$ *htpRam supFts lon*::miniTn*10* (Boyd *et al.*, 1987) harboring pGP1 and invasin-producing T7-promoter plasmids were grown to mid-log phase in 2 × YT medium containing kanamycin (25 μg/ml) and ampicillin (100 μg/ml), and induced at 42°C for 30 min. After 1−3 h at 30°C, cells were pelleted, washed once in 10 mM HEPES (pH 8.0) and resuspended in the 10 mM HEPES (pH 8.0), 100 μg/ml *p*-methylsulfonylfluoride (PMSF) at a concentration of ~10^{10} cells/ml. After lysis by sonication, extracts were centrifuged at 90 000 r.p.m. for 30 min in order to separate soluble from insoluble fractions. Pellets were washed 2 times with 10 mM HEPES (pH 8.0) and resuspended in the same buffer. Both soluble and insoluble fractions were stored at −70°C.

Purification of invasin deletion derivatives InvΔ53C and InvΔ254

The two invasin deletion derivatives encoded on the plasmids pJL225 and pRI254 were purified by an identical procedure. SW5AA2Q harboring pGP1 and either of the two invasin-encoding plasmids was grown in 10 l of 2 × YT medium (Messing, 1983) to an A_{600} = 1.0 at 30°C. The culture medium was shifted to 42°C for 30 min, before continued growth for 2 h at 30°C. The cells were washed once in 10 mM HEPES, pH 8.0, 150 μg/ml PMSF and lysed by passage in a French pressure cell at 14 000 p.s.i. Unlysed cells were removed by centrifugation at 2000 *g* for 10 min and the membranous debris was collected by centrifugation at 100 000 *g* for 1 h. Approximately 70% of the overproduced carboxyl-terminal fragment of invasin was found in aggregates that pelleted under these conditions. The pellet was washed once by resuspension in the same buffer followed by centrifugation at 100 000 *g*.

To purify the invasin derivative, the pelleted debris was resuspended in 200 ml of 6 M guanidine−HCl, 50 mM Tris−HCl (pH 8.0), 150 μg/ml PMSF, which dissolved the aggregated invasin. The guanidine−HCl extract was sequentially dialyzed against 0.5 M guanidine−HCl, 50 mM Tris−HCl (pH 8.0) followed by dialysis against 150 mM KCl, 50 mM Tris−HCl (pH 8.0) and 50 mM NaCl, 50 mM Tris−HCl (pH 8.0) in order to remove insoluble material that accumulated after dilution of guanidine−HCl. Before each buffer change, the precipitate that accumulated during dialysis was removed by centrifugation at 100 000 *g* for 30 min and the soluble supernatant which contains invasin was dialyzed further until a substantial precipitate formed. After completion of dialysis and no further precipitate formed the soluble fraction containing invasin was concentrated by precipitation in 35% ammonium sulfate and the resuspended pellet was dialyzed exhaustively against 50 mM Tris−HCl (pH 8.0), 50 mM NaCl. To gain further purification, the dialysate was loaded onto a DEAE−cellulose column (DE52, Whatman) equilibrated with 50 mM Tris−HCl (pH 8.0), 50 mM NaCl and eluted with a 50−250 mM linear NaCl gradient. The fractions containing the invasin derivative were pooled and stored frozen. Approximately 15 mg of protein was obtained from 10 l of culture. The purified preparation of invasin fragment InvΔ53C was able to support mammalian cell attachment (Table I) and block the entry of invasin-encoding *E.coli* whereas InvΔ254 was defective for these activities (Table I, Figures 6 and 9).

Purification of MPB−invasin fusion proteins

MBP−invasin fusion proteins were purified by affinity chromatography using cross-linked amylose as described (Guan *et al.*, 1988). *E.coli* 71-18 Δ(*lac-proAB*) *thi supE* (F′*proAB*$^+$ *lacI*^{q} *lacZΔM15*) (Yanisch-Perron *et al.*, 1985) or *E.coli* MC1000 *leu*$^+$ *ara*$^+$ *ΔphoA−Pvu*II *lpp5508 degP*::Tn*5* (F′*pro*AB$^+$ *lacI*^{q} *lacZΔM15*) (Strauch and Beckwith, 1988) were used to overproduce proteins. Bacteria harboring the plasmids encoding the fusion proteins were grown at 30°C in 500 ml of 2 × YT medium plus 100 μg/ml ampicillin to A_{600} = 0.50. The gratuitous inducer IPTG was added to a final concentration of 1.0 mM to induce the *lac* promoter and the culture was grown an additional 2 h at 30°C. The cells were washed once in 10 mM HEPES (pH 8.0) resuspended in 10 mM HEPES (pH 8.0), 150 μg/ml PMSF, 0.03 U/ml aprotinin and then lysed by two passes in a French pressure cell at 14 000 p.s.i. The insoluble fraction was removed by two successive centrifugations at 100 000 *g* for 30 min. The soluble fraction was loaded onto a 5 ml cross-linked amylose column (2 cm diameter). The column was washed with 30 ml of 10 mM HEPES (pH 8.0), 150 μg/ml PMSF, 0.03 U/ml aprotinin and then 15 ml of 10 mM HEPES (pH 8.0). The hybrid proteins were eluted in 6.0 ml 10 mM HEPES (pH 8.0), 10 mM maltose and stored at −70°C. Between 3 and 7 mg of each fusion protein was purified from 500 ml of cultured cells.

Production of mAbs and the Fab fragment directed against invasin

The mAbs were raised as previously described (Isberg and Leong, 1988). From ~180 wells that produced antibody against invasin, 32 hybridomas that produce anti-invasin mAb were cloned 2 times on soft agar and supernatants from these hybridomas were used in this study.

The mAbs were purified by affinity methods on a 3 ml Protein A−Sepharose 4B column (Pharmacia) as described (Underwood *et al.*, 1983). Only heavy and light chains were apparent when analyzed by gel electrophoresis. Concentrations of antibodies were determined by protein assay (Lowry *et al.*, 1951) or indirect ELISA assay (Zola, 1987). Purified mAb was cleaved with papain to generate the Fab fragment as described (Goding, 1986).

Quantitation of surface-exposed epitopes on whole bacteria

The set of mAbs generated against invasin was used to quantitate invasin epitopes of the surface of live bacteria that express invasin and invasin deletion derivatives. The epitopes for each of the 32 mAbs was mapped by immunoblotting of invasin deletion derivatives and MBP−invasin fusion proteins (see, e.g. Figure 3). Each epitope was also tested for exposure on the surface of whole bacteria, using immunofluorescence microscopy, slide aggutination assays, ELISA assays and immunoelectron microscopy (J.M.Leong, R.S. Fournier and R.R.Isberg, manuscript in preparation). Those mAbs that recognize surface-exposed epitopes were used in these experiments.

E.coli MC1000 harboring pGP1 and plasmids that express invasin derivatives from the T7 promoter were grown to mid-logarithmic phase at 30°C. At this temperature the invasin-producing plasmids are not fully induced but the *inv* gene (or gene fragment) is expressed at a low level. Cultures were washed 2 times in phosphate buffered saline (PBS) and the cells were resuspended in 10% of the original volume. Bacteria were incubated for 30 min at 37°C with a mixture of mAbs that recognize surface-exposed epitopes. For any single experiment, mAbs were chosen that recognized epitopes present in all of the invasin derivatives being tested, i.e. each of the derivatives being compared in a given experiment reacted with each of the mAbs in the mixture (as determined by immunoblotting). The mAbs were used at a concentration 5-fold greater than that which gives a half-maximal signal in a standard ELISA assay. Bacteria were then washed three times in immunofluorescence (IF) buffer, containing 100 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM HEPES (pH 7.0) and resuspended in IF buffer with 1% bovine serum albumin (BSA) and FITC-conjugated anti-mouse IgG antibody. Cells were then incubated in the dark at 30°C for 2 h and washed 3 times in IF buffer. Before the final wash, the OD_{600} of the cell suspensions was measured to determine cell number. Fluorescence was visually evaluated by fluorescence microscopy. The fluorescent-labeled antibody was then extracted from the surface of the bacteria by incubating in 0.1 M NaOH, 0.5% Na_2CO_3 at 30°C with gentle shaking, bacteria were pelleted, and the fluorescence in the supernatant was quantitated in a Perkin-Elmer LS-5 fluorescence spectrophotometer at an excitation wavelength of 490 nm and an emission wavelength of 530 nm.

The measured fluorescence was normalized to cell number by dividing by the OD_{600} determined before extraction of the bacteria, to give 'fluorescence per OD'. The fluorescence per OD for MC1000

(pGP1/pT7-12), which expresses no invasin derivative, was assigned the background value and was subtracted from all experimental values to give 'net fluorescence per OD'. Finally, this value for each plasmid-bearing strain was divided by the net fluorescence per OD determined for MC1000 (pGP1/pRI253*inv*$^+$), which expresses full-length invasin. This value of 'relative surface exposure' is given for each plasmid in Table I.

Assay for bacterial entry into mammalian cells

To assay the invasiveness of bacteria harboring *inv* deletion plasmids, 2×10^7 bacteria from a fresh overnight culture were used to infect 2×10^6 HEp-2 cells in 1 ml of RPMI 1640 medium in 24-well tissue culture dishes (Falcon). The bacteria were centrifuged onto the monolayer at 1000 r.p.m. for 10 min and infected cultures were incubated for 2 h at 37°C in 5% CO_2 atmosphere. Monolayers were washed 3 times with PBS to remove non-adherent bacteria and then 1 ml of RPMI 1640 containing 50 μg/ml gentamicin was added. Gentamicin efficienty kills extracelullarly-located bacteria, but bacteria that have entered the mammalian cells are protected from its action. After 1.5 h at 37°C the monolayers were washed 3 times with PBS and internalized bacteria were released from the monolayer by adding 0.5% Triton X-100 and titered on L agar plates.

To assay the invasiveness of bacteria harboring plasmids that produce MPB−invasin fusion proteins, fresh overnight cultures were diluted 1:50 in L broth plus 100 μg/ml ampicillin and grown to mid-logarithmic phase at 30°C. IPTG was added to a final concentration of 1.0 mM to induce expression of the fusion proteins from the *lac* promoter and cultures were grown for another 45 min at 30°C. A total of 10^7 bacteria in 0.015 ml were added to 2×10^5 HEp-2 cells and bacterial entry was assayed as described above.

Inhibition of bacterial entry by purified invasin derivatives

Samples of 10 μg of purified invasin deletion derivatives InvΔ53C or InvΔ254 were incubated with 2×10^5 HEp-2 cells in 0.20 ml of RMPI 1640, 0.4% BSA, 20 mM HEPES (pH 7.0) in 24-well tissue culture dishes for 1 h at room temperature. *E.coli* MC4100 *ara*D Δ(*lac*) *rps*L (pRI203*inv*$^+$) (Kumamoto and Beckwith, 1983) cells (4×10^6) from a fresh overnight culture were added to each well and the infections were allowed to proceed for 3 h at 37°C in a 5% CO_2 atmosphere. Bacterial invasion was quantitated by gentamicin-resistant viable counts as described above. Inhibition of bacterial entry by MBP-invasin fusion proteins was tested identically, except that the monolayer was incubated with 40 μg of purified protein in 0.2 ml of RPMI 1640, 0.4% BSA, 20 mM HEPES (pH 7.0), 2.5 mM maltose.

Immunoblotting and HEp-2 cell filter binding

Invasin-containing cell extracts were subjected to SDS−PAGE using standard techniques (Laemmli, 1970) with the modifications that samples were heated only to 37°C prior to loading and that gels were run at ≤11 mAmps to avoid excessive heating. After SDS−PAGE, proteins were electrophoretically transferred to Immobilon filters (PVDF; Millipore) and the filters were incubated overnight in 1% BSA/PBS to minimize non-specific cell binding to the filter (Towbin *et al.*, 1979). To detect all species of invasin in immunoblots we used a mixture of mAbs directed against invasin protein. Goat anti-mouse IgG antibody conjugated to alkaline phosphatase was used as the secondary antibody and immunoblots were processed as previously described (Isberg and Leong, 1988).

The HEp-2 cell filter-binding assay was performed as described (Isberg and Leong, 1988). Filters were incubated with 2×10^7 dispersed HEp-2 cells in 20 ml RPMI 1640 medium, 0.4% BSA, 10 mM HEPES (pH 7.0) at 37°C for 1 h. After washing in PBS, filters were fixed in 1% glutaraldehyde in 50 mM NaCl, 100 mM sodium phosphate (pH 7.2) for 10 min at 20°C. Filters were then stained for 2 min in 1% amido black, 40% methanol, 10% acetic acid and washed in 40% MeOH, 10% acetic acid as described (Bell and Engval, 1982; Hayman *et al.*, 1982). Alternatively, cells bound to the filter were stained by incubating the filters in veronal−acetate buffer (pH 9.6), 4 mM $MgCl_2$, 100 μg/ml nitro blue tetrazolium, 50 μg/ml bromo-chloro-indolyl-phosphate.

HEp-2 cell binding in microtiter wells and inhibition of binding by mAbs directed against invasin

Membrane (insoluble) fractions were crosslinked to 96-well microtiter dishes (Flow) as described (Pierschbacher *et al.*, 1983). Dilutions of soluble proteins were used to coat microtiter wells by incubation for 2 h at 37°C in 50 mM sodium bicarbonate (pH 9.6) followed by blocking overnight at 4°C in 0.2% BSA in PBS. To assay for cell attachment to invasin, HEp-2 cells were dispersed from confluent monolayers in trypsin, washed 2 times in soybean trypsin inhibitor (Hayman *et al.*, 1982; Ruoslahti *et al.*, 1983) and resuspended at 1×10^6 cells/ml in RPMI 1640 containing 20 mM HEPES (pH 7.0) and 0.4% BSA. 0.1 ml aliquots of HEp-2 cells were allowed to attach to the bottoms of microtiter wells for 1 h at 37°C and the number of attached cells was quantitated by hexosaminidase assay (Landgren, 1984) or by crystal violet staining and solubilization in sodium deoxycholate (Brasaemle and Attie, 1988).

To demonstrate inhibition of cell binding, invasin-coated wells were preincubated with 50 μl of hybridoma supernatant, purified mAb or Fab fragment for 1 h at room temperature. Dispersed HEp-2 cells (5×10^4) were then added in 100 μl of RPMI 1640 medium, 0.4% BSA, 10 mM HEPES (pH 7.0) and allowed to bind for 1 h at 37°C. After washing 3 times with PBS the bound HEp-2 cells were quantitated using substrate for the lysosomal enzyme hexosaminidase (Landegren, 1984). Cell binding is expressed as a percentage of maximal cell binding, i.e. binding in the absence of mAb.

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