INFECTION AND IMMUNITY, Oct. 1991, p. 3424–3433
0019-9567/91/103424-10$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 59, No. 10

Mapping and Topographic Localization of Epitopes of the *Yersinia pseudotuberculosis* Invasin Protein

JOHN M. LEONG,[1]† ROBERT S. FOURNIER,[1] AND RALPH R. ISBERG[1,2]*

Department of Molecular Biology and Microbiology[1] and Howard Hughes Medical Institute,[2] Tufts University School of Medicine, 136 Harrison Avenue, Boston, Massachusetts 02111

Received 3 June 1991/Accepted 9 July 1991

The *Yersinia pseudotuberculosis* invasin protein is a 986-amino-acid outer membrane protein that promotes bacterial penetration into mammalian cells by binding to β_1-chain integrin receptors. We previously showed that the integrin binding domain is encoded by the carboxyl-terminal 192 amino acids. To further investigate the structure of this protein, we characterized a set of 32 monoclonal antibodies (MAbs) directed against invasin. Invasin deletion derivatives and fusion proteins carrying different segments of invasin were used to map the epitopes of this set of MAbs into 10 overlapping but distinct intervals. Indirect immunofluorescence of intact bacteria expressing invasin demonstrated that two large regions of invasin contain epitopes exposed on the bacterial surface. To assess the role of these surface-exposed regions in the binding and invasion of mammalian cells, each of the MAbs was tested for its ability to inhibit these processes. All of the MAbs that recognized bacterial surface-exposed epitopes in the cell binding domain of invasin inhibited both cell attachment and cell penetration, and no other MAbs inhibited either activity.

The ability to enter normally nonphagocytic cells is an important property of many intracellular pathogens (for a review, see references 5 and 26). Several enteric bacterial pathogens penetrate nonphagocytic cells, either to grow within these cells or to spread into deeper tissues (31, 35). The ease of genetic manipulation of several of these pathogens, such as *Salmonella*, *Shigella*, and *Yersinia* species, has contributed to their use as models to study the mechanism of bacterial invasion (6, 8, 11, 23, 25). *Yersinia pseudotuberculosis* is an invasive enteric pathogen with multiple pathways for entry into host cells (11). The best-characterized pathway is mediated by the *inv* gene, which was initially isolated on the basis of its ability to convert *Escherichia coli* K-12 into an efficient invader of cultured mammalian cells (12). *inv* mutants of *Y. pseudotuberculosis* are impaired in their ability to penetrate cultured mammalian cells, suggesting that this pathway is the most efficient of the three pathways (15, 28).

The *inv* gene encodes a 986-amino-acid outer membrane protein, termed invasin, that promotes cellular penetration by avidly binding to multiple members of the β_1-chain integrin family of receptors (14). Integrin receptors may be ideally suited for mediating bacterial internalization, because they are thought to interact with the host cell cytoskeleton (1, 10, 29). We previously showed that the carboxyl-terminal 192 amino acids contain the integrin binding domain of invasin. A monoclonal antibody (MAb) that blocked attachment recognized an epitope in this region, and the carboxyl-terminal 192 amino acids of invasin could mediate cell binding when fused to *E. coli* maltose binding protein (MBP) (19). If this carboxyl-terminal domain interacts with receptors on the mammalian cell, it must be exposed on the surface of the bacterium. In this report, we directly test this prediction and identify other bacterial surface-exposed regions of invasin by characterizing the epitopes of a set of 32 MAbs directed against the protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains were grown as previously described (15). *E. coli* MC1000 is F$^-$ Δ(*ara-leu*) Δ(*lac*)*X74 rpsL galE galK* (16), *E. coli* MC4100 is *araD* Δ(*lac*) *rpsL* (16), and *E. coli* 71-18 is F′ *proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*/F$^-$ Δ(*lac-proAB*) *thi supE*. *E. coli* SR2, a derivative of KS330 (32), is F′ *proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*/F$^-$ Δ*phoA PvuII lpp-5508 degP*::Tn*5* and was used to limit degradation of MBP-invasin fusion proteins, and *E. coli* SW5AA2Q F$^-$ *htpR*(Am) *supF*(Ts) *lon*::mini Tn*10* (2) was used to stabilize some of the invasin deletion derivatives, as described previously (19).

The plasmid pGP1 contains the phage T7 RNA polymerase under the control of the phage λ p_L promoter (33). The plasmids used to express invasin deletion proteins under the control of the phage T7 RNA polymerase promoter ϕ10 in the vector pT7-7 or pT7-4 were described previously (19). The plasmids that overproduce MBP-invasin fusion proteins are derivatives of pCG806 and pCG807fX (21). Restriction fragments carrying various lengths from the 3′ end of the *inv* gene were inserted at the 3′ end of the *malE* gene, as described previously (19). The plasmid pJL282 is a derivative of pTU500-152*Eco*RI, a gift of Ulf Henning (7), and encodes the OmpA-invasin fusion protein OmpA-Inv288. This hybrid protein carries the amino-terminal 152 residues of the *E. coli* OmpA protein fused to the carboxyl-terminal 288 residues of invasin. pRI203*inv*::Tn*phoA*1-4, an *inv* derivative of pRI203 (15), was constructed as described previously, by using λ*b*221 *rex*::Tn*5-phoA* *c*I857 *Pam*80 as a transposon donor (22). The Tn*phoA* insertion is approximately 650 bp 3′ from the start of the *inv* gene (10a).

Overproduction of invasin and invasin derivatives. *E. coli* MC1000 harboring plasmids pRI253 and pGP1 was thermally induced to overproduce invasin as described previously (13). Bacteria were lysed and extracted with Triton X-100–EDTA, and the insoluble debris, which was enriched for invasin,

* Corresponding author.
† Present address: Division of Rheumatology and Immunology, Department of Medicine, Tufts-New England Medical Center Hospital, Boston, MA 02111.

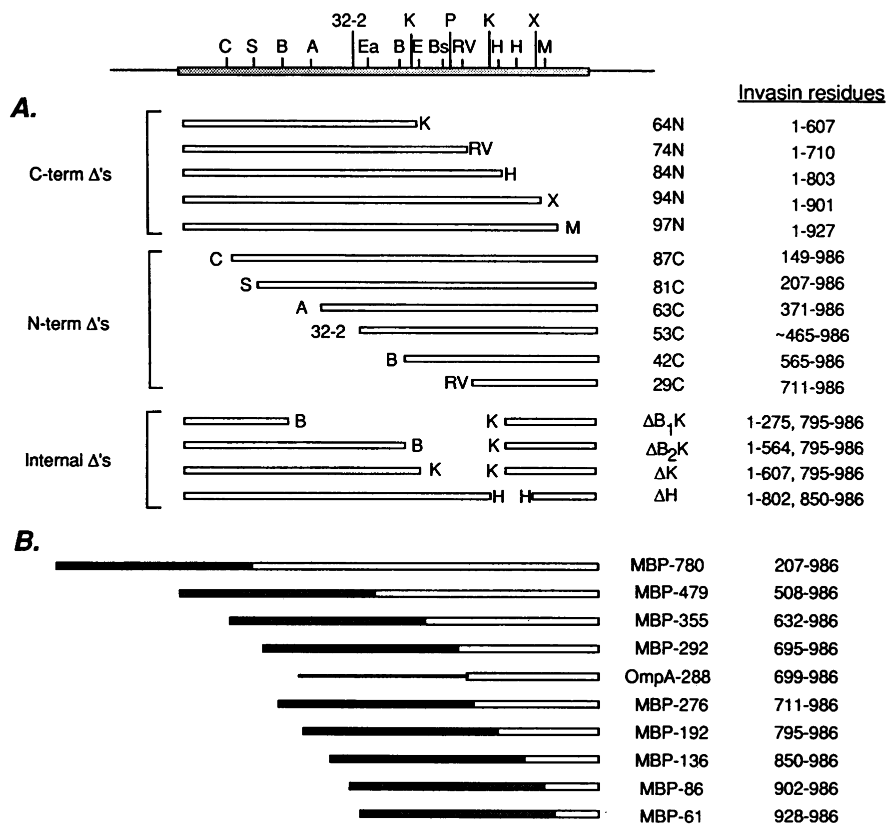

FIG. 1. Invasin derivatives. The *inv* gene is shown schematically above with the restriction sites that were used to generate the deletions and fusion proteins: C, *Cla*I; S, *Sna*BI; B, *Bss*HII; A, *Acc*I; 32-2, the site of a *Hin*dIII linker in an amino terminal deletion derivative of *inv* generated as described previously (15); Ea, *Eag*I; K, *Kpn*I; E, *Eco*0109; Bs, *Bst*YI; P, *Pfl*MI; 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. The designation of each protein fragment is given to the right. The number in each designation corresponds to the approximate molecular mass (in kilodaltons) for each fragment; the N or C proceding the number denotes the intact end of the invasin fragment produced. The four internal deletions are in frame. The invasin residues present in each invasin derivative are given to the far right. (B) Set of invasin fusion proteins analyzed. The MBP portion of each MBP-invasin fusion protein is denoted by shaded bar. The OmpA portion of each OmpA-invasin fusion protein is denoted by a line. The designation of each fusion protein is given to the right. The number in each designation denotes the number of invasin residues present in each protein. The invasin residues present in each hybrid protein are given to the far right.

was used for immunization and for cell attachment experiments. In addition, *E. coli* membrane fractions were similarly prepared from MC1000 harboring pT7-4 and pGP1 to generate negative control membranes that did not contain invasin.

The invasin deletion derivatives were overproduced by thermal induction of *E. coli* MC1000 harboring the *inv* deletion plasmids and pGP1. OmpA-Inv299 was produced by isopropyl-β-D-thiogalactopyranoside induction of a mid-logarithmic culture of *E. coli* 71-18/pJL282. *E. coli* SR2 harboring *malE-inv* gene fusion plasmids was similarly induced to express MBP-invasin fusion proteins (19). Purification of MBP-invasin hybrid proteins by using cross-linked amylose in affinity chromatography was performed as described previously (19, 21). Bacterial extracts containing invasin derivatives were prepared as described previously (19).

Coating microtiter wells with invasin and invasin derivatives. Invasin-containing and control membrane fractions were fixed to 96-well microtiter plates (ICN Flow Laboratories, Costa Mesa, Calif.) for cell binding assays and enzyme-linked immunosorbent assays (ELISAs) as follows. One hundred microliters of 10-μg/ml poly-L-lysine (Sigma Chemical Co., St. Louis, Mo.) in phosphate-buffered saline (PBS) was added to each well and incubated for 30 min at room temperature. The wells were washed two times in PBS, and 50 μl of 100-μg/ml membrane fraction in PBS was added. The microtiter plates were centrifuged for 10 min at 1,000 rpm, and incubated overnight at 4°C. Then 50 μl of 0.25% glutaraldehyde (Sigma) was added per well. After incubation for 10 min at room temperature, the plates were washed in PBS and stored in 100 mM glycine–0.1% bovine serum albumin (BSA) in PBS at 4°C. To prepare microtiter wells coated with whole *E. coli* MC4100/pRI203 cells, bacteria were grown in 80 ml of L broth supplemented with 100 μg of ampicillin per ml to an optical density at 600 nm of 0.4, washed once in PBS, and resuspended in 50 ml of PBS. Then 50 μl of bacteria was added to polylysine-treated microtiter wells, and the plates were centrifuged for 10 min at 1,000 rpm in an IEC tabletop centrifuge fitted with microtiter plate carriers and fixed in glutaraldehyde as described above.

Microtiter wells were also coated with the 53-kDa carboxyl-terminal invasin fragment InvΔ53C for cell binding studies. InvΔ53C (100 μl), purified as described previously (19), was used to coat wells at a concentration of 2 μg/ml in

50 mM sodium bicarbonate (pH 9.6) for 2 h at 37°C. The plates were then washed three times with PBS and blocked overnight at 4°C in 0.2% BSA in PBS.

Production of MAbs directed against invasin. Membrane fractions enriched for invasin were used to immunize RBF/DnJ mice. Spleen cells from two immune mice, as determined with the ELISA (4), were fused to the Fox-NY myeloma cells (34). Nine hundred and sixty wells were tested for production of antibody directed against invasin by using standard ELISAs with either invasin-containing or negative control (without invasin) membrane fractions as the antigen. Immunoblotting (36) confirmed that approximately 180 wells produced antibody against invasin. Thirty-two hybridomas that produce anti-invasin MAb were cloned two times on soft agar (3), and supernatants from these hybridomas were used in this study. The titers of hybridoma supernatants containing anti-invasin MAbs were determined by using microtiter wells coated with full-length invasin, and supernatants were adjusted to identical relative concentrations for the assays described in this work. Immunoglobulin isotypes were determined with the ELISA by using a commercially available kit according to the instructions of the supplier (Bio-Rad Laboratories, Richmond, Calif.).

MAbs were purified by affinity methods on a 3-ml protein A-Sepharose 4B column (Pharmacia LKB Biotechnologies, Piscataway, N.J.) as described previously (37). Only the heavy and light chains were apparent when analyzed by gel electrophoresis. The concentrations of antibodies were determined by using a protein assay (20).

Mapping epitopes recognized by anti-invasin MAbs. The epitopes recognized by the anti-invasin MAbs were mapped by using standard ELISA and immunoblotting techniques in which the invasin deletion derivatives and fusion proteins were the antigens. Both soluble and insoluble extracts were prepared from bacteria that overproduce invasin derivatives, as described previously (19).

The bacterial extracts were also spotted onto nitrocellulose membranes to test for reactivity with hybridoma supernatants. One to four microliters, containing approximately 10 μg of extract, was spotted onto a nitrocellulose filter (Millipore Corp., Bedford, Mass.) and allowed to dry. After the reaction was blocked in PBS with 5% nonfat dry milk, the filter was washed three times in 0.5% Tween 20 in PBS and incubated for 2 h at room temperature in hybridoma supernatant diluted in RPMI 1640 to a relative final concentration of 5 arbitrary units, defined as fivefold the concentration required for half-maximal binding in a standard ELISA. After the filter was washed two times in 0.5% Tween 20 in PBS, bound MAb was revealed by using a secondary antibody, goat anti-mouse antibody conjugated to alkaline phosphatase, as recommended by the manufacturer (Promega Biotech, Madison, Wis.).

Immunoblot assays were performed to confirm the preliminary mapping data derived from the ELISA and the filter spot assay. Invasin-containing cell extracts were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by standard techniques (17), with the modification that samples were heated only to 37°C before loading. After SDS-polyacrylamide gel electrophoresis, proteins were electrophoretically transferred to Immobilon filters (PVDF; Millipore), and the filters were processed as previously described (19). As in the filter spot assays, hybridoma supernatants were diluted to a relative concentration of 5 arbitrary units to allow direct comparison of each MAb.

Identification of surface-exposed epitopes on whole bacteria. Each MAb was tested for reactivity with whole Inv^+ bacteria to identify those that recognize bacterial surface-exposed epitopes. *E. coli* MC4100 harboring the invasin-encoding plasmid pRI203 (12) was grown to saturation in 1 ml of L broth supplemented with 100 μg of ampicillin per ml, washed two times in PBS, and resuspended in the original volume of immunofluorescence (IF) buffer (100 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.0]). The bacteria were incubated for 30 min at 37°C with hybridoma superna-

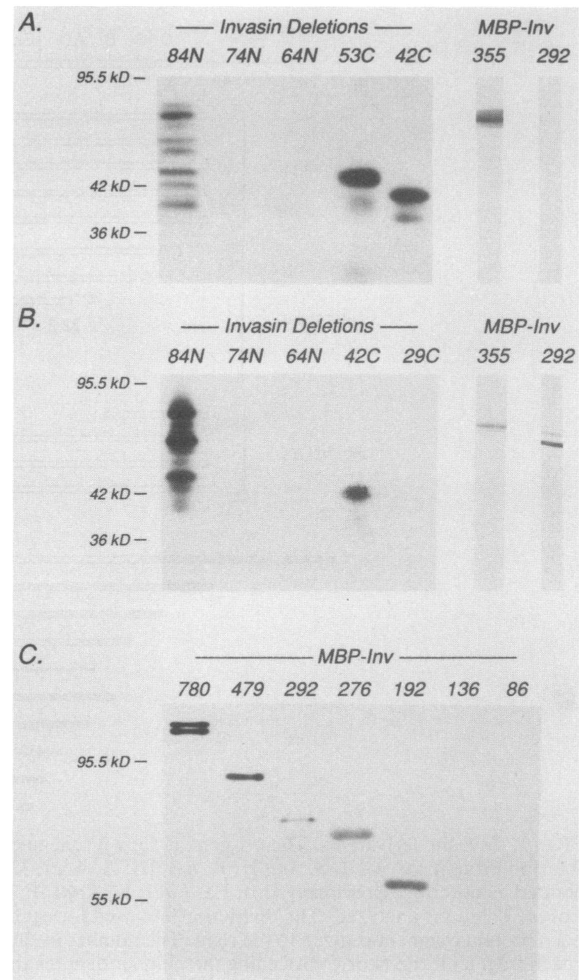

FIG. 2. Immunoblots of invasin deletion derivatives and fusion proteins. The invasin derivative loaded in each lane is given above the lane. The electrophoretic migration of molecular weight markers is shown at left. (A) Immunoblot with MAb 6H10-4. Approximately 5 μg of cell extract containing the designated invasin derivative was loaded in each lane. Each filter was incubated with MAb 6H10 supernatant diluted to a final concentration of 5 arbitrary units, i.e., fivefold more concentrated than the dilution required to give a half-maximal signal in a standard ELISAs. Bound antibody was revealed by using goat anti-mouse antibody linked to alkaline phosphatase (see Materials and Methods). The filter strips at right (MBP-Inv355 and MBP-Inv292) were each prepared from a different gel than that used for the deletion derivative immunoblot. (B) Immunoblot with MAb 1B3-3. Filters were prepared as in panel A. (C) Immunoblot with MAb 2G2-4. Approximately 0.2 μg of each designated purified MBP-invasin hybrid protein was loaded in each lane. The filter was incubated with 0.8 μg of MAb 2G2-4 per ml, and bound antibody was revealed as described above.

TABLE 1. Immunoblot reactivities of MAbs to invasin deletion fragments

MAb	Immunoreactivity[a] of MAb to:															
	WT[b]	N-terminal Inv fragments[c]					C-terminal Inv fragments						Internal deletions[d]			
		97N	94N	84N	74N	64N	87C	81C	63C	53C	42C	29C	ΔB_1K	ΔB_2K	ΔK	ΔH
2F4-2	+	+	ND[d]	+	+	+	ND	ND	+	−	ND	ND	−	+	+	+
1E8-2	+	+	ND	+	+	+	ND	ND	+	−	−	ND	−	+	+	+
1D11-1	+	+	+	+	+	+	+	+	+	−	ND	ND	ND	+	+	+
3H2-1	+	ND	ND	ND	+	+	ND	+	+	−	−	ND	−	+	+	ND
4A7-8	+	ND	ND	ND	ND	+	ND	+	+	−	−	ND	−	+	+	ND
6A3-1	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+	+
4H11-1	+	ND	+	+	+	+	ND	ND	+	+	ND	ND	ND	ND	+	ND
2C8-2	+	ND	ND	ND	ND	+	ND	ND	ND	+	−	ND	ND	−	ND	ND
4C1-4	+	+	+	+	+	+	+	+	+	+	−	ND	−	−	+	+
4D4-3	+	ND	ND	ND	ND	+	ND	ND	ND	+	−	ND	−	−	+	ND
3C8-1	+	ND	ND	ND	ND	+	ND	ND	ND	+	−	−	ND	−	−	ND
9G3	+	+	ND	+	+	−	ND	+	ND	+	ND	ND	ND	ND	−	+
6H10-4	+	ND	ND	+	−	−	ND	ND	ND	+	+	−	−	−	ND	+
10G2-20-1	+	ND	ND	+	−	−	ND	ND	ND	+	+	−	ND	ND	ND	+
1B3-3	+	ND	ND	+	−	−	ND	ND	ND	+	+	−	−	−	−	+
2A1-6	+	+	+	+	−	−	ND	+	+	+	+	−	ND	−	−	+
1D1-1	+	ND	ND	+	−	−	ND	ND	+	+	+	−	−	−	−	ND
1H6-1	+	ND	ND	+	−	−	ND	ND	ND	+	+	ND	ND	ND	−	+
2B10-1	+	ND	ND	+	−	−	ND	ND	ND	+	+	ND	−	−	−	ND
1A4-4	+	+	ND	+	−	−	ND	ND	+	+	+	−	−	−	−	+
3H10-1	+	ND	ND	+	−	−	ND	ND	ND	+	+	−	−	−	−	+
3E1-4	+	ND	ND	+	−	ND	ND	ND	ND	+	+	ND	ND	ND	−	+
2D11-4	+	ND	ND	+	−	−	ND	ND	ND	ND	+	−	ND	−	−	+
2F11-12-1-2	+	ND	ND	+	−	ND	ND	ND	ND	ND	+	−	ND	−	ND	+
2A9-1	+	+	+	+	−	−	ND	+	+	+	+	+	−	−	−	+
1D8-2	+	−	ND	−	ND	ND	ND	+	+	+	+	ND	ND	−	−	−
2G2-4	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	−
3A2-1	+	ND	ND	−	−	ND	ND	+	+	+	+	+	ND	+	+	−
1B10-8	+	−	ND	−	−	−	ND	+	ND	+	+	+	+	+	+	−
6D7-1	+	−	ND	−	ND	ND	+	+	+	+	+	+	+	+	+	−
4B10-9	+	−	ND	−	−	−	ND	+	+	+	+	ND	ND	+	+	−
9G11-9A	+	ND	ND	ND	ND	ND	ND	ND	+	+	+	+	+	+	ND	−

[a] +, immunoreactive; −, not immunoreactive; ND, not determined.
[b] Wild-type (full-length) invasin.
[c] Protein fragment designations are as given in Fig. 1. The number in each designation corresponds to the approximate molecular mass in kilodaltons. N or C denotes the intact amino-terminal or carboxyl-terminal end of each fragment.
[d] Internal deletion designations are as shown in Fig. 1.

tants at a concentration of 5 arbitrary units, as defined above. Bacteria were washed three times in IF buffer and resuspended in IF buffer with 1% BSA and fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G antibody diluted 1:100. Cells were then incubated in the dark at 30°C for 2 h and washed three times in IF buffer. Fluorescence was visually evaluated by using fluorescence microscopy. MC4100/pRI203*inv*::Tn*phoA* was used as a negative control for all experiments.

Inhibition of HEp-2 cell binding by MAbs directed against invasin. To demonstrate invasin-mediated cell binding and inhibition by hybridoma supernatants, 96-well microtiter dishes (ICN) were coated with either invasin fragment InvΔ53C purified as described previously (19), membrane fractions containing full-length invasin, or whole bacteria expressing invasin prepared as described above. The results of blocking experiments were similar for all three methods of microtiter well coating. Invasin-coated wells were preincubated for 90 min at room temperature with 50 μl of supernatant or purified MAbs. HEp-2 cells, dispersed from confluent monolayers as described previously (9, 30), were resuspended at 5×10^5 cells per ml in RPMI 1640 containing 20 mM HEPES (pH 7.0) and 0.4% BSA. One hundred microliters of this cell suspension was then added to each well, and cells were allowed to bind for 30 min at 37°C. After HEp-2 cells were washed three times with PBS, bound cells were quantitated by using a substrate for the lysosomal enzyme hexosaminidase (18). Cell binding was expressed as a percentage of maximal cell attachment, i.e., attachment in identical buffer but in the absence of MAb.

Inhibition of HEp-2 cell penetration by MAbs directed against invasin. Inhibition of cellular entry was determined using the previously described gentamicin protection assay (12). Briefly, approximately 10^7 MC4100/pRI203 cells were incubated for 30 min at 37°C in hybridoma supernatants. The bacteria then were allowed to bind to HEp-2 cell monolayers for 90 min on ice and then to penetrate the HEp-2 cells during a 1-h incubation at 37°C in a 5% CO_2 atmosphere. Monolayers were washed three times with PBS to remove nonadherent bacteria, and RPMI 1640 containing 20 mM HEPES (pH 7.0), 0.4% BSA, and 50 μg of gentamicin per ml was added. After 1 h at 37°C, monolayers were washed three times in PBS; intracellular bacteria, which are protected from gentamicin treatment, were quantitated by lysing the monolayers in 0.5% Triton X-100 and plating on standard bacteriological medium.

TABLE 2. Immunoblot reactivities of MAbs to invasin fusion proteins

MAb	Immunoreactivity[a] of MAb to invasin fusion protein[b]:									
	MBP-780 (207)	MBP-479 (508)	MBP-355 (632)	MBP-292 (695)	OmpA-288 (699)	MBP-276 (711)	MBP-192 (795)	MBP-136 (850)	MBP-86 (902)	MBP-61 (928)
2F4-2	ND	−	−	−	−	−	−	−	ND	−
1E8-2	ND	−	ND	ND	−	−	−	ND	ND	ND
1D11-1	ND	−	ND	ND	ND	ND	ND	ND	ND	ND
3H2-1	ND	−	ND	ND	ND	ND	ND	ND	ND	ND
4A7-8	ND	−	ND	ND	ND	ND	ND	ND	ND	ND
6A3-1	ND	−	ND	ND	ND	ND	ND	ND	ND	ND
4H11-9	ND	+	ND	ND	ND	−	−	ND	ND	ND
2C8-2	ND	+	ND	ND	ND	ND	ND	ND	ND	ND
4C1-4	ND	+	ND	ND	ND	ND	ND	ND	ND	ND
4D4-3	ND	+	ND	ND	ND	ND	ND	ND	ND	ND
3C8-1	ND	+	ND	ND	ND	ND	ND	ND	ND	ND
9G3	ND	+	ND	−	−	−	−	ND	ND	ND
6H10-4	ND	ND	+	−	−	ND	ND	ND	ND	ND
10G2-20-1	ND	ND	+	−	−	ND	ND	ND	ND	ND
1B3-3	ND	ND	+	+	−	ND	ND	ND	ND	ND
2A1-6	ND	ND	+	+	−	ND	ND	ND	ND	ND
1D1-1	ND	ND	+	+	−	ND	ND	ND	ND	ND
1H6-1	ND	ND	+	+	−	−	−	ND	ND	ND
2B10-1	ND	ND	+	+	−	ND	ND	ND	ND	ND
1A4-4	ND	ND	ND	ND	+	−	−	ND	ND	ND
3H10-1	ND	ND	+	+	+	−	ND	ND	ND	ND
3E1-4	ND	ND	+	+	+	ND	ND	ND	ND	ND
2D11-4	ND	ND	−	+	+	(+)	−	ND	ND	ND
2F11-12-1-2	ND	ND	ND	ND	+	+	−	ND	ND	ND
2A9-1	ND	ND	ND	ND	+	ND	ND	ND	ND	ND
1D8-2	ND	ND	ND	ND	+	+	+	ND	ND	ND
2G2-4	+	+	+	+	+	+	+	−	−	ND
3A2-1	+	+	+	+	+	+	+	−	−	ND
1B10-8	ND	ND	ND	ND	+	+	+	−	ND	ND
6D7-1	ND	+	+	+	+	+	+	ND	ND	ND
4B10-9	ND	ND	ND	+	+	+	+	ND	ND	ND
9G11-9A	ND	ND	ND	ND	+	ND	+	(+)	ND	ND

[a] +, immunoreactive; (+), weakly immunoreactive; −, not immunoreactive; ND, not determined.
[b] Protein fragment designations are as shown in Fig. 1. The number in each designation corresponds to the number of invasin residues in each fusion protein. The numbers within parentheses indicate invasin residue numbers at the junction of the fusion proteins.

RESULTS

Mapping epitopes of anti-invasin MAbs. A set of invasin deletion derivatives and fusion proteins was used to map the epitopes recognized by 32 MAbs raised against bacterial membrane fractions containing multiple species of invasin (see Materials and Methods). The deletion series, described previously (19) and shown schematically in Fig. 1A, consisted of amino-terminal, carboxyl-terminal, and internal deletion derivatives. The MBP-invasin and OmpA-invasin fusion proteins consisted of various lengths of the carboxyl terminus of invasin fused to the carboxyl terminus of MBP and OmpA protein, respectively (19) (Fig. 1B). Immunoblotting studies with anti-invasin antiserum or a mixture of anti-invasin MAbs confirmed the presence of an invasin derivative of the predicted molecular weight in each of the bacterial extracts used in these experiments.

To localize epitopes preliminarily, bacterial extracts of the invasin derivatives were bound to microtiter wells or nitrocellulose filters and probed with dilutions of hybridoma supernatants (data not shown). Immunoblots were then used to confirm that the MAbs reacted with expected invasin species. Three examples are shown in Fig. 2. The results of these analyses are summarized in Tables 1 and 2. Ten distinct but overlapping epitope-containing regions were identified (Table 3). All of the epitopes were localized to the carboxyl-terminal 616 amino acids (Table 3; Fig. 3). This result is perhaps a reflection of the heterogeneity of the invasin species used for immunization (see Materials and Methods).

At least four of the regions contained epitopes that required structural information from each of two distant regions of the protein, i.e., from both the amino-terminal and the carboxyl-terminal boundaries of a relatively large region of invasin. For example, MAb 2G2-4 recognized an epitope in the amino acid interval 795 to 986, and altering either the amino-terminal boundary to amino acid 850 (MBP-Inv136) or the carboxyl-terminal boundary to amino acid 928 (InvΔ97N) resulted in a loss of recognition (Fig. 2; Tables 1 and 2). Heating the invasin derivatives to 100°C, as opposed to 37°C, in 0.1% SDS also resulted in the loss of the 2G2-4 epitope (data not shown). These observations suggest that at least four epitopes of invasin are discontinuous (as opposed to sequential).

Identification of epitopes exposed on the bacterial surface. Indirect immunofluorescence was used to determine which epitopes were located in recognizable form on the surface of Inv^+ bacteria. *E. coli* MC4100 harboring the *inv*-encoding plasmid pRI203 was incubated with hybridoma supernatants, and bound antibody was revealed with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G antibody (Materials and Methods). Bacterium-associated fluorescence was assessed visually (Fig. 4) and is summa-

TABLE 3. Epitopes of invasin protein

MAb group	Epitope coordinates[a]	MAb(s)	Isotype	Surface exposure[b]	Blocking of:	
					Binding[c]	Invasion[d]
1a[e]	371–564	2F4-2, 1E8-2	IgG2B	+	−	−
1b[e]	371–564	1D11-1, 3H2-1, 4A7-8	IgG1	−	−	−
2	371–607[f]	6A3-1	IgG1	±	−	−
3	508–607	4H11-9, 2C8-2, 4C1-4	IgG2A	±	−	−
		4D4-3	IgG1	−	−	−
		3C8-1	IgG2B	−	−	−
4	508–710	9G3	IgG2B	±	−	−
5	632–803[f]	6H10-4	IgG2A	±	−	−
		10G2-20-1	IgG2B	−	−	−
6	695–803[f]	1B3-3	IgG2B	+	−	−
		2A1-6, 1D1-1, 1H6-1, 2B10-1	IgG2B	±	−	−
7	699–803	1A4-4, 3H10-1	IgG2A	+	−	−
		3E1-4	IgM	ND[g]	−	−
8	711–803	2D11-4	IgG2A	+	−	−
		2F11-12-1-2	IgG1	+	−	−
		2A9-1	IgG2B	+	−	−
9a[h]	795–986[f]	1D8-2	IgG2B	±	−	−
9b[h]	795–986[f]	2G2-4, 3A2-1, 1B10-8, 6D7-1	IgG2A	+	+	+
		4B10-9	IgG1	+	+	+
10	850–986	9G11-9A	IgG1	+	+	+

[a] Epitope coordinates were determined from the data in Tables 1 and 2.
[b] The surface exposure of an epitope was determined using indirect immunofluorescence with the Inv^+ bacterium MC4100/pRI203*inv*$^+$ (see Materials and Methods). +, reactive with whole bacteria; ±, only weakly reactive with whole bacteria or reactivity varied from experiment to experiment; −, nonreactive with whole bacteria.
[c] Ability of hybridoma supernatants to block attachment of HEp-2 cells to microtiter wells coated with purified invasin fragment InvΔ53C, Inv^+ bacteria, or invasin-containing membrane fractions (see Materials and Methods). A given MAb was designated as positive (+) for blocking of binding if the supernatant consistently inhibited cell attachment by fivefold or more, compared with that in hybridoma medium alone.
[d] Ability of hybridoma supernatants to inhibit cellular penetration of the Inv^+ bacterium MC4100/pRI203*inv*$^+$ in the gentamicin protection assay (Materials and Methods). A given MAb was designated as positive (+) for blocking of invasion if supernatant inhibited entry by 50-fold or more. MAbs that were designated as negative (−) for blocking inhibited entry less than threefold compared with that in hybridoma medium alone.
[e] Group 1 MAbs were subdivided into groups 1a and 1b on the basis of their unambiguous differences in bacterial surface reactivity.
[f] Epitope(s) within this group represent(s) discontinuous (as opposed to sequential) antigenic site(s); i.e., protein sequence information from both the amino-terminal and carboxyl-terminal boundaries is required for MAb recognition (derived from the data in Tables 1 and 2; see text). Other epitope interval groups contain epitopes that may or may not be sequential antigenic sites.
[g] ND, not determined.
[h] Group 9 MAbs were subdivided into groups 9a and 9b on the basis of their ability to block binding and penetration.

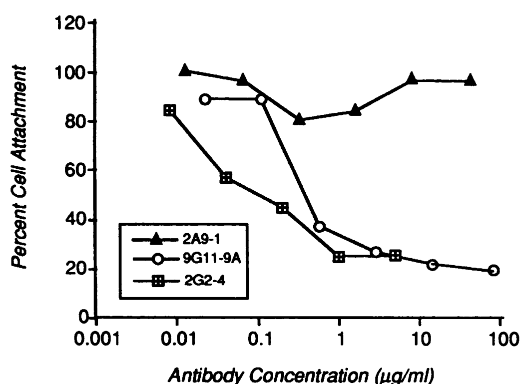

FIG. 3. Inhibition of cell attachment by anti-invasin MAbs. Microtiter wells were coated with purified invasin fragment InvΔ53C and then incubated with appropriate dilutions of MAbs. Live, dispersed HEp-2 cells were added to each well and allowed to attach for 30 min at 37°C (see Materials and Methods). After cells were washed with PBS, cell binding was quantitated by using the colorimetric substrate for hexoseaminidase and measuring optical density at 600 nm. Cell attachment is expressed relative to binding in the absence of antibody. Each point for MAb 2A9-1 represents the average of two determinations. Each point for MAbs 2G2-4 and 9G11-9A represents the average of three determinations.

rized in Table 3. Fourteen of the MAbs consistently recognized whole Inv^+ bacteria, 6 consistently did not, and 12 recognized whole bacteria poorly or only intermittently upon repeated experiments. The ELISA, agglutination assays, and immunoelectron microscopy with whole bacteria gave similar results (data not shown). The MAbs that did not bind whole bacteria may recognize epitopes that are exposed only upon protein unfolding or epitopes that are buried in the bacterial membrane. It is also possible that these MAbs recognize peptide sequences that are located on the surface of the bacterium but are not in a conformation recognized by that MAb.

From these experiments, it is apparent that at least two regions of invasin contain epitopes exposed on the surface of bacteria. The interval of amino acids 371 to 564 contains the surface-exposed epitopes for MAbs 2F4-2 and 1E8-2 (Table 3). These MAbs were classified as group 1a antibodies to distinguish them from group 1b antibodies, which recognize epitopes in this interval that are not present on the bacterial surface (Table 3). The second region of invasin that contains surface epitopes is the carboxyl-terminal 292 amino acids. This region, which contains all of the other 12 unambiguously surface-exposed epitopes of invasin, includes the cell binding domain of invasin.

Inhibition of cell binding. We tested each MAb for the ability to block invasin-mediated cell attachment. Invasin-coated microtiter wells were preincubated with superna-

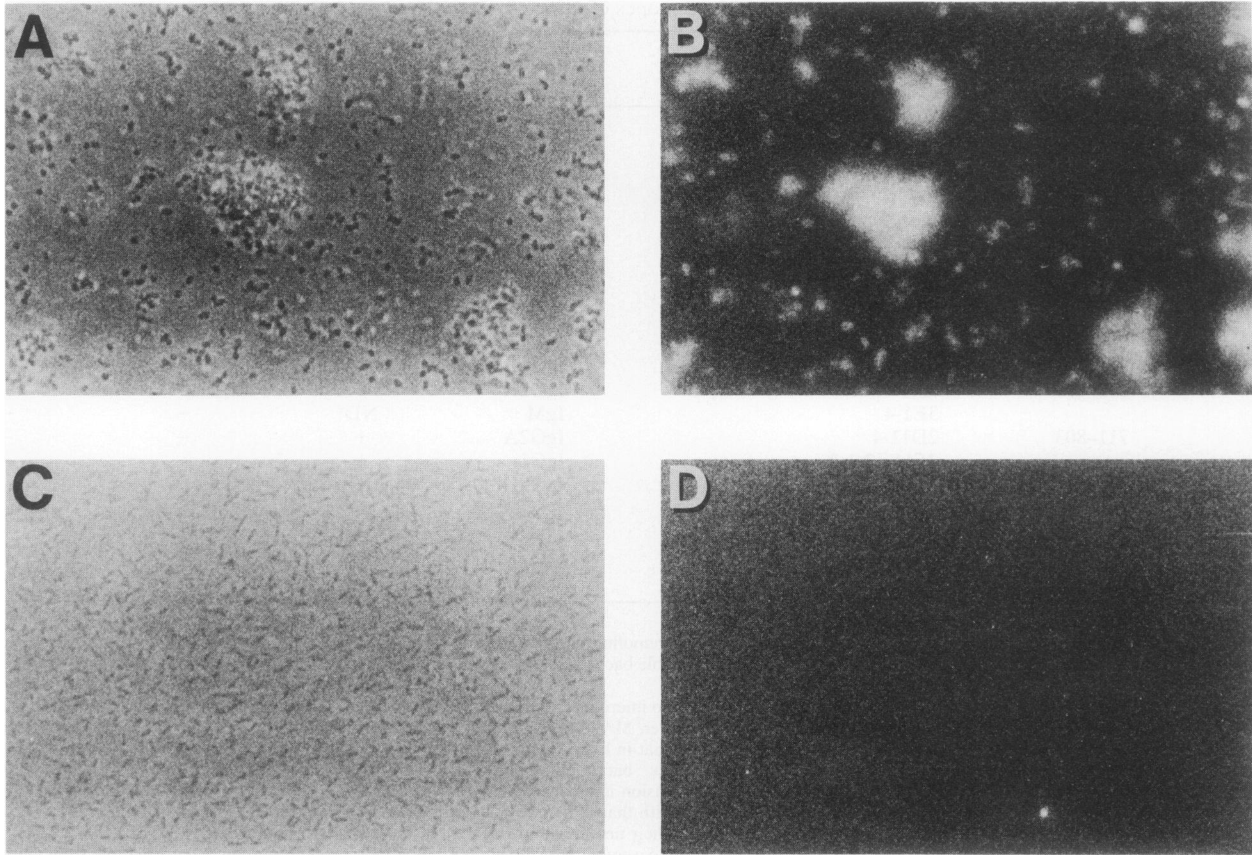

FIG. 4. Indirect immunofluorescent staining of live Inv^+ bacteria. *E. coli* MC4100/pRI203 was incubated for 30 min with hybridoma supernatant diluted to a final concentration of 5 arbitrary units (see Materials and Methods). After washing, bound antibody was revealed by using fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G, and bacteria were visualized with phase-contrast (A and C) or fluorescence (B and D) microscopy at ×400. (A) and (B) MAb 1B10-8, which recognizes a surface-exposed epitope, was used. Bacteria are clumped because MAb 1B10-8 agglutinates Inv^+ bacteria. (C) and (D) MAb 4H11-9, which does not recognize a surface-exposed epitope, was used.

tants, and then HEp-2 cells were added and allowed to bind (Materials and Methods). Six of the 32 supernatants blocked invasin-mediated cell attachment at least 5- to 10-fold (Fig. 3; Table 3). This inhibition was not dependent on the bivalent nature of these immunoglobulin G antibodies, because we showed previously that the Fab fragment of one of the blocking MAbs, 3A2-1, also inhibited cell attachment (19). One of these MAbs, 9G11-9A, recognized an epitope in the carboxyl-terminal 137 residues of invasin, and the other five blocking MAbs, belonging to group 9, recognized epitopes in the last 192 amino acids. MAb 1D8-2 was the only group 9 MAb that did not block cell attachment. This antibody was also the only group 9 MAb that did not efficiently recognize invasin expressed on the surface of whole bacteria. Therefore, all of the MAbs that efficiently bound whole bacteria and recognized an epitope in the cell attachment domain of invasin were able to inhibit invasin-mediated attachment, and no other MAbs blocked this activity.

Inhibition of bacterial invasion. We have previously shown that invasin-mediated binding of mammalian cells is necessary for penetration (13, 14). One would predict, then, that all of the MAbs that inhibit attachment would also inhibit entry. Additionally, if domains other than the binding domain of invasin were also necessary for penetration, MAbs recognizing other regions of invasin might also inhibit entry. Therefore, each hybridoma supernatant was tested for the ability to inhibit invasion of HEp-2 cell monolayers by MC4100/pRI203*inv*$^+$. Bacteria were preincubated with supernatants and then used to infect HEp-2 cells, and intracellular bacteria were quantitated by their survival of gentamicin treatment (Materials and Methods). The six MAbs that blocked cell attachment in microtiter wells also inhibited entry at least 50-fold (Table 3). No other MAbs, including those that recognized whole bacteria, inhibited entry more than two- to threefold relative to that of the control supernatant (containing no MAb). These experiments, then, gave no evidence for the involvement of other domains of invasin in the entry process.

DISCUSSION

We describe in this report the characterization of 32 MAbs directed against the *Y. pseudotuberculosis* invasin protein. The epitopes for each of the MAbs were delineated by using immunoblots with invasin deletion derivatives and fusion proteins, and 10 different but overlapping epitope-containing intervals were identified. Indirect immunofluorescence with whole Inv^+ bacteria identified epitopes that were present on the bacterial surface. Two large regions of invasin, the intervals from amino acids 371 to 564 and 695 to 986,

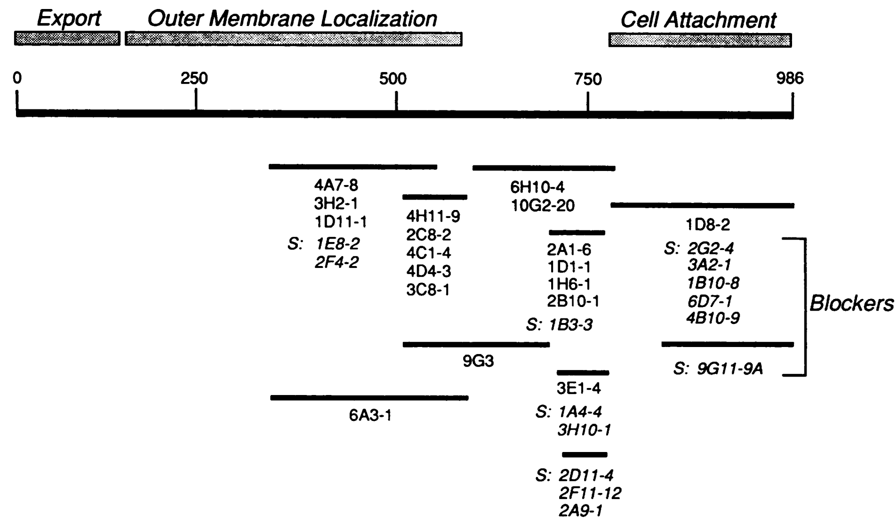

FIG. 5. Epitope map of invasin protein. The linear map of invasin protein (solid horizontal bar) is shown with numbers indicating amino acid residues. Regions of invasin (shaded bars) that have been assigned particular functions include the following: export, the region of protein required for export that can functionally be replaced by MBP; outer membrane localization, the region sufficient to promote localization of invasin to the outer membrane; cell attachment, the region sufficient to promote attachment to cultured mammalian cells (19) (see Discussion). Each of the epitope-containing intervals is represented below by a solid bar, and the MAbs whose epitopes are contained within each interval are listed below each bar. The MAbs in each group that recognize regions of the protein exposed on the bacterial cell surface are listed together in italics (*S*:). The six MAbs that block cell attachment and penetration are indicated by the bracket to the right.

contained surface-localized epitopes (Table 3). Finally, each MAb was tested for the ability to inhibit cell attachment to invasin-coated microtiter wells and bacterial invasion of cultured cell monolayers. All of the MAbs that recognized surface-localized epitopes in the carboxyl-terminal 192 amino acids of invasin blocked both binding and penetration, and no other MAbs inhibited either event.

None of the 32 MAbs recognized epitopes in the amino terminal one-third of the protein. The preparation of invasin used to immunize the mice contained multiple invasin species (some of which lack the amino terminus), and the absence of amino-terminal epitopes identified here may simply reflect an underrepresentation of this region during immunization. Alternatively, the carboxyl-terminal two-thirds of invasin could contain immunodominant epitopes; e.g., perhaps the amino terminus is buried when the protein is in its native conformation. The hydrophobicity analysis of the primary sequence of this region of invasin did not reveal any particularly striking features, although secondary structure analyses indicated a high probability of amphiphilic β sheets as is found in other outer membrane proteins (14a).

A homologous invasin protein has also been identified in the related enteric pathogen *Yersinia enterocolitica* (24). This invasin protein is 73% identical to *Y. pseudotuberculosis* invasin and has also been shown to tightly bind to cultured mammalian cells (38). Given the structural homology, it is not surprising that some MAbs described here reacted with both proteins. The MAbs 1B10-8 and 2A9-1 recognized the *Y. enterocolitica* invasin protein on immunoblots (27). It is likely that this structural homology reflects similarity in the mechanisms of penetration mediated by these two proteins.

We have previously assigned functions to different regions of invasin by analyzing deletion mutants and hybrid proteins of invasin fused to MBP (Fig. 5) (19). The first 148 amino acids contain information required for transport across the inner membrane, because the deletion fragment lacking this region, InvΔ87C, was not exported. This region of the protein can at least partially be replaced functionally by the periplasmic protein MBP, because the MBP-invasin fusion protein MBP-Inv838 (which lacks the amino-terminal 148 residues of invasin) was exported to the surface of the bacterium (19). A deletion mutant containing only the amino-terminal 608 residues was also exported to the outer membrane. These last two observations, taken together, suggest that if an invasin derivative is transported across the inner membrane, the signal(s) sufficient for outer membrane localization lies between residues 149 and 608. Finally, the carboxyl-terminal 192 amino acids, residues 795 to 986, were sufficient to bind mammalian cells (19). Interestingly, the remaining region with no assigned function is apparently largely dispensable, because the interval corresponding to amino acids 594 to 692 is not present in the invasin protein of *Y. enterocolitica* (38).

We have extended these studies by using the set of MAbs described here to identify regions of invasin protein that are exposed on the surface of the bacterium. It should be emphasized that the inability of a MAb to bind invasin on the surface of bacteria does not necessarily indicate that its epitope is buried in the bacterial membrane or in the interior of the folded protein. The MAb may simply recognize an alternative conformation of invasin that is not found when the protein is localized in the outer membrane. Nevertheless, given that the integrin-binding domain is at the carboxyl terminus of invasin, it is not surprising that the last 292 residues contain several epitopes exposed on the outer surface of the bacterium. Most of the epitopes that are unambiguously exposed on the bacterial surface mapped to this region; conversely, most of the epitopes that mapped to this region were surface exposed (Table 3). The region implicated in cell attachment is not the only surface-exposed region, however, because the interval corresponding to amino acids 371 to 564, identified by MAb group 1a, also contains sequences that are exposed on the cell surface.

Potentially, any of the surface-exposed epitopes of invasin could interact with the animal cell surface and play a role in penetration. To assess the involvement of these regions in invasion, we tested each MAb for the ability to inhibit attachment or penetration. Only the MAbs that recognize the integrin-binding domain of invasin were found to block either process. For instance, MAb 2A9-1 recognizes a cell surface-exposed region in the amino acid interval 711 to 803 but was unable to inhibit cellular entry. This suggests that the primary function of the amino-terminal 800 residues is to provide export signals and a stable anchor for the carboxyl-terminal binding domain, which is located on the bacterial surface and thus able to interact with its receptor.

Six of seven MAbs that recognize epitopes in the cell binding domain blocked attachment and entry (Table 3). Only MAb 1D8-2 recognizes the carboxyl terminus of invasin but did not interfere with binding. Apparently this MAb recognizes an epitope that is distinct from the structure on invasin that is recognized by invasin receptors. Consistent with this idea, the 1D8-2 epitope is not efficiently expressed in recognizable form on the surface of Inv$^+$ bacteria. The specific peptide sequence(s) within the cell-binding domain that are critical for binding and entry have not been identified, and the cellular events after invasin binding are unknown. The set of MAbs described here, particularly those that recognize epitopes in the last 192 amino acids, should aid in the further analysis of the structure(s) of invasin recognized by integrin receptors and the mechanism by which invasin promotes cellular attachment and penetration.

ACKNOWLEDGMENTS

We thank Paul Riggs for plasmids pCG806 and pCG807fX, Ulf Henning for plasmid pTU500-152*Eco*RI, and Stanley Tabor and Charles Richardson for pT7-7 and pT7-4. We also thank John Forger of the Harvard Monoclonal Antibody Facility for performing hybridoma fusions and Guy Tran Van Nhieu and Lina Moitoso de Vargas for careful review of the manuscript.

This work was supported by Public Health Service grant R01-AI23538 from the National Institutes of Health, the Center for Gastroenterology Research on Absorptive and Secretory Processes, Public Health Service grant 1 P30 DK39428 awarded by the National Institute of Diabetes and Digestive and Kidney Diseases, and a National Science Foundation Presidential Young Investigator Award to R.R.I. J.M.L. was a Helen Hay Whitney Postdoctoral Fellow and is now a Charles King Trust Research Fellow. R.R.I. was a Searle-Chicago Community Trust Scholar.

REFERENCES

1. **Argraves, W. S., K. Dickerson, W. H. Burgess, and E. Ruoslahti.** 1989. Fibulin, a novel protein that interacts with the fibronectin receptor β subunit cytoplasmic domain. Cell **58:**623–629.
2. **Boyd, D., C. Manoil, and J. Beckwith.** 1987. Determinants of membrane protein topology. Proc. Natl. Acad. Sci. USA **84:** 8525–8529.
3. **Civin, C. I., and M. L. Banquerigo.** 1986. Cloning of murine hybridoma cells in ultra-low gelation temperature agarose. Methods Enzymol. **121:**322–327.
4. **Engval, E., and P. Perlmann.** 1971. Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. Immunocytochemistry **8:**871–874.
5. **Finlay, B. B., and S. Falkow.** 1989. Common themes in microbial pathogenicity. Microbiol. Rev. **53:**210–230.
6. **Finlay, B. B., M. N. Starnbach, C. L. Francis, B. A. Stocker, S. Chatfield, G. Dougan, and S. Falkow.** 1988. Identification of Tn*phoA* mutants of *Salmonella* that are unable to pass through a polarized MDCK epithelial cell monolayer. Mol. Microbiol. **2:**757–766.
7. **Freudl, R., S. MacIntyre, M. Degen, and U. Henning.** 1986. Cell surface exposure of the outer membrane protein OmpA of *Escherichia coli* K-12. J. Mol. Biol. **188:**491–494.
8. **Galan, J. E., and R. Curtiss.** 1989. Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA **86:**6383–6387.
9. **Hayman, E. G., E. Engval, E. A'Hearn, D. Barnes, M. Pierschbacher, and E. Ruoslahti.** 1982. Cell attachment on replicas of SDS polyacrylamide gels reveals two adhesive plasma proteins. J. Cell. Biol. **95:**20–23.
10. **Horwitz, A., K. Duggan, C. Buck, M. C. Beckerle, and K. Burridge.** 1986. Interaction of plama membrane fibronectin receptor with talin—a transmembrane linkage. Nature (London) **320:**531–533.

10a. **Isberg, R. R.** Unpublished data.

11. **Isberg, R. R.** 1990. Pathways for the penetration of enteropathogenic *Yersinia* into mammalian cells. Mol. Biol. Med. **7:**73–82.
12. **Isberg, R. R., and S. Falkow.** 1985. A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K-12. Nature (London) **317:**262–264.
13. **Isberg, R. R., and J. M. Leong.** 1988. Cultured mammalian cells attach to the invasin protein of *Yersinia pseudotuberculosis*. Proc. Natl. Acad. Sci. USA **85:**6682–6686.
14. **Isberg, R. R., and J. M. Leong.** 1990. Multiple β1 chain integrins are receptors for invasin, a protein that promoted bacterial penetration into mammalian cells. Cell **60:**861–871.

14a. **Isberg, R. R., and Y. Lu.** Unpublished data.

15. **Isberg, R. R., D. L. Voorhis, and S. Falkow.** 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50:**769–778.
16. **Kumamoto, C. A., and J. Beckwith.** 1983. Mutations in a new gene, *secB*, cause defective localization in *Escherichia coli*. J. Bacteriol. **154:**253–260.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
18. **Landegren, U.** 1984. Measurement of cell numbers by means of the endogenous enzyme hexosaminidase: application to detection of lymphokines and cell-surface antigens. J. Immunol. Methods **67:**379–388.
19. **Leong, J. M., R. Fournier, and R. R. Isberg.** 1990. Identification of the integrin binding domain of the *Yersinia pseudotuberculosis* invasin protein. EMBO J. **9:**1979–1989.
20. **Lowry, O. H., N. J. Roseborough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
21. **Maina, C. V., P. D. Riggs, A. G. I. Grandea, B. E. Slatko, L. S. Moran, J. A. Tagliamonte, L. A. McReynolds, and C. Guan.** 1988. A vector to express and purify foreign proteins in *Escherichia coli* by fusion to, and separation from, maltose binding protein. Gene **74:**365–373.
22. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82:**8129–8133.
23. **Maurelli, A. T., B. Baudry, H. Dhautevi, T. L. Hale, and P. J. Sansonetti.** 1985. Cloning of plasmid DNA sequences involved in invasion of HeLa cells by *Shigella flexneri*. Infect. Immun. **49:**164–171.
24. **Miller, V. L., and S. Falkow.** 1988. Evidence for two genetic loci in *Yersinia enterocolitica* that can promote invasion of epithelial cells. Infect. Immun. **56:**1242–1248.
25. **Miller, V. L., B. B. Finlay, and S. Falkow.** 1988. Factors essential for the penetration of mammalian cells by *Yersinia*. Curr. Top. Microbiol. Immunol. **138:**15–39.
26. **Moulder, J. W.** 1985. Comparative biology of intracellular parasitism. Microbiol. Rev. **49:**298–337.
27. **Pepe, J. C., and V. L. Miller.** 1990. The *Yersinia enterocolitica inv* gene product is an outer membrane protein that shares epitopes with *Yersinia pseudotuberculosis* invasin. J. Bacteriol. **172:**3780–3789.
28. **Rosqvist, R., S. M., and H. Wolf-Watz.** 1988. Increased virulence of *Yersinia pseudotuberculosis* by two independent mutations. Nature (London) **334:**522–524.

29. **Ruoslahti, E.** 1988. Fibronectin and its receptors. Annu. Rev. Biochem. **57:**375–413.
30. **Ruoslahti, E., E. G. Hayman, M. Pierschbacher, and E. Engval.** 1983. Fibronectin: purification, immunochemical properties, and biological activities. Methods Enzymol. **82:**803–831.
31. **Sansonetti, P. J., P. Clerc, A. T. Maurelli, and J. Mounier.** 1986. Multiplication of *Shigella flexneri* within HeLa cells—lysis of the phagocytic vacuole and plasmid-mediated contact hemolysis. Infect. Immun. **51:**461–469.
32. **Strauch, K. L., and J. Beckwith.** 1988. An *Escherichia coli* mutation preventing degradation of abnormal periplasmic proteins. Proc. Natl. Acad. Sci. USA **85:**1576–1580.
33. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
34. **Taggart, R. T., and S. I. M. Samloff.** 1983. Stable antibody-producing murine hybridomas. Science **219:**1228–1230.
35. **Takeuchi, A.** 1967. Electron microscope studies of experimental *Salmonella* infection. I. Penetration into the intestinal epithelium by *Salmonella typhimurium*. Am. J. Pathol. **50:**109–136.
36. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
37. **Underwood, P. A., J. F. Kelly, D. F. Harmon, and H. M. MacMillan.** 1983. Use of Protein A to remove immunoglobulin from serum in hybridoma culture media. J. Immunol. Methods **60:**33–45.
38. **Young, V., V. Miller, S. Falkow, and G. K. Schoolnik.** 1990. Sequence, localization and function of the invasin protein of *Yersinia enterocolitica*. Mol. Microbiol. **4:**1119–1128.