INFECTION AND IMMUNITY, Sept. 1992, p. 3909–3912
0019-9567/92/093909-04$02.00/0

Vol. 60, No. 9

The Integrin-Binding Domain of Invasin Is Sufficient To Allow Bacterial Entry into Mammalian Cells

SUSANNAH RANKIN,[1] RALPH R. ISBERG,[1,2]* AND JOHN M. LEONG[1]†

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

Received 2 April 1992/Accepted 15 June 1992

***Yersinia pseudotuberculosis* is able to enter normally nonphagocytic host cells by multiple pathways, the most efficient of which is mediated by invasin, a 986-amino-acid bacterial outer membrane protein. It has previously been shown that the C-terminal 192 amino acids of invasin are sufficient to bind mammalian cells. To determine if additional regions of the invasin protein are necessary to promote entry, we developed a novel assay that tests the ability of various invasin derivatives to confer on *Staphylococcus aureus* the ability to enter animal cells. We determined that the 192-amino-acid cell-binding region of invasin, when used to coat the bacterial cell surface, was also sufficient to promote cellular penetration. These results suggest that the simple binding of invasin to its receptors is sufficient to mediate entry and that the bacterium plays a largely passive role in the entry process.**

Invasin binds to multiple β_1-chain integrins on the host cell surface prior to bacterial uptake (3–6). Integrins that act as invasin receptors include receptors for extracellular matrix components as well as those involved in intercellular interactions (2, 13, 14). The integrin-binding domain of invasin was previously identified by analyzing the products of gene fusions between various regions of the *inv* gene and the *Escherichia coli malE* gene, which codes for maltose-binding protein (MBP). Mammalian cells are able to bind to plastic surfaces coated with hybrid proteins containing at least the C-terminal 192 amino acids of invasin (8). Other regions of the protein might be required to trigger the internalization of bound bacteria. Delineation of the exact region of the invasin protein that is required for entry to occur, however, is technically difficult. Mutations of invasin that remove the amino-terminal end invariably result in proteins that are not localized to the bacterial surface (8), making assessment of entry-promoting activity impossible. We therefore developed a novel technique to identify the region of the invasin protein that is necessary to promote efficient bacterial entry.

Staphylococcus aureus is a gram-positive bacterium that is not efficiently internalized by cultured nonphagocytic cells. Protein A, which is expressed on the surface of the bacterium, binds to the Fc portion of immunoglobulin G, making it possible to coat the bacteria with anti-MBP serum and MBP-invasin hybrids (Fig. 1). For each protein to be tested, 5×10^9 fixed *S. aureus* cells (Pansorbin; Calbiochem) were washed twice in phosphate-buffered saline (PBS) and incubated with 0.5 ml of 2% antiserum in PBS for 1 h at 4°C. The bacterial cells were then washed three times with PBS and incubated for 1 h at 4°C with 0.5 ml of purified protein (50 μg/ml) in PBS. Approximately 10^7 bacteria were then added to 2×10^5 subconfluent HEp-2 cells and incubated for 90 min at 37°C in binding buffer (RPMI 1640, 0.4% bovine serum albumin [BSA], 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.0]). The monolayers were gently washed three times with PBS and fixed in modified Brenner's broth (12) at 4°C for 1 h. After being washed in 0.1 M cacodylate buffer (pH 7.4), the monolayers were dehydrated in ethanol and embedded in Epon 812 (Polysciences). The samples were sectioned and stained with Reynolds lead citrate (11). MBP-invasin hybrid protein was purified as described previously (8).

MBP-invasin attached in this fashion is able to promote cellular entry of *S. aureus* (Fig. 2). Bacteria coated with MBP-Inv479, which contains the C-terminal 479 amino acids

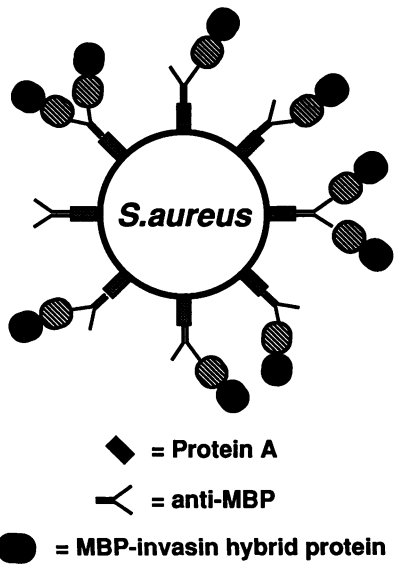

FIG. 1. Use of *S. aureus* to test entry proficiencies conferred by various hybrid proteins. *S. aureus* was grown to mid-logarithmic phase and washed several times. The bacteria were then coated with antiserum directed against MBP by incubating them in antiserum diluted in PBS, washing again with PBS, and coating with MBP-invasin fusion protein. After additional PBS washes, the bacteria were incubated with HEp-2 cells and tested for animal cell penetration either by microscopic observation or in a gentamicin protection assay.

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

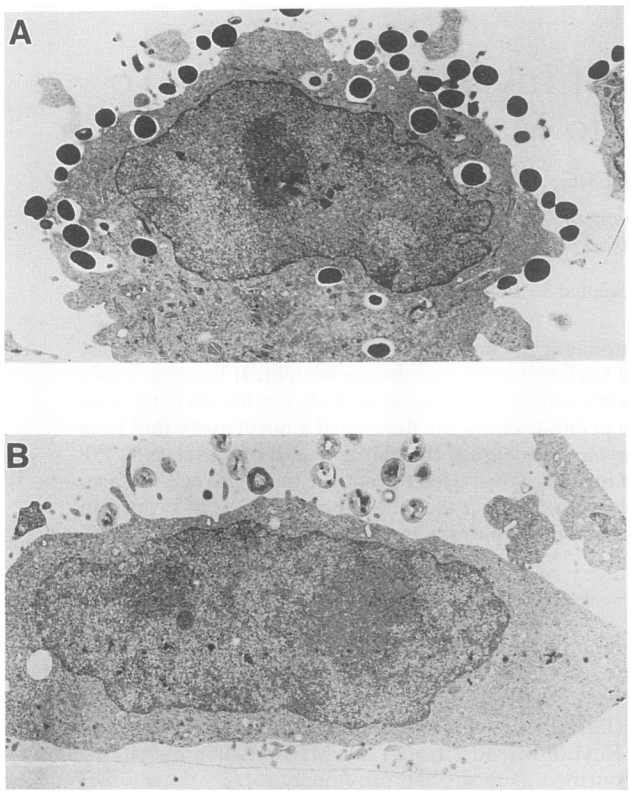

FIG. 2. Bacteria coated with MBP-invasin hybrid protein enter mammalian cells. *S. aureus* coated with MBP-Inv479 bound and entered animal cells (A), whereas the same bacteria coated with fibronectin were able to bind to the microvillar surface but were not able to enter the animal cells (B). Bacteria coated with MBP or left uncoated did not adhere to animal cells (not shown). Rabbit polyclonal anti-MBP serum (a generous gift of C. Kumamoto) was used for the MBP-invasin hybrid (A), and bovine anti-human fibronectin (Calbiochem) was used to bind fibronectin (120-kDa chymotryptic fragment of human fibronectin containing the cell-binding domain [10]; Telios Pharmaceuticals) (B).

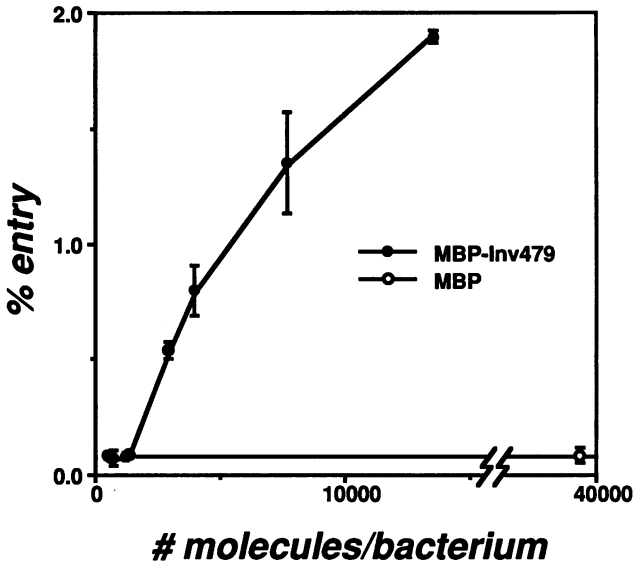

FIG. 3. MBP-invasin hybrid proteins specifically mediate entry of bacteria into cultured mammalian cells. Live *S. aureus* cells were coated with hybrid protein as described for Fig. 2. Entry efficiencies are expressed as percent survival relative to the initial inoculum. Data are plotted to show entry efficiency as a function of the number of molecules of protein per bacterium. Values shown are the averages of four individual assays; error bars indicate the standard deviations of the data.

of invasin, were efficiently internalized by HEp-2 cells. In contrast, untreated *S. aureus* or *S. aureus* coated with MBP did not penetrate the cultured cells. Bacteria coated in a similar manner with fibronectin were able to bind to the surface of HEp-2 cells but were not observed inside of these cells (Fig. 2B). The interaction of MBP-Inv-coated *S. aureus* with HEp-2 cells appeared similar to that seen with *Yersinia pseudotuberculosis* or with *E. coli* expressing the *inv* gene (7). Live *S. aureus* showed similar results, but levels of extracellular binding of uncoated bacteria were higher (data not shown).

The observed cellular penetration by *S. aureus* coated with MBP-Inv479 was confirmed by using a previously described uptake assay, which is based on the observation that bacteria inside of animal cells are protected from killing by the antibiotic gentamicin (1a, 17). In the assay for which results are shown in Fig. 3, 2% of the initial inoculum of bacteria coated with a high surface concentration of MBP-Inv479 were protected from gentamicin. In contrast, only 0.05% of *S. aureus* coated with MBP alone survived the antibiotic treatment (Fig. 3). The entry efficiency of MBP-Inv-coated *S. aureus* was comparable to that of wild-type *Y. pseudotuberculosis* or *E. coli* harboring the *inv* gene (7) and was dependent upon the presence of anti-MBP serum to coat the bacteria with hybrid protein (Fig. 4).

Cellular entry by *S. aureus* coated with MBP-Inv479 consistently required less than 10^4 MBP-invasin molecules on the bacterial surface. Bacteria were coated with different surface concentrations of fluorescently labeled MBP-Inv479, and entry assays were performed. Purified proteins were labeled with carboxytetramethyl rhodamine, succinimidyl ester (Molecular Probes, Inc.), as described by Pagliaro and Taylor (9). The amount of MBP-invasin on the *S. aureus* cell surface was manipulated by mixing 5×10^9 bacteria with serial twofold dilutions of anti-MBP serum, starting with 0.4% antiserum, washing in PBS, and adding saturating amounts of rhodamine-labeled MBP-Inv479 (approximately 5 μg). These constructions were tested for efficiency of entry in a modification of the gentamicin protection assay described previously (1a, 17). Briefly, 2×10^7 coated *S. aureus* cells were centrifuged at $120 \times g$ for 10 min onto 2×10^5 subconfluent HEp-2 cells and allowed to bind and enter for 90 min at 37°C in RPMI 1640 containing 0.4% BSA and 20 mM HEPES (pH 7.0). Monolayers were washed three times with PBS and treated with 50 μg of gentamicin per ml in binding buffer for 15 min at 37°C to kill extracellular bacteria. After five additional washes with PBS, the animal cells were lysed with 0.5% Triton X-100 in distilled H_2O and CFU were quantitated and compared with the number of bacteria originally added to the monolayer. A portion of each preparation was reserved for quantitation of the hybrid protein on the bacterial surface by analysis in a Titertek Fluoroskan II fluorometer.

The results indicated that approximately 0.5×10^4 to 1×10^4 molecules of MBP-Inv479 per bacterium were sufficient to mediate efficient entry (Fig. 3). By densitometric scanning of immunoblots, it was estimated that a similar number of

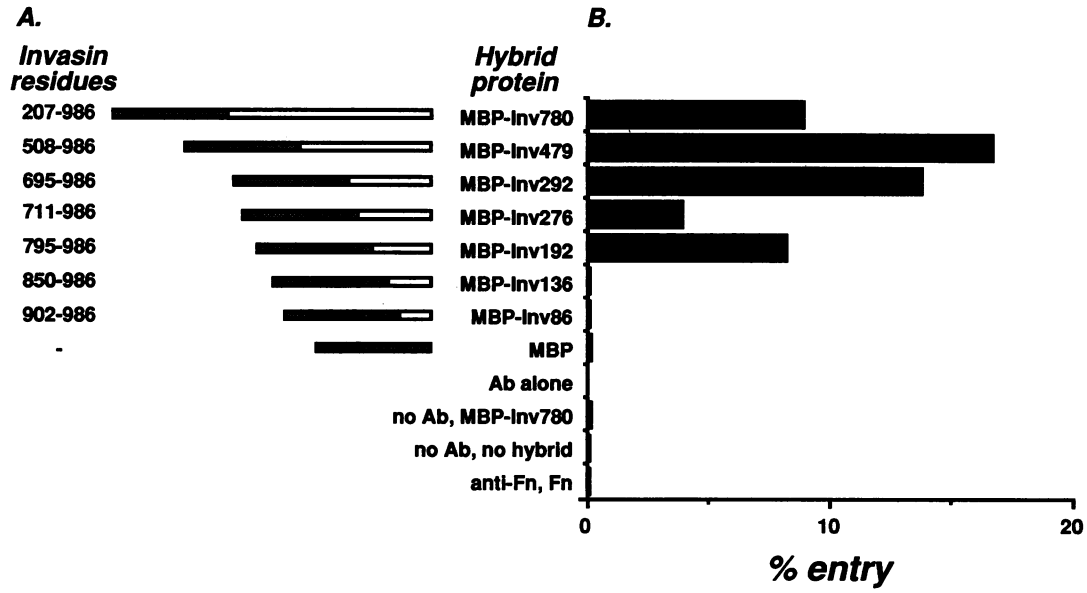

FIG. 4. The C-terminal 192 amino acids of invasin are sufficient to promote entry into mammalian cells. (B) The bar graph shows the efficiency of uptake conferred on *S. aureus* by various MBP-invasin hybrid proteins. (A) Hybrid proteins used in the assay; hatched bars represent MBP, and open bars represent C-terminal fragments of invasin. The experiment was performed four times in duplicate, and experiments done on different days showed similar relative efficiencies of entry. Invasin residues: amino acid sequences from the carboxyl-terminal end of invasin present in the hybrid protein. Hybrid protein: MBP-invasin hybrids containing intact MBP at the amino-terminal end and successively shorter carboxyl-terminal fragments of invasin. Fn, 120-kDa chymotryptic fragment of human fibronectin; Ab, polyclonal anti-MBP serum made in a rabbit; % entry, percentage of bacteria which survive gentamicin killing as described in the text. High efficiencies of entry, compared with those in Fig. 3, are a reflection of lower multiplicities of infection in this particular experiment.

invasin molecules was expressed in wild-type *Y. pseudotuberculosis* YPIII (data not shown).

The assay described above allows a test of whether the cell-binding C-terminal 192 amino acids of invasin are sufficient to promote bacterial entry or whether other regions of the protein are required. MBP-Inv hybrid proteins containing various portions of the C terminus of invasin were tested for their ability to promote the entry of *S. aureus* into HEp-2 cells (Fig. 4). *S. aureus* was coated with previously described MBP-invasin hybrids (8) as shown in Fig. 2 and used in a gentamicin protection assay. *S. aureus* coated with hybrid proteins that include at least the C-terminal 192 amino acids of invasin entered cultured cells efficiently (>4% entry). In contrast, only 0.05% of the infecting bacteria coated with nonbinding hybrids (those that do not mediate cell attachment) survived the gentamicin treatment. This result is comparable to the survival rates of uncoated *S. aureus* and *E. coli* K-12 (Fig. 4) (7). That none of these shorter invasin derivatives mediated entry suggests that an intact cell-binding domain is both sufficient and necessary for entry. Certainly, if invasin has an entry-promoting region in addition to, and separate from, its cell-binding region, we have not been able to identify it with the set of hybrid proteins described here. We cannot rule out the possibility that higher-resolution deletion mapping could reveal two separate activities, or that the smaller hybrid proteins, such as MBP-Inv136, result in unfolding of the entire C-terminal domain, abolishing both activities simultaneously.

The entry efficiencies conferred by binding-proficient hybrids varied from 4 to 18% of the initial inoculum. These differences are reminiscent of differences seen in cell-binding activities (8) and may reflect unequal protein stabilities or affinities or both. For example, MBP-Inv276, which mediated 4% entry, binds HEp-2 cells less efficiently than the other binding-proficient hybrids and is more susceptible to cycles of freezing and thawing (14a).

Invasin-mediated entry, then, appears to result from the microorganism passively binding the appropriate host cell receptor, which causes the host cell to internalize the microorganism. This implies that the only bacterial role in this entry process is to express the protein on its surface, which is consistent with the previous observations that inert particles coated with purified invasin fragments are efficiently internalized by animal cells (1). It is conceivable that *S. aureus* could be contributing to the entry process by providing an uptake signal which requires bacterial adhesion for activity. This is inconsistent with our observation that simply coating *S. aureus* with an adhesin, e.g., fibronectin, does not allow the bacterium to enter mammalian cells. Invasin on the surface of *S. aureus* then apparently does not potentiate the activity of an *S. aureus* factor to allow uptake by the mammalian cells but rather is itself sufficient to mediate the uptake process.

Although the binding of invasin to β_1-chain integrins on HEp-2 cells appears to be sufficient to promote bacterial entry, fibronectin binding to the same receptors is not (Fig. 4) (16). A possible explanation lies in the different binding affinities of invasin and fibronectin. The dissociation constant for invasin binding to $\alpha_5\beta_1$ is 100-fold lower than that for fibronectin (15). Preliminary results with *S. aureus* coated with a collection of anti-integrin antibodies spanning a range of binding affinities are consistent with this affinity-dependent model of β_1-integrin-mediated entry (14a).

It should be noted that the assay described here has allowed us to measure easily the cellular entry promoted by invasin derivatives that are not secreted to the bacterial cell

surface. The assay using *S. aureus* is quantitative, sensitive, and reproducible. The results can be scored with ease by the previously standardized technique of gentamicin protection. As the assay does not require the expression of potential "invasins" or their derivatives on the bacterial cell surface, it may be useful particularly in the study of host cell binding or entry by organisms unrelated to common laboratory bacteria.

We thank Silvie Verdon and Judith Reichler for electron microscopy, Carol Kumamoto for anti-MBP antiserum, Paul Riggs for the MBP fusion vectors, and Guy Tran Van Nhieu for review of the text.

This work was supported by the Center for Gastroenterology Research on Absorptive and Secretory Processes, PHS grant 1 P30 DK39428 awarded by NIDDK, and grant RO1-A123538 from the National Institutes of Health to R.R.I. J.M.L. was a Helen Hay Whitney Fellow. R.R.I. was a Searle-Chicago Community Trust Scholar. S.R. was supported by NIH training grant 5T32GM0730.

REFERENCES

1. **Chen, S. Y., and A. Skvorak.** Unpublished observations.
1a. **Devenish, J. A., and D. A. Schiemman.** 1981. HeLa cell infection by *Yersinia entercolitica*: evidence for lack of intracellular multiplication and development of a new procedure for quantitative expression of infectivity. Infect. Immun. **32:**48–55.
2. **Hynes, R. O.** 1987. Integrins: a family of cell surface receptors. Cell **48:**549–554.
3. **Isberg, R. R.** 1990. Pathways for the penetration of enteroinvasive Yersinia into mammalian cells. Mol. Biol. Med. **7:**73–82.
4. **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.
5. **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.
6. **Isberg, R. R., and J. M. Leong.** 1990. Multiple β_1 integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell **60:**861–871.
7. **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.
8. **Leong, J. M., R. S. Fournier, and R. R. Isberg.** 1990. Identification of the integrin binding domain of the *Yersinia pseudotuberculosis* invasin protein. EMBO J. **9:**1979–1989.
9. **Pagliaro, L., and D. L. Taylor.** 1988. Aldolase exists in both the fluid and solid phases of the cytoplasm. J. Cell Biol. **107:**981–991.
10. **Pierschbacher, M. D., E. G. Hayman, and E. Ruoslahti.** 1981. Location of the cell-attachment site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. Cell **26:**259–267.
11. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17:**208–212.
12. **Sandow, B. A., N. B. West, R. L. Norman, and R. M. Brenner.** 1979. Hormonal control of apoptosis in hamster uterine luminal epithelium. Am. J. Anat. **156:**15–35.
13. **Takada, Y., C. Huang, and M. E. Hemler.** 1987. Fibronectin receptor structures in the VLA family of heterodimers. Nature (London) **326:**607–609.
14. **Takada, Y., J. L. Strominger, and M. E. Hemler.** 1987. The very late antigen family of heterodimers is part of a superfamily of molecules involved in adhesion and embryogenesis. Proc. Natl. Acad. Sci. USA **84:**3239–3243.
14a. **Tran Van Nhieu, G.** Unpublished observations.
15. **Tran Van Nhieu, G., and R. R. Isberg.** 1991. The Yersinia pseudotuberculosis invasin protein and human fibronectin bind to mutually exclusive sites on the $\alpha_5\beta_1$ integrin receptor. J. Biol. Chem. **266:**24367–24375.
16. **Van de Water, L., A. T. Destree, and R. O. Hynes.** 1983. Fibronectin binds to some bacteria but does not promote their uptake by phagocytic cells. Science **220:**201–204.
17. **Vesikari, T., J. Bromirska, and M. Mäki.** 1982. Enhancement of invasiveness of *Yersinia enterocolitica* and *Escherichia coli* in HEp-2 cells by centrifugation. Infect. Immun. **36:**834–836.