

The Invasin Protein of *Yersinia enterocolitica*: Internalization of Invasin-bearing Bacteria by Eukaryotic Cells Is Associated with Reorganization of the Cytoskeleton

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Abstract. *Yersinia enterocolitica*, a facultative intracellular pathogen of mammals, readily enters (i.e., invades) cultured eukaryotic cells, a process that can be conferred by the cloned *inv* locus of the species. We have studied the mechanism by which the product of *inv*, a microbial outer membrane protein termed "invasin," mediates the internalization of bacteria by HEp-2 cells and chicken embryo fibroblasts. Invasin-bearing bacteria initially bound the filopodia and the leading edges of cultured cells. Multiple points of contact between the bacterial surface and the surface of the cell ensued and led to the internalization of the bacterium within an endocytic vacuole; the same multi-step process could be induced by an inert particle coated with invasin-containing membranes. Both adherence and internalization were blocked by an anti-sera directed against the β_1 integrin cell-adherence

molecule. Ultrastructural studies of detergent-insoluble cytoskeletons from infected cells and immunofluorescence microscopy of phalloidin-labeled cells showed alterations in the structure of the cytoskeleton during the internalization process including the accumulation of polymerized actin around entering bacteria. Bacterial entry was prevented by cytochalasin D indicating that the internalization process requires actin microfilament function. Possible linkages between β_1 containing integrins and the cytoskeleton were examined during the internalization process through the use of protein-specific antibodies and immunofluorescence microscopy. Like actin, the actin-associated proteins filamin, talin and the β_1 integrin subunit were also found to accumulate around entering bacteria. These findings suggest that the invasin-mediated internalization process is associated with cytoskeletal reorganization.

SEVERAL species of the tribe Enterobacteriaceae enter epithelial cells and other non-professional phagocytes while infecting mammalian hosts (Devenish and Scheimann, 1981; Formal et al., 1983; Finlay and Falkow, 1989). This process, which has been termed "invasion," appears to be a crucial pathogenic trait. The invasive phenomenon is exemplified by *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* and contributes to their capacity to cause a variety of gastrointestinal infections in humans (Weber et al., 1970; Feeney et al., 1987). Examination of infected tissues from patients with yersinosis shows the intracellular location of these bacteria; their invasive ability can also be demonstrated experimentally by in vitro studies with a variety of cultured mammalian cells (Miller et al., 1988). While the *Yersiniae* can enter cells by several different mechanisms (Isberg, 1989), the best defined pathway is through the action of "invasin." The invasins of *Y. enterocolitica* and *Y. pseudotuberculosis* are homologous, but non-identical outer membrane proteins specified by the *inv* loci. The capacity of these proteins to confer the invasive phenotype has been proven conclusively by experiments showing that the acquisition of a plasmid containing the *inv* locus by a normally non-

invasive *Escherichia coli* yields a recombinant strain which is fully invasive of cultured cells (Isberg et al., 1987; Young et al., 1990).

The invasins of *Y. pseudotuberculosis* interacts with members of the integrin superfamily of eukaryotic cell adhesion molecules (Isberg and Leong, 1990). The integrins are large, membrane-spanning cell surface α/β heterodimers that are involved in cell-adhesion processes including the attachment of cells to the extracellular matrix (Hynes, 1987; Buck and Horwitz, 1987). As such, the integrins are ideally deployed to form a transmembrane link between the extracellular environment of the cell and the cytoskeleton (Burrige et al., 1988; Geiger, 1989). These structural relationships and the repertoire of functions now attributed to the cytoskeleton indicate that several of the proteins that comprise it might participate in the internalization of invasins-bearing bacteria. Indeed, structural changes in the cytoskeleton are evident during the entry of several other invasive pathogens. The penetration of HeLa cells by *Shigella flexneri* is accompanied by actin polymerization and myosin accumulation around the internalized bacteria (Clerc and Sansonetti, 1987). Similar rearrangements in actin are seen during the

invasion, movement and spread of *Listeria monocytogenes* in eukaryotic cells (Tilney and Portnoy, 1989). In contrast, little is known about whether or how the invasin proteins of the *Yersiniae* might interact with the cytoskeleton during the internalization process.

In the present study, we have examined the interaction between an invasin-expressing recombinant strain harboring the *inv* locus of *Y. enterocolitica* and cultured epithelial cells. Biochemical and morphological studies of the cytoskeleton of infected cells were undertaken and revealed that the invasin-mediated internalization process is accompanied by reorganization of the cytoskeleton.

Materials and Methods

Bacteria, Plasmids, and Cell Culture

E. coli strains were maintained at -70°C in Luria broth (LB)¹ medium (Miller, 1972) containing 40% (vol/vol) glycerol or on LB agar plates. Strains carrying recombinant plasmids were maintained on LB agar plates containing 100 $\mu\text{g}/\text{mL}$ ampicillin.

The invasin overproducing plasmid pYIT1 (Young et al., 1990), carries the *inv* gene under the control of the inducible *tac* promoter. Overproduction of invasin was accomplished by adding 1 mM IPTG (Sigma Chemical Co., St. Louis, MO) to the growth medium of cells carrying pYIT1. Preparation of bacterial outer and cytoplasmic membranes was performed as described earlier (Young et al., 1990).

Human laryngeal epithelial (HEp-2) cells were maintained in DME supplemented with 10% FBS, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (pen/strep). Chicken embryo dermal fibroblasts (CEFs) were obtained from 8–12-d-old embryos and grown in DME supplemented with non-essential amino acids (NEAA), 10% FBS, and pen/strep. CEFs were used for a maximum of five passages before being discarded.

For immunofluorescence, 5×10^4 CEFs were seeded onto 12-mm-round 2 coverslips in a 24-well microtiter plate 12 h before infection with bacteria.

Antibodies

Rabbit anti-*E. coli* antiserum was obtained from Axell (Accurate Chemical, Westbury, NY). Monoclonal anti-*E. coli* OmpF was obtained from Biodesign International (Kennebunkport, ME). Monospecific, polyclonal goat anti- β_1 serum was a gift of Martin Hemler (Dana Farber Cancer Institute, Boston, MA) (Chan et al., 1991). This serum had been elicited against a purified very late antigen protein and the serum refined by affinity purification and shown by immunoprecipitation experiments to be specific for the M_r 130,000 beta subunit. Rabbit antiserum directed against the cytoplasmic domain of the β_1 integrin subunit was a gift of Richard Hynes (Marcantonio and Hynes, 1988). Rabbit anti-talin serum was kindly provided by Keith Burridge (Department of Anatomy, University of North Carolina, Chapel Hill, NC). Monoclonal anti-filamin was purchased from Sigma Chemical Company. mAb directed against α -tubulin, Texas red, and FITC donkey anti-rabbit IgG and Texas red sheep anti-mouse IgG were purchased from Amersham (Arlington Heights, IL). Dichlorotrazinylaminofluorescein (DTAF)-labeled donkey anti-mouse sera was purchased from Jackson ImmunoResearch (West Grove, PA). Optimal concentration for each antibody was determined by a dilution experiment.

Adherence and Invasion Assays

Adherence of mammalian cells to invasin-containing outer membranes and fibronectin was performed as described earlier (Young et al., 1990). Briefly, cells were labeled with ^{35}S methionine and then removed from plates with EDTA. Suspended cells were then added to 48-well microtiter dishes previously coated with outer membranes or fibronectin. Adherence was determined by the number of input counts that remained bound after 30 min at 37°C . Blocking of adherence using a goat anti- β_1 antiserum was accom-

plished by preincubating the suspended cells with dilutions of the serum, which was also present during the assay.

Entry into cultured cells was measured in a manner similar to that described previously (Finlay and Falkow, 1988). 24-well microtiter plates were seeded with 5×10^4 HEp-2 cells/well in media without antibiotics 24 h before the assay. 1×10^7 bacteria were added to the cell monolayers and incubated for 2 h at 37°C , 5% CO_2 . The wells were then washed $2 \times$ with PBS and extracellular bacteria were killed by adding fresh tissue culture media to which 100 $\mu\text{g}/\text{mL}$ gentamicin was added. After 2-h incubation at 37°C , 5% CO_2 the monolayers were washed $3 \times$ with PBS, lysed with 0.1 ml 1% Triton X-100, and then viable counts determined by titration on LB agar with 100 $\mu\text{g}/\text{mL}$ ampicillin. To determine cell-associated bacteria (i.e., adherent and internalized) the gentamicin treatment was omitted. The monolayers were instead washed $7 \times$ with PBS after infection and then immediately lysed and plated.

In experiments where cytochalasin was used, tissue culture media containing dilutions of cytochalasin D (Sigma Chemical Co.) prepared from a 1 mg/ml stock in DMSO (Sigma Chemical Co.) was added to wells and the monolayers incubated at 37°C , 5% CO_2 for 30 min before the addition of bacteria.

Coating of Latex Microspheres with Bacterial Membranes

Bacterial membranes were added at the rate of 50 μg total outer membrane protein/cm of bead surface area. 5×10^8 1 μm -diam latex microspheres (Polysciences Inc., Warrington, PA) were mixed with bacterial membranes in 1 ml PBS, sonicated briefly and allowed to incubate for 2 h at 37°C before being added to HEp-2 cell monolayers grown on coverslips.

Immunofluorescence

Coverslip-grown CEFs were incubated for 2, 5, 10, 20, 60, and 120 min after the addition of bacteria. After interaction with bacteria, coverslips were washed seven times with PBS and then fixed and permeabilized with -20°C acetone for 2 min on ice followed by four washes with PBS. Blocking of non-specific sites was accomplished by 1-h incubation at room temperature in PBS with 0.5% BSA (PBS/BSA).

Coverslips were then inverted onto 50- μl drops of primary antibody diluted appropriately in PBS/BSA and incubated 1 h at room temperature. Excess antibody was washed away with PBS/BSA and the coverslips then inverted onto 50 μl of appropriate fluorochrome-linked secondary antibody for 1 h at room temperature. After a final wash in PBS/BSA, coverslips were rinsed in deionized water to remove salts, and then mounted in 10% Mowiol in 0.1 M Tris (pH 8.5) with Citifluor (Amersham) added as an anti-fade agent. BodipyTM phalloidin was obtained from Molecular Probes (Eugene, OR) and used as directed.

Epifluorescence microscopy was performed on a Zeiss Axiophot using a Zeiss Planapo 100 \times objective (Carl Zeiss, Inc., Thornwood, NY) and recorded on TMAX 400 film processed at 400 ASA with TMAX developer (Eastman Kodak Co., Rochester, NY). Negatives were printed on Ilford Multigrade III RC paper using an Ilford enlarger and print processor (Ilford, Inc., Paramus, NJ).

Confocal laser scanning microscopy was performed on a Sarastro (Ypsilanti, MI) confocal microscope system coupled to a Zeiss Axiocrop using a Zeiss Planapo 100 \times objective. Image processing was performed using the Vanis program (Sarastro) and photographs taken from the screen of the coupled Silicon Graphics computer screen using Kodak Gold 200 ASA print film or Ektachrome 160T slide film.

Video Light Microscopy and Image Processing

An inverted microscope (model IM-35; Carl Zeiss, Inc., Thornwood, NY) was set up for differential interference contrast (DIC) observation as described previously (Forscher and Smith, 1988). Observation was through a $63 \times /1.4$ NA Planapo objective. A Newvicon tube equipped video camera (model C2400; Hamamatsu Corp., Middlesex, NJ) was used to generate video signals which were processed with a digital image processor (series 151; Imaging Technology, Inc., Woburn, MA) controlled by an IBM PC-AT host computer. DIC images were processed to eliminate background mottle and shading by digitally subtracting an out-of-focus reference frame from the frames of interest.

Sample Preparation and Electron Microscopy

HEp-2 and CEF's were grown on Formvar-coated nickel electron micro-

1. Abbreviations used in this paper: CEF, chicken embryo fibroblast; DIC, differential interference contrast; DTAF, dichlorotrazinylamino fluorescein; IPTG, isopropylthiogalactoside; LB, Luria broth; NEAA, non-essential amino acids.

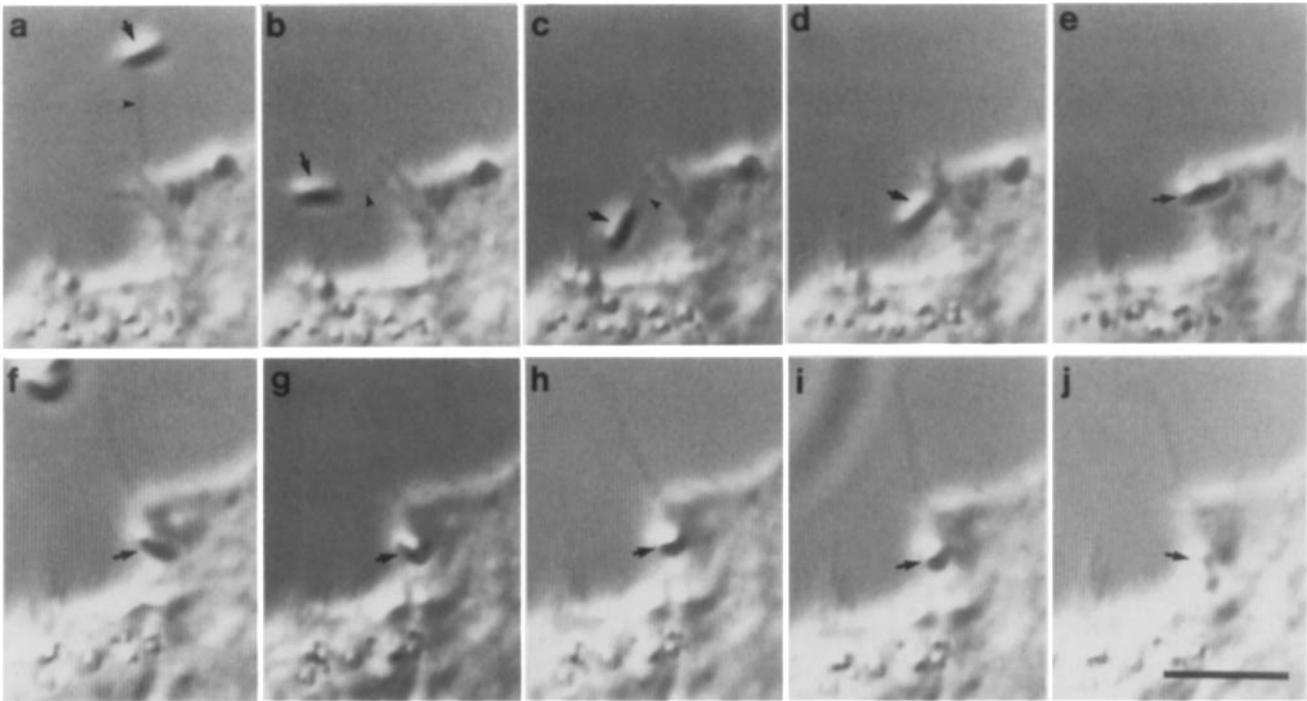


Figure 1. Video-enhanced DIC time-lapse microscopy of the interaction between invasin-bearing bacteria and mammalian cells. The interaction between invasin-bearing bacteria and HEp-2 cells was monitored by video-enhanced DIC time-lapse microscopy. Single cells or small groups of cells were monitored by a sequence of frames taken three seconds apart before and after the addition of bacteria (DH5 α /pYIT1). Selected frames from one representative sequence are presented here. A single bacterium (*a-j*, arrows) is seen adhering initially to a filopodium (*a-c*, arrowheads) and then to the surface (*d-g*) of the host cell by a focal type of attachment around which the bacterium is free to pivot. Following the development of multifocal contacts between the bacterium and the cell (*g-i*), the bacterium is internalized by the cell (*j*). Bar, 10 μ m.

scope grids mounted on glass coverslips and detergent-insoluble cytoskeletons prepared as follows: after infection of the grid-grown cells, the grids were washed 3 times in Hanks Balanced Salt Solution (HBSS; Gibco Laboratories, Grand Island, NY) and then prefixed/solubilized in 0.5% Triton X-100/0.25% glutaraldehyde/5 mM EGTA/5 mM MgCl₂/0.5 mM PMSF in HBSS for 2 min at room temperature. The grids were then washed 3 \times in 5 mM EGTA/0.5 mM PMSF in HBSS followed by a 20-min fixation with 1% glutaraldehyde in HBSS at room temperature. The grids were then rinsed 3 \times in distilled water and then stained with 0.2% tannic acid (Sigma Chemical Co.) in distilled water for 5 min at room temperature. The grids were then washed 5 \times with distilled water, 5 min for each wash at room temperature. The grids were then postfixed in 0.5% OsO₄ (Polysciences Inc.) for 15 minutes on ice, washed 3 \times with distilled water and then dehydrated with a series of graded ethanols (50%, 80%, 95%, 100% \times 3). The grids were then treated with hexamethyldisilazane (HMDS, Polysciences Inc.) as a substitute for critical-point drying 3 \times for 10 min at room temperature after which they were allowed to dry.

Embedding and sectioning of infected cells for transmission EM was performed as previously described (Isberg and Falkow, 1985).

Results

Entry of Invasin-bearing Bacteria into HEp-2 Cells Occurs As A Sequence of Discrete Steps

E. coli strain DH5 α /pYIT1, which carries the *inv* gene from *Y. enterocolitica* on a *tac* expression plasmid, expresses invasin on its surface when grown in the presence of isopropylthiogalactoside (IPTG) (Young et al., 1990). DH5 α /pYIT1 was propagated in a medium containing IPTG, the induced bacteria added to HEp-2 cells grown on glass coverslips mounted on the heated stage (37°C) of an inverted microscope and the interaction between the bacteria and the HEp-2

cells then observed using video-enhanced DIC time-lapse microscopy (Forscher and Smith, 1988).

The HEp-2 cells were plated at a density that resulted in their growing as single cells or in isolated groups of two to four cells. The cells were plated 24 h before infection, by which time they were well spread on the substratum, expressing numerous filopodia on their surfaces. After the addition of the invasin-expressing DH5 α /pYIT1 strain to the cell culture, time-lapse microscopy showed bacteria drifting in the medium, randomly colliding with the HEp-2 cells, either with the main cell body, or with one of the filopodia extending from the cell. The majority of these interactions were transient, but a limited number (<1%) resulted in the adherence of a bacterium to a cell. One of the filopodia was the site to which the bacteria attached in \sim 95% of cases, even though the estimated available surface area of all the filopodia of a particular cell was <10% of the cell's entire surface area. Contact between a bacterium and the filopodium to which it had attached was highly focal, involving only a small part of the bacterial surface (Fig. 1 *a*). As a result, an attached bacterium appeared to be tethered through this point of contact and its corresponding filopodium to the cell body (Fig. 1, *a-c*). When the site of initial adherence was not with a filopodium, it was generally along one of the margins of the cell. In control experiments, using an isogenic bacterial strain that did not express invasin, bacterial-cell contact was observed, but adherence was never seen (data not shown).

A tethered bacterium remained attached to its filopodium

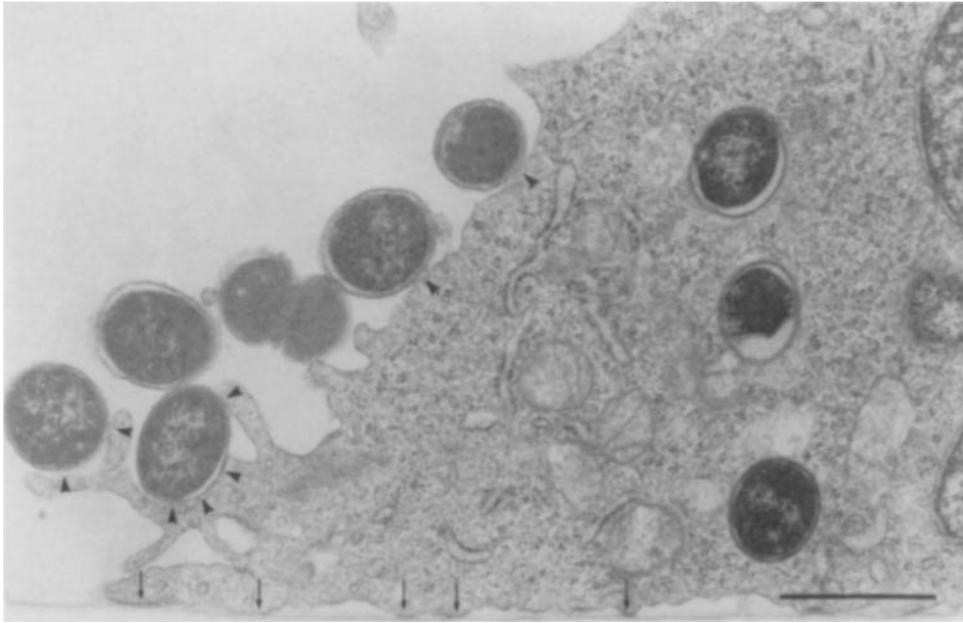


Figure 2. Thin section electron micrograph of internalized invasin-bearing bacteria. HEP-2 cells infected with the DH5 α /pYIT1 invasin-expressing recombinant strain were fixed, embedded and sectioned for electron microscopy as described in Materials and Methods. Bacteria can be seen attached to the surface of the cell by single or multiple sites of close juxtaposition between the bacterial and cell membrane (*arrowheads*). Other bacteria are internalized within individual endosomes. Focal contacts, areas of close association between the cell membrane and the underlying substratum, are also visible (*arrows*). Bar, 1 μ m.

for a variable length of time ranging from several seconds to several minutes. Thereafter, owing to motion of the filopodium, one to several contacts between the bacterium and the surface of the cell occurred, leading to the adherence of the bacterium to the cell body (Fig. 1, *b-d*). Initially, the point of contact between the bacterium and the cell body was also quite focal, only one part of the bacterium appearing to be bound to the surface of the cell (Fig. 1, *e* and *f*). As a result, the bacterium appeared to pivot around this point of contact. However, within a few minutes, multiple points of contact were established leading to a firmly adherent, immobile bacterium on the surface of the cell (Fig. 1 *g*). Transmission EM studies of bacterially infected cell cultures at this stage confirmed this observation and showed that the bacterial outer membrane and the plasma membrane of the cell are closely juxtaposed (Fig. 2).

Internalization of the bacterium by the cell then occurred; once internalized, electron microscopic examination of thin

sections showed bacteria within membrane-bound endocytic vesicles (Fig. 2). The internalization process was frequently accompanied by blebbing of the plasma membrane near the site of bacterial entry (Fig. 3). Blebbing was most evident when several bacteria simultaneously entered the same region of a particular cell.

To examine the ultrastructure of the cytoskeleton during different stages of the internalization process, HEP-2 cells were grown on Formvar-coated electron microscope grids and then infected with the invasin-expressing recombinant strain. Subsequently, the infected HEP-2 cells were prefixed with glutaraldehyde, extracted with Triton X-100, and then fixed and stained for EM (Spudich, A., and J. T. Wrenn, manuscript submitted for publication). This procedure solubilizes the lipid bilayers of the cell, including endosomal membranes, yielding clear images demonstrating the spatial relationships between attached and internalized bacteria and the cytoskeleton.

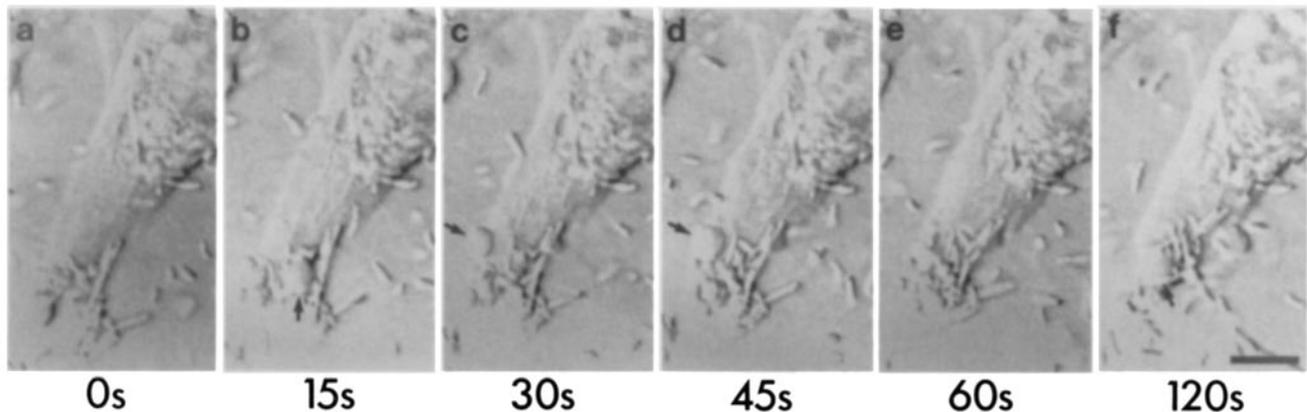


Figure 3. Video-enhanced DIC time-lapse microscopy of host cell membrane blebbing in response to invasin-bearing bacteria. HEP-2 cells were prepared as in Fig. 1, but infected with a 10-fold greater inoculum of bacteria. A series of representative frames from a 2-min sequence, each frame having been taken 7.5 s apart, are shown. A region of the host cell in contact with a group of invading bacteria appears to be disrupted, with the rapid formation of at least three large (2–3- μ m diam) membrane blebs (*arrows*). Bar, 10 μ m.

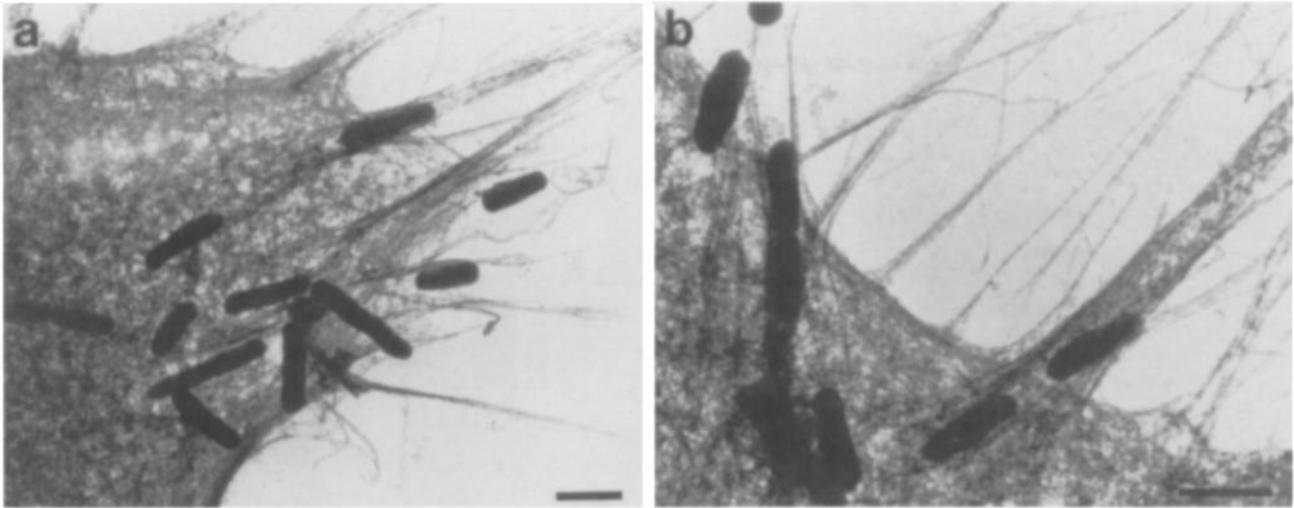


Figure 4. Transmission electron micrographs of detergent-insoluble cytoskeletons of HEp-2 cells infected with invasin-bearing bacteria. HEp-2 cells grown on Formvar-coated electron microscopy grids were infected with invasin-expressing *E. coli*. Following infection the grids were simultaneously fixed and permeabilized to solubilize all cellular membranes, but preserve the underlying cytoskeletal architecture. The grids were then fixed and stained for transmission EM. (a) Infected cell with internalized and filopodia-associated bacteria. (b) Infected cell showing filaments of the filopodia extending into the main body of the cell, still associated with bacteria. Accumulations of electron-dense material are seen around the internalized bacteria. Bars, 1 μm .

The ultrastructural appearance of these detergent-extracted cells could be correlated with the same stages of the internalization process as revealed by time-lapse video microscopy. Bacteria were again seen to be associated with filopodia (Fig. 4 a). Bacteria located more towards the center of the cell were frequently, but not invariably associated with bundles of cytoskeletal filaments or accumulations of granular, electron-dense material (Fig. 4 b). Taken together, these findings indicate that the internalization process is associated with local changes in the organization of the cytoskeleton.

Invasin-coated Microspheres Bind to and Are Internalized by HEp-2 Cells

As demonstrated above, the adherence of invasin-expressing bacteria to HEp-2 cells leads to their internalization, a process that might require energy derived from the metabolic activity of the microbe. This possibility was examined experimentally by determining if the invasin-mediated internalization process requires living bacteria or can be carried out by the invasin molecule per se, presented to the cell in an inert form. Invasin-containing outer membranes were isolated from *E. coli* DH5 α /pYIT1 and used to coat 1- μm latex beads. As a control, beads were coated with invasin-negative outer membranes prepared from DH5 α carrying the parent plasmid pJF119EH. Beads were allowed to interact with cultured HEp-2 cells in the same manner as described above for intact bacteria; then, the presence or absence of internalized beads was monitored by transmission electron and light microscopy. As had been observed with invasin-expressing *E. coli* and *Y. enterocolitica* (Fig. 5 a), the beads not only bound (Fig. 5 b), but were internalized by the HEp-2 cells (Fig. 5 c). In contrast, beads coated with control membranes were rarely associated with cells (data not shown). Thus, inert particles coated with invasin-containing membranes both adhere to and are internalized by, HEp-2 cells.

Antibodies against the β_1 Integrin Subunit Block the Adherence of HEp-2 Cells to Invasin-coated Surfaces

The results described above suggest that the interaction of invasin with the surface of the HEp-2 cell not only results in bacterial adherence, but also initiates changes in the physiology and structure of the cell that lead directly to bacterial internalization.

Integrins containing the β_1 subunit can serve as mammalian cell-surface receptors for the invasin of *Y. pseudotuberculosis* (Isberg and Leong, 1990). To determine if receptors for the invasin of *Y. enterocolitica* are also integrins and to examine the adherence phase of the internalization process in greater detail, a monospecific polyclonal goat antiserum to the β_1 integrin subunit (Chan et al., 1991) was tested for its ability to block the adherence of HEp-2 cells to the *Y. enterocolitica* invasin. This experimental approach was based on a previous study from this laboratory showing that cultured mammalian cells bind specifically to surfaces coated with outer membranes prepared from bacteria expressing the *Y. enterocolitica* invasin (Young et al., 1990). In the present study, the addition of the anti- β_1 integrin serum was found to cause a concentration-dependent inhibition of this binding (Fig. 6) indicating that the invasin of *Y. enterocolitica* and *Y. pseudotuberculosis* probably bind similar receptors. Since the fibronectin receptor is also an integrin which belongs to the β_1 family, the binding of HEp-2 cells to a fibronectin-coated surface was examined as well and found to exhibit the same concentration-dependent inhibition of binding with this serum.

Internalization of Bacteria Is Blocked by Cytochalasin D and Is Coupled with A Rearrangement of Actin but not Tubulin

The integrin cytoplasmic domain is thought to be associated

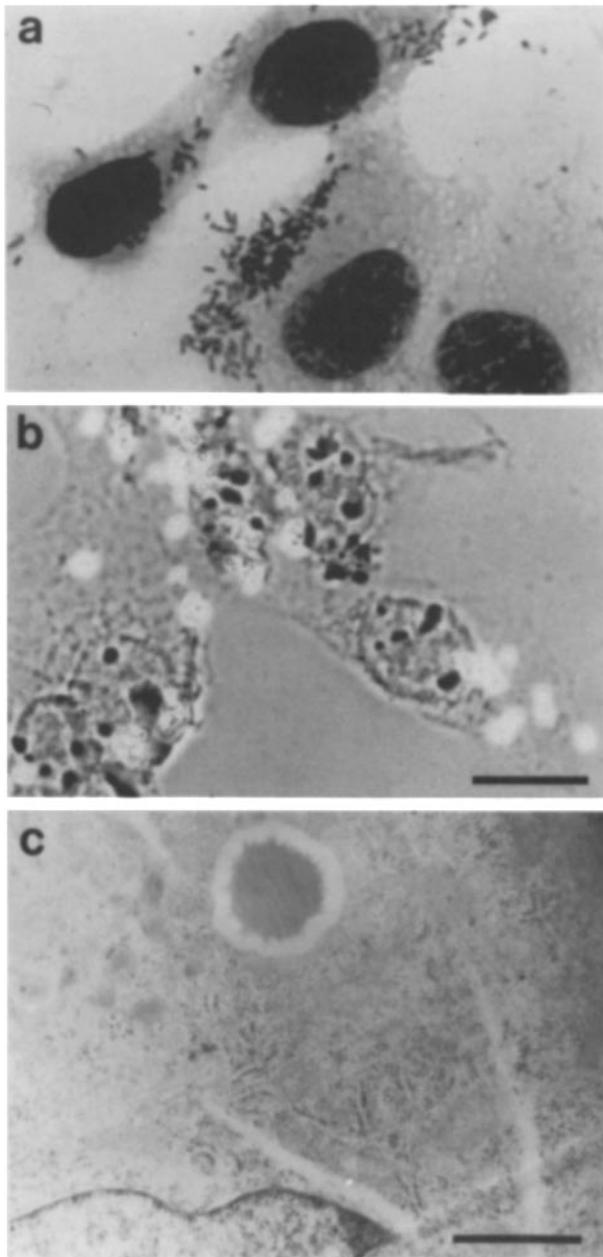


Figure 5. Interaction between invasin-bearing particles and mammalian cells. (a) Giemsa stain of HEP-2 cells infected for two hours with DH5 α /pYIT1 which carries the cloned *inv* gene from *Y. enterocolitica*. Multiple intracellular bacteria are evident. (b) Wright-Giemsa stain of HEP-2 cell to which 1- μ m latex beads coated with outer membranes from *E. coli* DH5 α /pYIT1 were added for 2 h. Intracellular latex beads are seen. (c) Transmission electron micrograph of a cell from b showing an invasin-coated latex bead within an endosome. Bars: (a and b) 10 μ m; (c) 1 μ m.

with the underlying actin-based cytoskeleton (BurrIDGE et al., 1988). This structural relationship, the interaction between invasin and the β_1 -containing integrins that was demonstrated above (Fig. 6) and the appearance of filamentous cellular structures near internalized bacteria (Fig. 4) all suggest an active role for the cytoskeleton in the internalization process. This hypothesis was evaluated by determining the effect of cytochalasin D on invasin-mediated bacterial ad-

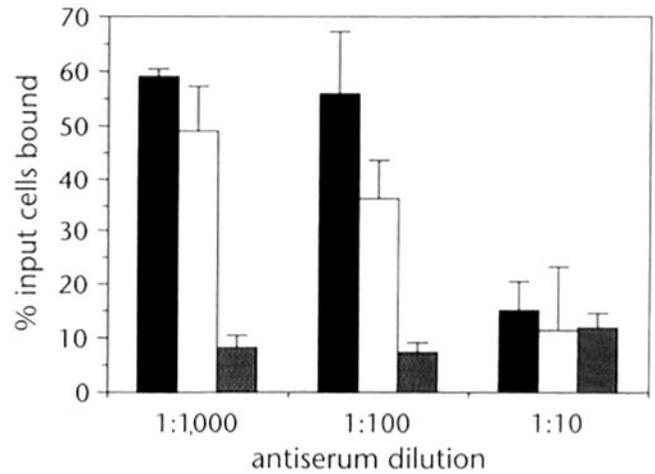


Figure 6. Inhibition of invasin-mediated binding of mammalian cells by an anti- β_1 integrin serum. HEP-2 cells labeled with 35 [S]-methionine were detached from culture dishes and incubated for 30 min at 37°C with the indicated dilutions of a goat monospecific, polyclonal serum to the purified β_1 integrin subunit. Then, the cells were tested for their ability to adhere to microtiter wells coated with fibronectin (■) or with bacterial outer membranes from an *E. coli* recombinant strain expressing (□) or not expressing (▨) the invasin protein. After 30 min, the number of adherent cells was deduced from the amount of radioactivity that remained bound after washing away non-adherent cells.

herence and internalization. Cytochalasin D is a fungal metabolite that binds to the barbed end of actin filaments; as a result the association and dissociation of actin monomers at that end are inhibited (Cooper, 1987). HEP-2 cells were incubated with different cytochalasin D concentrations before their use in the invasion assay (see Materials and Methods). Increasing concentrations of cytochalasin D resulted in a dose-dependent decrease in the number of internalized bacteria, a 300-fold decrease being produced by a cytochalasin D concentration of 5 μ g/ml (Fig. 7). In contrast, the addition of cytochalasin D over the same concentration range resulted in only a three-fold decrease in the total number of cell-associated bacteria (i.e., adherent plus internalized bacteria), providing further evidence that adherence and internalization are discrete events and that only the latter is dependent on actin microfilament function.

Since cytochalasin D was observed to inhibit the internalization of invasin-bearing bacteria, the organization of actin microfilaments within HEP-2 cells during infection was studied in greater detail. HEP-2 cells were stained with phalloidin labeled with the green fluorochrome Bodipy (Polysciences Inc.) following the interaction of these cells with the invasin producing *E. coli* strain. Phalloidins bind tightly to polymeric forms of actin, but not to actin monomers (Faulstich et al., 1988). Within the same HEP-2 cell, the location of adherent or internalized bacteria was determined using a polyclonal rabbit antiserum against an *E. coli* cell wall antigen followed by a goat anti-rabbit antibody that had been labeled with Texas red. Confocal microscopy was then used to visualize the two different fluorochromes. As a control, duplicate slides were stained only with the anti-*E. coli* antibody and the Texas red-linked second antibody. Examination of these controls using the two-color filter set demon-

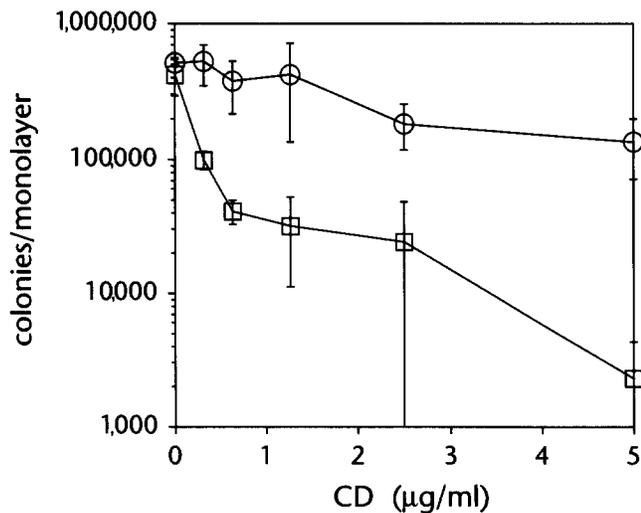


Figure 7. Effect of cytochalasin D on invasin-mediated adherence and invasion. HEP-2 cells were seeded in 24-well microtiter plates 24 h before the assay. The monolayers were pretreated with various concentrations of cytochalasin D (CD) 30 min before the addition of bacteria. Approximately 5×10^8 colony forming units of the DH5 α /pYIT1 recombinant strain were grown in the presence of IPTG to induce production of invasin; these bacteria were then added to the monolayer and allowed to interact for two hours. The total number of cell-associated bacteria (i.e., adherent plus internalized bacteria) was determined by washing the monolayers after two hours of infection; then the cells comprising the monolayer were lysed with detergent and the viable counts of the released adherent bacteria were determined by plating on selective media (○). The number of internalized bacteria was also determined by incubating washed, infected monolayers with media containing gentamicin in order to kill adherent, but non-internalized bacteria; then the monolayers were lysed and the number of internalized bacteria determined by viable counts (□).

strated an absence of detectable fluorescence from the Texas red fluorochrome in the channel used to detect the labeled phallotoxin. Thus, the confocal images could be used to determine the locations of and the spatial relationships between, polymerized actin and the internalized bacteria during the internalization process.

Figure 8. Distribution of actin in a HEP-2 cell infected with invasin-bearing bacteria. Confocal micrographs of a HEP-2 cell infected with the recombinant invasin-bearing strain DH5 α /pYIT1. Shown are look-through projections constructed from six confocal planes spaced 0.2- μ m apart. The distribution of actin was determined by staining with Bodipy-labeled phalloidin while the location of the bacteria was determined by indirect immunofluorescence microscopy using a polyclonal rabbit serum against *E. coli* and secondary labelling with a Texas red-conjugated goat anti-rabbit serum. (a) Composite projection showing the location of both bacteria (red) and actin (green). Areas of extensive overlap appear yellow. (b) Projection showing only the location of the bacteria from a. (c) Pseudocolor projection showing only the location of polymerized actin from a. The brightest area of actin staining (white) is seen in areas corresponding to the location of internalized bacteria. (d) Pseudocolor projection of uninfected HEP-2 cells showing the location of polymerized actin. The brightest areas of staining (white) are along stress fibers and the leading edges of the cells. Bars, 5 μ m.

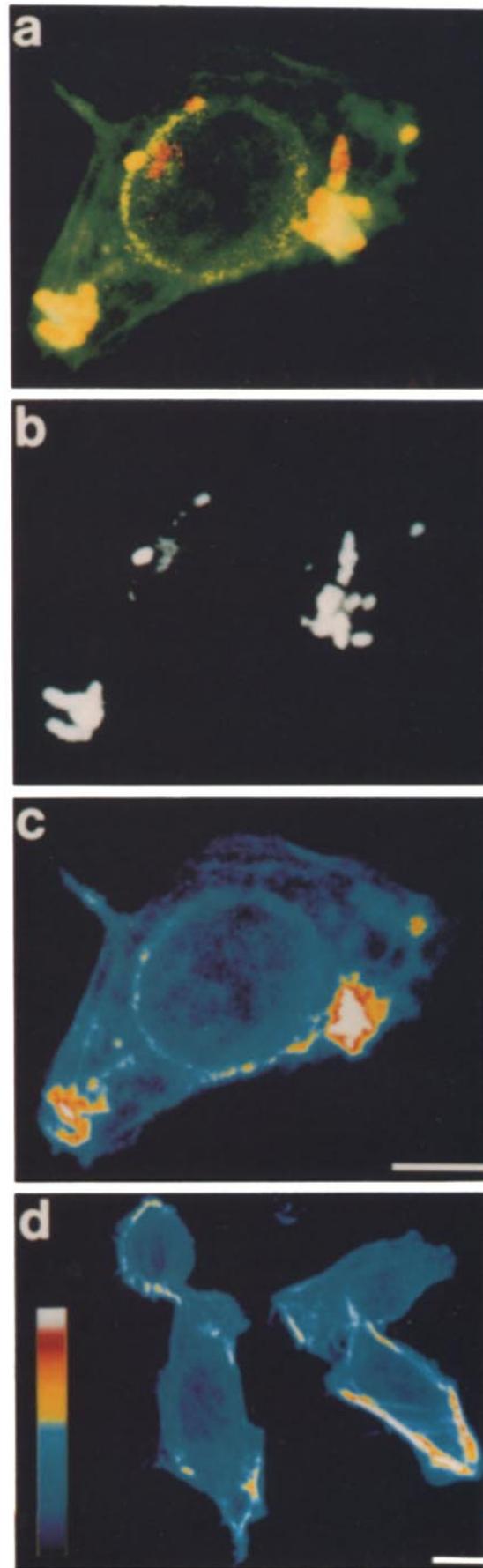


Fig. 8 shows a series of look-through projections constructed from six confocal planes, each separated by 0.2 μm , starting near the ventral surface and moving towards the dorsal surface of an infected HEP-2 cell, 20 min. after the addition of bacteria. The projections show the staining patterns of the two fluorochromes superimposed (Fig. 8 *a*), Texas red alone, revealing the location of bacteria (Fig. 8 *b*), and Bodipy alone, showing the organization of actin polymers (Fig. 8 *c*). Polymerized actin can be seen to accumulate around the entering bacteria. The phalloidin image is rendered in pseudocolor to allow comparison of the amount of fluorescence—and thus the amount of polymerized actin—in different regions of the cell. Bright staining of polymerized actin with phalloidin is apparent in areas that contain bacteria. In contrast to normal stress fibers that are also evident in this figure, the bacteria-associated accumulations of actin are more amorphous, indicating that the polymerized actin may be in the form of short filaments or possibly nonfilamentous aggregates.

The structural changes in the cytoskeleton depicted in Figure 8 were most apparent early in the internalization process, especially between 5 and 30 min after the addition of bacteria, when most of the cell-associated bacteria were surrounded by actin accumulations. However, two hours after the addition of bacteria, >90% of the cells were found to contain internalized bacteria, but only a small percentage (<10%) of the bacteria were associated with actin accumulations (data not shown). Thus, the rearrangement of actin into new polymeric configurations may be a transient phenomenon occurring early in the internalization process analogous to the transient assembly of actin seen in neutrophils that are actively phagocytosing particles (Sheterline et al., 1984).

In contrast to the effect of cytochalasin D on the internalization process, the microtubule inhibitors colchicine, vincristine and vinblastine do not block the internalization of *Y. enterocolitica* (Finlay and Felkow, 1988). However, to determine if microtubules might be involved at some time following internalization, indirect immunofluorescence microscopy using a mouse mAb to the α -tubulin subunit was used to examine bacterially infected HEP-2 cells. The location of bacteria within the same cell was also determined by indirect immunofluorescence as described above. During the early, mid and late phases of the internalization process, no change in the structure or location of microtubules was detected (Fig. 9). No accumulations of tubulin around entering bacteria were apparent, nor did there appear to be any disruption of existing microtubules.

Actin-associated Proteins Accumulate near Entering Bacteria during the Internalization Process

The studies described above showed that rearrangement of actin microfilaments occurs in cells infected with invasin-expressing bacteria and that this effect is probably mediated by the interaction of invasin with its integrin receptor. Consequently, the spatial relationships between bacteria, the cytoplasmic tail of the β_1 integrin subunit, talin, and filamin within infected cells was determined using specific antibodies and confocal fluorescence microscopy. Each of these antibodies had been elicited to an actin-associated protein prepared from chicken tissues, or in the case of the anti- β_1 integrin subunit antibody, to a synthetic peptide derived from the sequence of a chicken integrin. Accordingly, for all ex-

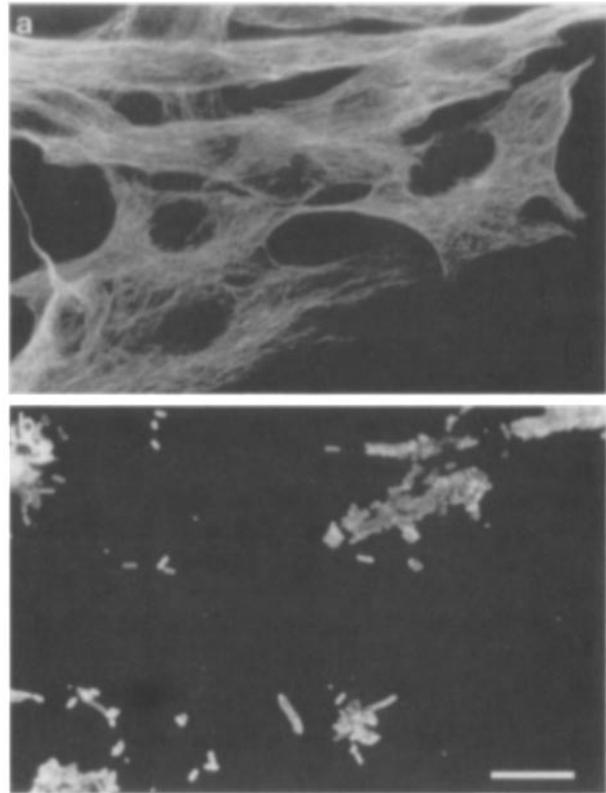


Figure 9. Organization of microtubules in HEP-2 cells infected with bacteria expressing invasin. Infected HEP-2 monolayers were fixed, incubated with a tubulin antibody, and then examined by indirect immunofluorescence microscopy. (*a*) Hep-2 cells stained with a mAb to the α tubulin subunit 20 min after infection with the invasin-expressing DH5 α /pYIT1 recombinant strain. (*b*) Corresponding field showing bacteria demonstrated with a rabbit polyclonal serum to an *E. coli coli* cell-wall antigen. Bar, 10 μm .

periments using these antibodies, chick embryo fibroblasts (CEF) infected with invasin-bearing bacteria were used rather than HEP-2 cells. However, before these experiments it was determined that bacterially infected CEFs were similar to HEP-2 cells with respect to the kinetics of infection, effects of cytochalasin D and changes in the ultrastructure of infected cells during the internalization process (data not shown).

Fig. 10 depicts a series of projections from confocal planes that show the location of bacteria (*b*, *f*, and *j*); and the distribution of integrin (*c*), filamin (*g*), and talin (*k*) in CEFs 30 min after infection with invasin bearing bacteria. Fig. 10 *a*, *e*, and *i* show the superimposition of the bacteria and the actin-associated proteins. Amorphous accumulations of each of these proteins were evident in regions of the cell containing entering bacteria and were in a pattern that was not seen in uninfected control cells (*d*, *h*, and *l*). These accumulations were most evident early in the internalization process and disappeared over time (data not shown).

Discussion

Video microscopy, coupled with EM of detergent-insoluble cytoskeletal whole mounts and thin sections of infected cells have shown in this study that the internalization of invasin-

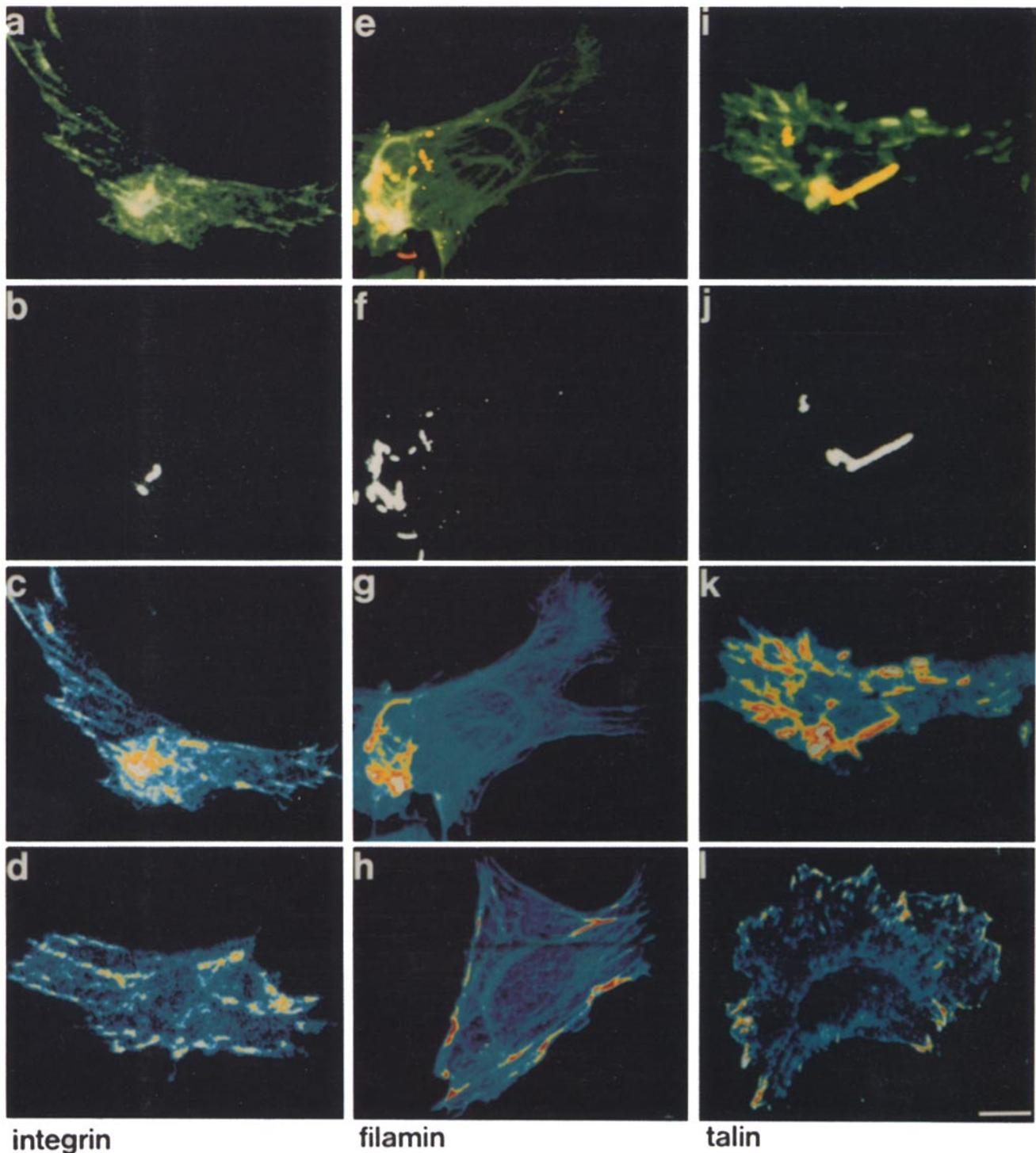


Figure 10. Organization of actin-associated proteins in chicken embryo fibroblasts infected with the invasin-expressing recombinant strain. Infected chicken embryo fibroblasts were fixed and then examined by indirect immunofluorescence confocal microscopy using antibodies against chicken gizzard filamin, a synthetic peptide derived from the cytoplasmic domain of the chicken β_1 integrin subunit and chicken gizzard talin. Shown are look-through projections composed from series of confocal planes separated by $0.2 \mu\text{m}$. (a, e, and i) Composite projection showing the location of bacteria (red) and actin-associated proteins (green). The bacteria were stained with polyclonal or monoclonal antibodies against bacterial surface components and then with the appropriate Texas red-labeled secondary antibody. Integrin (a), filamin (e), and talin (i) were stained with polyclonal or monoclonal antibodies (see Materials and Methods) and then with the appropriate FITC- or DTAF-labeled secondary antibody. (b, f, and j) Projections showing only the position of bacteria from the corresponding images above. (c, g, and k) Corresponding pseudocolor projections showing only the location of the various actin-associated proteins. (d, h, and l) Control cells showing the typical staining patterns found in uninfected cells. Bar, $10 \mu\text{m}$.

expressing bacteria by HEp-2 cells is a rapid process that occurs in three stages. Internalization begins with the focal attachment of a bacterium to the cell, initially to a filopodium and then to the main body of the cell. This stage is reversible and appears to involve a limited area of the bacterial surface. Within a few minutes the second stage occurs, resulting in a bacterium that is irreversibly bound to the cell through multiple points of contact between the organism's outer membrane and the plasma membrane of the cell. The third stage rapidly ensues leading to the presence of the bacterium within a membrane-bound vesicle. The entire internalization phenomenon, including the adherence and entry phases of the process, can be demonstrated using invasin-coated latex beads. Thus, invasin can direct the internalization of bacteria without the de novo synthesis of bacterial proteins or energy sources.

The involvement of the cytoskeleton during this internalization process is evident in the electron micrographs of the detergent extracted cells shown in Fig. 4; in these images, internalized bacteria are seen to be associated with accumulations of electron-dense filamentous, and granular structures. Fluorescence microscopy studies of these cells with Bodipy-labeled phalloidin shows that these accumulations contain polymerized actin. Further evidence that actin polymerization is involved comes from the observation that cytochalasin D will block internalization. Changes in the organization of actin and perhaps other elements of the cytoskeleton during the early phase of the internalization process could also account for the apparent increased fluidity and occasional blebbing of the cell membrane that was seen by video microscopy. If so, this may indicate a partial depolymerization and repolymerization of the cytoskeleton in the affected area of the cell cortex.

The mammalian cell receptors for the homologous invasin proteins of *Y. enterocolitica* (Young et al., 1990) and *Y. pseudotuberculosis* (Isberg et al., 1987) are members of the integrin superfamily of cell adhesion proteins (Fig. 6, this report, and Isberg and Leong, 1990). Integrins are large, heterodimeric cell surface molecules that span the plasma membrane and which have a large ligand-binding extracellular domain and a small intracellular domain; present evidence indicates that they are involved in a variety of adhesive interactions (Hynes, 1987). The integrins were originally divided into three families based on the three different β subunits first described. Each β chain can associate with a variable number of α chains. Based on direct binding assays (Isberg and Leong, 1990) and antibody blocking studies (Fig. 6) the invasin proteins of these *Yersinia* species bind certain integrins that contain the β_1 chain. This interaction appears to be an early event that precedes the internalization of invasin-bearing bacteria by eukaryotic cells.

The interaction between integrins and the cytoskeleton has been examined (for reviews see Burridge et al., 1988; Geiger, 1989), giving rise to a model that specifies a chain of interacting proteins—including talin, vinculin and α -actinin—that are thought to couple microfilaments to integrins. This model also predicts that integrins, through their membrane-spanning and ligand-binding properties, may in turn provide a linkage between the extracellular matrix and the cytoskeleton. According to this model, the invasin-mediated internalization process would be initiated when invasin binds to the extracellular domain of a β_1 -integrin, followed by an

interaction between the cytoplasmic domain of the integrin and proteins comprising the cytoskeleton.

In this study, the possible participation of integrin, talin, and filamin during the invasin-mediated internalization process was studied. Integrin was examined because of its direct interaction with the invasin protein. Talin was investigated because its aggregation may be one of the first events induced by the binding of fibronectin to integrin (Mueller et al., 1989). Moreover, talin is found early in developing focal adhesions (DePasquale and Izzard, 1987; Izzard, 1988). Filamin was examined because of its proposed role as an actin-organizing protein (Weihsing, 1985).

According to the immunofluorescence microscopy studies depicted in Figs. 8 and 10, actin, integrin, talin, and filamin are associated with entering bacteria. The co-localization of each of these proteins with internalized bacteria leads us to propose a model for the invasin-mediated penetration of eukaryotic cells. The central thesis of this model is that contact between invasin molecules on the surface of a bacterium and integrins on the surface of a host cell initiates a variety of cellular functions involving filopodia and the leading edge of the cell. Filopodia, which are the preferred sites for the initial contact of the cell with invasin-bearing bacteria (Figs. 1 and 4), are thought to have an exploratory function especially during cell movement; they are generally concentrated at the leading edge of a cell (Albrecht-Buehler, 1976). Filopodial tips (footpads) have specialized membranes (Robinson and Karnovsky, 1980; Tsui et al., 1985) containing integrins; in addition to their concentration in footpads, integrins also appear to be present along the length of the filopodia (Letourneau and Shattuck, 1989). Filopodia are able to generate a traction force which appears to be sufficient to move small objects or (in the case of a filopodium attached to a fixed surface) to pull the leading edge of a cell forward (Heidemann et al., 1990). Thus during cell spreading a filopodium, once in contact with a surface which is suitable for the cell to spread on, first adheres to the surface and then the rest of the leading edge of the cell follows.

Our model predicts that when a filopodium comes in contact with an invasin-bearing bacterium, a similar chain of events may occur. However, because of differences in the size and the radius of curvature between a bacterium and a flat surface, a cell that is proceeding to "spread" over a bacterium may engulf it instead. This idea has been examined experimentally through morphological studies of fibroblasts interacting with fibronectin-coated beads of increasing diameter (Grinnell, 1984). Fibroblasts were shown to internalize fibronectin-coated beads with diameters of 1 to 6 μm , but were unable to internalize completely beads that were greater than 15 μm in diameter. The fibroblasts appeared to spread over the surface of these larger beads; during this process they began to resemble cells growing on flat surfaces. These experiments with fibronectin-coated beads are especially pertinent to our model because the fibronectin receptor is known to belong to the β_1 family (Hynes, 1987). The internalization of fibronectin-coated beads may be analogous to the internalization of invasin-bearing bacteria in several other important respects as well. These include the observation that the internalization of fibronectin-coated beads is blocked by cytochalasins (Schwartz and Juliano, 1984); that internalized, fibronectin-coated beads associate with actin filaments (Wagner and Hynes, 1982); and that focal contact

proteins accumulate around internalized fibronectin-coated beads (Grinnell and Geiger, 1986; Mueller et al., 1989). However, at least one difference is apparent between the invasin-mediated and the fibronectin-mediated internalization process, the persistence of actin accumulations around fibronectin-coated beads for at least six hours (Grinnell and Geiger, 1986). This difference notwithstanding, the many similarities between the two systems suggest that the invasin-mediated internalization process may not be a unique achievement of the *Yersiniae*. Instead we hypothesize that this phenomenon could be an adaptation by these intracellular pathogens that allows them to exploit two normal cellular processes—motility and surface attachment—to gain access to the interior of a host cell.

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