Mutations in the Cytoplasmic Domain of the Integrin β₁ Chain Indicate a Role for Endocytosis Factors in Bacterial Internalization*

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Mutations that result in defective β₁-integrin focal adhesion formation were analyzed for effects on bacterial internalization. Mutations in the cytoplasmic domain of the β₁ chain that disrupt the sequence NPIY resulted in integrins deficient in bacterial uptake. Other mutations in the β₁ chain that reduced cytoskeletal association showed enhanced bacterial uptake. Replacement of the NPIY sequence of the β₁ subunit by the endocytosis internalization sequence PPYG resulted in integrin receptors highly proficient in bacterial internalization, yet severely defective in focal contact localization. Electron microscopy indicated that coated structures associated specifically with bacteria-binding β₁-integrins, with an apparent recruitment of coated pits from ventral cell surfaces to apical surfaces corresponding to nascent bacterial phagosomes. Clathrin inhibition studies indicated a role for the adaptor molecule AP2 as well as clathrin in integrin-mediated bacterial internalization. These results indicate that association of β₁-integrins with the cytoskeleton at focal contacts interferes with integrin-mediated bacterial internalization. Also, although actin polymerization is required for bacterial uptake, clathrin is probably involved in bacterial uptake promoted by β₁-integrins.

Integrins are heterodimeric receptors involved in numerous cellular processes including migration, differentiation, and adhesion to extracellular matrix and cell-surface proteins (1–3). Members of this family are αβ heterodimeric transmembrane proteins that are involved in both inside-out and outside-in signaling (2, 4, 5). Ligand specificity of each receptor is determined by the particular α and β chains found in the heterodimer. The unique cytoplasmic domains of the different subunits allow diversity and regulation of receptor function (6). For example, the cytoplasmic domain of the β₁ subunit interacts with the cytoskeleton by binding to actin-binding proteins such as talin (7) and α-actinin (8). Mutational analysis as well as peptide inhibition studies have identified regions of the cytoplasmic domain of the β₁ chain important for this cytoskeletal association (9–11). The β₁ chain cytoplasmic domain also plays a prominent role in transduction of signals originating either from outside or inside the cell (12–14). The cytoplasmic domain of the α subunit appears to regulate ligand affinity (15) and participates in processes such as gel contraction (16).

Integrins also promote the internalization of various microorganisms. In the macrophage, the integrin receptor CR3 (or αMβ₂) can mediate internalization of complement-coated particles (17). In normally non-phagocytic cells, viruses and bacteria have been shown to be internalized by integrins containing β₁, β₃, and β₇ chains (18, 19). The uptake of the enteroinvasive bacterium Yersinia pseudotuberculosis by β₁-integrins has been studied in detail. Uptake of this microorganism occurs via the bacterial surface protein invasin (20), which binds multiple β₁-integrins (18). Invasin-mediated bacterial uptake is accompanied by a local rearrangement of the actin network (21) and is inhibited by drugs that antagonize actin polymerization (22). This has led to speculation that direct association of integrins with the cytoskeleton is required during internalization.

By using various anti-integrin antibodies to coat the Gram-positive bacterium Staphylococcus aureus, we have been able to mimic the phenotype of invasin-expressing bacteria with respect to uptake. We have shown that integrin-mediated internalization following bacterial attachment to the cell is primarily dependent on the affinity of the bacterial ligand for the integrin (23, 24). In this study, we use this technique to study the role of the cytoplasmic domain of the integrin β₁ subunit. We identify a region of the β₁ subunit cytoplasmic domain that is critical for internalization and show a potentially antagonistic relationship between cytoskeletal association of the cytoplasmic domain and bacterial uptake.

MATERIALS AND METHODS

Bacterial Strains, Cell Lines, and Media—To assay bacterial uptake mediated by chicken integrin β₁ chains, S. aureus strain 377 McCo:1 was grown in Penassay broth and coated with the anti-chicken mAb₁ CSAT as described (24). MC4100/pRI203 is Escherichia coli harboring the inv gene (25). It was grown at 37 °C in L broth containing ampicillin at a 100 μg/ml final concentration. Salmonella typhimurium strains 1344 and EL-451, obtained from Dr. Catherine Lee (Harvard Medical School, Boston), were grown as described (61) in L broth at 37 °C without agitation. Human cultured HEp-2 cells were grown in RPMI 1640 medium containing 5% newborn calf serum, and mouse cultured 3T3 fibroblastic cells were grown in Dulbecco’s modified Eagle’s medium containing 10% calf serum at 37 °C in an incubator containing 5% CO₂.

Plasmid Constructions—Plasmid pRSVneoβ₁ containing the full-length CDNA encoding the chicken integrin β₁ subunit under the control of the Rous sarcoma virus promoter was used as the selectable marker for the bacterial strain MC4100/pRI203.

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1 The abbreviations used are: mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; β₁, chicken integrin β₁ chain.
neomycin was described previously (10). Plasmids containing single amino acid substitutions (F766A, F771L, E784L, N785I, P786A, Y788A, Y788F, N797I, and Y800A) or deletions between residues 759 and 771 and between residues 771 and 790 in the cytoplasmic domain of the chicken integrin β1 subunit were described previously (10). WAFB was obtained by replacing the 1.1-kilobase pair ClaI-Sall fragment containing the chicken integrin β1 chain cDNA of plasmid pRSVneo-c,-N785I was introduced into plasmid pSV5select (Promega) using the Sall restriction enzyme. The same transformation protocol was performed in E. coli MC4100/pRI203 cells. The mutant construct was verified by restriction digestion.

The reason for the low frequency of G418-resistant clones expressing the chicken integrin β1 chain and for the low level of expression of the chicken integrin β1 chain with PPGY or to chicken integrin β1 chain derivatives was performed as described (23) by electroporation at 450 V and 500 microfarads in a Gene Pulse (Bio-Rad). For each transfection, between 24 and 48 individual G418-resistant transfectants were cloned and tested for chicken integrin receptor expression by an ELISA-based procedure (24). Briefly, individual clones were plated in 96-well tissue culture plates to obtain confluent monolayers, and the cells were fixed with 3.7% paraformaldehyde for 15 min at 22°C. The wells were washed three times with PBS containing 10 mM HEPES, 0.1% BSA, and 0.1% NaN3. The cultures were shifted to 37°C for 15 min to allow bacterial internalization. The tannic acid post-fixation, which has been described as an efficient technique to enhance clathrin-coated membrane visualization, could not be unambiguously determined because of numerous cell areas of dense cytoplasmic background.

The percentage of coated membranes over the ventral plasma membrane could not be unambiguously determined because of numerous cell areas of dense cytoplasmic background.

Bacterial Depletion and Analysis of Bacterial Internalization and Transferrin Uptake—The potassium depletion treatment, previously shown to inhibit clathrin-mediated endocytosis, was described (29) with modifications that allowed quantitation of bacterial internalization. Mondays of HEp-2 cells seeded in 24-well plates at 50% confluency were incubated overnight; rinsed twice in RPMI 1640 medium containing 20 mM HEPES, pH 7.0, and 1% BSA; and incubated in this buffer for 30 min at 37°C. Triplicate monolayers were then washed twice in different buffers and incubated in the appropriate buffer for 30 min at 37°C to test their effects on bacterial uptake. The following buffers were used: buffer K− (140 mM NaCl, 0.5 mM CaCl2, 1 mM MgCl2, 3 mM KCl, and 50 mM HEPES, pH 7.0) and buffer K−Δ (140 mM NaCl, 0.5 mM CaCl2, 1 mM MgCl2, and 50 mM HEPES, pH 7.0). To test the effect of K− depletion, after 30 min of incubation in buffer K−, the cells were subjected to a hypotonic shock for 5 min in buffer K−Δ diluted 1:1 with H2O prior to incubation in buffer K−Δ. The cell monolayers were then analyzed for bacterial internalization and transferrin uptake. Bacterial uptake was quantitated by challenging the treated cells either with Mc4100/pRI203 (inv+) or with S. aureus coated with the anti-β1-integrin mAb 1A11B1 (gift of Dr. Caroline Damsky) or with the anti-αvβ3 mAb VD1 as described previously (23) at a multiplicity of infection of 10 bacteria/cell for 30 min at 37°C. The percentage of internalized bacteria was determined by the gentamicin protection assay (25). Transferrin uptake was determined as described previously (30). Briefly, cells were incubated with 125I-transferrin (20 μg/ml, 2.4 × 107 cpm/ng) in the corresponding buffer for 5 min at 37°C, and transferrin uptake was removed with three washes of ice-cold PBS. The monolayers were treated with 0.3 μg/mL of senegalese pneumococcal N-acetyl muramidase (Difco Laboratories) in L1/10 for 15 min at 0.3% final concentration for 1 h on ice. The cells were then transferred to Eppendorf tubes and centrifuged for 2 min, and the pellet and the supernatant were counted for γ-radiations. Nonspecific internalization was determined by incubation with 125I-transferrin for 5 min on ice. Specific internalization of 125I-transferrin, corresponding to the Pseudomonas-resistirnt cell-associated counts, was obtained by subtracting the nonspecific counts and is expressed as a percentage of total cell-associated counts.

Syringe Loading of HEp-2 Cells with Anti-clathrin mAb—HEp-2 cells were loaded with mAb as described previously (31). Briefly, monolayers of HEp-2 cells were trypsinized, washed once in RPMI 1640 medium containing 5% newborn calf serum, and resuspended in RPMI 1640 medium containing 5% newborn calf serum to contain 106 cells/ml. The cell suspension was aliquoted in a microcentrifuge tube containing 20 μg of mAb. The suspension was subjected to a 5 min, 17,000 × g centrifugation. For each sample, 70 μl of the cell suspension was aliquoted in a microcentrifuge tube containing 20 μg of mAb. The suspension was subjected to a 5 min, 17,000 × g centrifugation. The cell Newsletter was added to the saline, and the pellets were centrifuged for 1 h on ice. The cell Newsletter was added to the saline, and the pellets were centrifuged for 1 h on ice. The cell Newsletter was added to the saline, and the pellets were centrifuged for 1 h on ice. The cell Newsletter was added to the saline, and the pellets were centrifuged for 1 h on ice.
kan II, Perkin-Elmer) and ~50% of the initial input of cells adhering to plastic. After loading, the cells were washed twice with RPMI 1640 medium containing 20 mM HEPES, pH 7.0, and 1% BSA and infected with E. coli MC4100(pRI203 at a multiplicity of infection of 1 bacterium/cell or with S. typhimurium SL1344 at a multiplicity of infection of 10 bacteria/cell. The plates were centrifuged for 10 min at 1000 × g and incubated for 30 min at 37°C. The percentage of internalized bacteria was determined using the gentamicin protection assay (25). In each experiment, the results are expressed as a percentage of the number of gentamicin-resistant counts obtained when loading the cells with the irrelevant mAb CSAT. In three experiments performed, the samples were diluted before plating to obtain a number of gentamicin-resistant counts ranging from 100 to 600 viable counts for the CSAT mAb control.

RESULTS

The Chicken Integrin β1 Subunit Forms Chimeric Receptors in HEp-2 Cells and Mediates Bacterial Uptake—With the exception of β2β2-integrins (3) that act as receptors for the bacterial protein invasin (18). To overcome this problem and to study the effect of integrin β1 chain mutations on bacterial internalization, we took advantage of the fact that species-specific high affinity antibodies can promote bacterial internalization when used to coat the bacterium S. aureus (24). Human HEp-2 cells transfected with mutant derivatives of the chicken β1 chain were challenged with S. aureus coated with the chicken-specific anti-integrin β1 chain mAb CSAT. This allowed bacterial internalization by hybrid integrin receptors containing the transfected mutant chicken β1 subunit to be analyzed in the presence of wild-type endogenous human β1-integrins (24).

The chicken integrin β1 subunit expressed in mammalian cells is reported to associate with the endogenous α chains, forming heterodimers functional in adhesion and cytoskeletal association (3, 9). After transfection with the chicken β1 clone, the expression levels of the chicken/human hybrid integrins on the surface of various transfected clones of HEp-2 cells were determined by an ELISA-based assay (see “Materials and Methods”). From immunoprecipitation experiments, the levels of chicken/human hybrid integrins in the various transfectants were generally <25% of the endogenous levels of human integrin β1 chains (data not shown). Transfectants expressing various derivatives of the chicken β1 chain bearing the F768A, P786A, or N785I substitution in the β1 cytoplasmic domain (Table I) were also analyzed by immunoprecipitation using the CSAT mAb. No obvious qualitative or quantitative differences in association with the endogenous α subunits could be observed among the different mutants when compared with the wild-type chicken β1 chain (data not shown). The values obtained by scanning the intensity of the band corresponding to the chicken β1 chain were in good agreement with the values obtained in the ELISA-based assay, and these ELISA-based determinations were used to quantitate the expression levels of chicken/human hybrid receptors at the surface of the various transfectants (Table I).

Transfectants expressing the wild-type chicken β1 chain internalized significant levels of S. aureus coated with the anti-chicken β1-integrin mAb CSAT (Table I, β1-c-wild type, uptake expression equal to 4.1) when compared with nontransfected HEp-2 cells. Therefore, the chicken β1/human hybrids could be used to analyze the effect on bacterial uptake of mutations located in the cytoplasmic domain of the integrin β1 chain.

The NPIY Sequence Located at Residues 785–788 of the Cytoplasmic Domain on bacterial uptake

Table I

| Clone | Expressiona | Uptakeb,cd | Uptake/expressionb,cd |
|-------|-------------|------------|-----------------------|
| HEP-2 | 0.00        | 0.00       | 0.00                  |
| β1c-wild type | 0.67 ± 0.49 | 1.8 ± 0.6  | 4.1 ± 2.1             |
| F768A | 1.90 ± 1.07 | 39.3 ± 24.7| 14.7 ± 2.9            |
| F771L | 1.08 ± 0.68 | 16.4 ± 3.9 | 11.8 ± 1.5            |
| P786A | 0.14 ± 0.12 | 5.0 ± 0.5  | 22.0 ± 6.5            |
| N785I | 1.24 ± 0.75 | 0.00       | 0.00                  |
| P786A | 1.22 ± 0.15 | 0.00       | 0.00                  |
| Y788E | 0.17 ± 0.03 | 0.00       | 0.00                  |
| Y788F | 1.11 ± 0.43 | 15.8 ± 5.3 | 14.7 ± 7.3            |
| S790D | 0.32 ± 0.02 | 1.5 ± 0.5  | 12.5 ± 4.5            |
| N797I | 0.13 ± 0.03 | 4.8 ± 2.5  | 36.9 ± 15.8           |
| Y800A | 0.11 ± 0.03 | 4.6 ± 1.8  | 41.8 ± 12.3           |
| N785I/N797I | 0.73 ± 0.45 | 0.00 | 0.00 |
| Δ759–771 | 0.58 ± 0.27 | 0.00 | 0.00 |
| Δ771–790 | 1.65 ± 0.85 | 0.00 | 0.00 |
| NPIY → PPGY | 0.85 ± 0.32 | 11.7 ± 2.3 | 18.0 ± 5.6 |

* The expression levels of chicken β1-integrin were determined by an ELISA-based procedure (see “Materials and Methods”). Each value is the mean of three to six determinations from independently derived clones.

† The percentage of bacterial uptake was determined by the gentamicin protection assay (25) after normalization to the cell density (24). Units are defined under “Materials and Methods.” Each value is the mean of three to six determinations from independently derived clones.

‡ Uptake levels of 0.00 represent uptake efficiency at or lower than levels of internalization by nontransfected controls.

§ The relative percentage of bacterial uptake was obtained by arbitrarily dividing the values of “% bacterial uptake” by the values of “relative chicken β1-integrin expression.” ✓ refers to standard deviation.

Table II

| Clone | Expression | Uptakeb,cd | Uptake/expressionb,cd |
|-------|------------|------------|-----------------------|
| HEP-2 | 0.00       | 0.00       | 0.00                  |
| β1c-wild type | 0.67 ± 0.49 | 1.8 ± 0.6  | 4.1 ± 2.1             |
| F768A | 1.90 ± 1.07 | 39.3 ± 24.7| 14.7 ± 2.9            |
| F771L | 1.08 ± 0.68 | 16.4 ± 3.9 | 11.8 ± 1.5            |
| P786A | 0.14 ± 0.12 | 5.0 ± 0.5  | 22.0 ± 6.5            |
| N785I | 1.24 ± 0.75 | 0.00       | 0.00                  |
| P786A | 1.22 ± 0.15 | 0.00       | 0.00                  |
| Y788E | 0.17 ± 0.03 | 0.00       | 0.00                  |
| Y788F | 1.11 ± 0.43 | 15.8 ± 5.3 | 14.7 ± 7.3            |
| S790D | 0.32 ± 0.02 | 1.5 ± 0.5  | 12.5 ± 4.5            |
| N797I | 0.13 ± 0.03 | 4.8 ± 2.5  | 36.9 ± 15.8           |
| Y800A | 0.11 ± 0.03 | 4.6 ± 1.8  | 41.8 ± 12.3           |
| N785I/N797I | 0.73 ± 0.45 | 0.00 | 0.00 |
| Δ759–771 | 0.58 ± 0.27 | 0.00 | 0.00 |
| Δ771–790 | 1.65 ± 0.85 | 0.00 | 0.00 |
| NPIY → PPGY | 0.85 ± 0.32 | 11.7 ± 2.3 | 18.0 ± 5.6 |

* The expression levels of chicken β1-integrin were determined by an ELISA-based procedure (see “Materials and Methods”). Each value is the mean of three to six determinations from independently derived clones.

† The percentage of bacterial uptake was determined by the gentamicin protection assay (25) after normalization to the cell density (24). Units are defined under “Materials and Methods.” Each value is the mean of three to six determinations from independently derived clones.

‡ Uptake levels of 0.00 represent uptake efficiency at or lower than levels of internalization by nontransfected controls.

§ The relative percentage of bacterial uptake was obtained by arbitrarily dividing the values of “% bacterial uptake” by the values of “relative chicken β1-integrin expression.” ✓ refers to standard deviation.

The role in integrin function is unclear. To determine whether these residues of the β1-integrin cytoplasmic domain are critical for bacterial internalization, transfectants of HEp-2 cells expressing derivatives of the chicken/human hybrid integrins were tested for their ability to internalize S. aureus coated with the anti-chicken integrin mAb CSAT as described (24).

Transfectants were cloned, and the efficiency of bacterial internalization as well as the levels of expression of chicken/human hybrid integrins were normalized relative to nontransfected HEp-2 cells. Several substitutions in the cytoplasmic region cyto-2 (NPIY) (Fig. 1) (10) resulted in no detectable levels of bacterial internalization when compared with non-

Fig. 1. Effect of mutations in the integrin β1 chain cytoplasmic tail on bacterial uptake and focal contact localization. The amino acid residues of the integrin β1 chain cytoplasmic tail are numbered according to Tamkun et al. (62). Amino acid substitutions are depicted with arrows.Deletions and the PPGY substitution are represented as boxes. Except for the PPGY mutation, data concerning focal contact localization are derived from Reszka et al. (10). Bacterial uptake: -, receptor deficient in bacterial uptake; +, wild-type levels of internalization; +; internalization levels superior to wild-type levels. Focal contact localization: -, receptor deficient in focal contact localization; +, intermediate levels of localization; +; wild-type levels of focal contact localization.

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transfected HEp-2 cells (Table I, N785I, P786A, and Y788E). In addition, the deletion (residues 759–771) removing the cyto-1 region as well as the deletion (residues 771–790) removing the cyto-2 region also abolished the ability of integrin-mediated bacterial internalization (Table I). In contrast, the single amino acid substitutions F768A, F771L, and E784L as well as other mutations in the region called cyto-3 (Fig. 1) (10) resulted in increased efficiency of bacterial uptake relative to wild-type transfected cells. Several of the mutants that were more efficient at promoting bacterial uptake than the parental integrin β1 chain exhibited a reduction in focal contact association (Table I) (10).

These results indicate that residues in the membrane-proximal NPIY (cyto-2) region as well as the integrity of the cyto-1 region are critical for integrin-mediated bacterial internalization. The fact that single amino acid substitutions in the cyto-1 and cyto-3 regions depressing cytoskeletal association resulted in an increased uptake efficiency suggests that cytoskeletal association of integrins at focal contacts interferes with bacterial internalization.

The NPIF and PPGY Internalization Sequences Can Substitute for the Integrin β1 Chain NPIY Sequence in Integrin-mediated Bacterial Internalization—NXY internalization sequences are presumed to allow localization of receptors mediating endocytosis to clathrin-coated pits, perhaps by interacting with adaptors or associated molecules (34). In the case of the low density receptor receptor, replacement of NPVY by the NPVF sequence allows rapid endocytosis (35) because the Tyr → Phe change does not disrupt the tight-turn conformation of the wild type (36). As shown in Table I, substitution of Tyr788 with Phe resulted in integrins that were more efficient at promoting bacterial uptake than the parental integrin β1 chains (Table I). This is consistent with the proposition that the presence of an aromatic amino acid at residue 788 allows internalization.

To determine if the membrane-proximal NPIY sequence could be substituted with another internalization sequence, the cyto-2 NPIY sequence of the integrin β1 chain was replaced by PPGY, which determines rapid internalization of the lysosomal acid phosphatase via coated pits (37, 38). As shown in Table I, the integrin receptor containing the PPGY sequence was competent to mediate bacterial internalization (Table I, NPIY → PPGY) with an efficiency comparable to that of other cytoplasmic mutants of the β1 chain that were more proficient than the wild type. Binding studies performed on cells expressing mutant integrins bearing the PPGY or N785I substitution indicated that these receptors bound the CSAT mAb at a similar efficiency (Fig. 2), ruling out the possibility that the variations in bacterial uptake were due to a change in ligand-receptor affinity (23).

To analyze the ability of this integrin derivative to localize to focal contacts, immunofluorescence staining of 3T3 transfected with the anti-chicken β1-integrin mAb W1B10 followed by an anti-mouse IgG antibody linked to fluorescein isothiocyanate (see “Materials and Methods”). Shown are 3T3 transfected expressing β1c/PGPY (A), β1c/N785I (B), and β1c/wild type (C). The staining for the β1c/PGPY → PPGY clone appears punctated. β1c/N785I shows a slight defect in localization of β1-integrin to focal contacts. The β1c-wild type clone done shows a typical focal contact staining pattern. Bar = 10 μm.
with phagosomes in the process of internalizing latex beads (39). Indirect immunofluorescence staining of HEp-2 cells indicated that clathrin associates with E. coli (inv+) during the initial events of internalization (data not shown). To characterize the potential role of clathrin during integrin-mediated bacterial internalization, the distribution of clathrin was analyzed by thin-section transmission electron microscopy.

HEp-2 cell monolayers were incubated with E. coli MC4100/pRI203 (inv+) for 2 h at 22°C to allow bacterial binding. After washing to remove unbound bacteria, the cells were shifted at 37°C for 10 min to allow internalization (see "Materials and Methods"), and the samples were prepared using standard protocols (28).

In uninfected HEp-2 cells, coated pits appeared to be associated primarily with the basolateral surface of the cell with a density of ~11.2 pits/mm compared with 3.4 pits/mm for the apical surface (Table II, column A). In HEp-2 cells challenged with bacteria, the distribution of coated pits was dramatically different. In these cells, most of the coated pits appeared to be located on the apical surface with an incidence of 11.4 pits/mm of membrane, whereas the density of basolaterally located pits decreased to 5.8 pits/mm of membrane (Table II, column B). The vast majority of the pits (83%) that were on the apical surface appeared to associate with nascent bacterial phagosomes (Table II and Fig. 4C, curved arrows). Also, large coated membrane lattices (~500 nm) were often visible at the cell membrane region juxtaposing the engulfed bacteria (Fig. 4, A and B, arrows). In uninfected cells, such structures were detected primarily at the basal surface (Table II). Consistent with these results, quantitation of coated membranes indicated that in uninfected cells, clathrin structures associated with ~0.3% of the apical surface (Table II), whereas 4.2% of the apical surface of phagocytizing cells was covered by clathrin-coated membranes, with 90% of these coated membranes associated with bacterial phagosomes (Table II).

In these experiments, the thin sections showed an average of 3.85 cell-associated bacteria/cell section, with ~25% of the cell-bound bacteria associated with clathrin-coated membranes (Table II). This number increased to 50% for cell sections showing only 1 or 2 bound bacteria (data not shown). Some bacteria were internalized in phagosomes showing several coated pit-like structures (Fig. 4D, arrows), whereas other phagosomes were devoid of such structures (Fig. 4D, arrowhead).

Immunostaining with the anti-adaptor molecule AP2 antibody AP-6 also showed the formation of large adaptin lattices specifically associated with the plasma membrane juxtaposing the bacteria that were not detected in uninfected control cells (data not shown). These data indicate that clathrin tends to redistribute to the apical surface of HEp-2 cells that are internalizing invasin-expressing bacteria and that the majority of

![FIG. 4. Invasin-promoted uptake generates the formation of large coated membrane lattices containing coated pit-like structures.](image)
ranging from 90 to 95% when compared with cells treated in
endocytosis (29, 40). To determine the effects of potassium
depotonic shock and incubated for 30 min in K−-free medium prior to infection with MC4100/pRI203 (inv+),
bar was transferred to Eppendorf tubes and centrifuged for 2 min, and the pellet was resuspended in
sucrose. Cells were infected with S. typhimurium strain SL1344; solid bars, cells infected without
Staphylococcus aureus (strain INV) or with anti-chicken integrin mAb AIIBII; α1, monolayers challenged with S.
aureus coated with the anti-β1 mAb AIIBII; α5, monolayers challenged with S. aureus coated with the anti-α5β1 mAb VD1; INV, monolayers challenged with MC4100/pRI203 (inv+). Hatched bars, buffer containing potassium: solid bars, buffer without potassium as described under "Materials and Methods." Each bar represents the mean ± S.D. of three independent determinations.
Cell loading with anti-clathrin antibody results in inhibition of integrin-mediated bacterial uptake. HEp-2 cells were potassium-depleted as described above, and the uptake of transferrin was determined as described previously (30). Cells were incubated with 125I-labeled transferrin (TFN) for 30 min at 37°C. The percentage of internalized bacteria was determined by the gentamicin protection assay (25) after normalization to the value obtained for the CSAT mAb control (see "Materials and Methods"). Each bar represents the mean ± S.D. of three independent determinations. Hatched bars, buffer containing potassium: solid bars, buffer without potassium as described under "Materials and Methods." Each bar represents the mean ± S.D. of three independent determinations.

The results presented here clearly indicate that integrin-mediated bacterial internalization and focal contact localization have different requirements. Previous studies demonstrated that the cytoplasmic domain of the β1 subunit allows association of integrins with the cytoskeleton as well as localization of integrins to focal contacts (9, 32). Three clusters of amino acids distributed along the cytoplasmic domain of the β1 subunit, denoted cyto-1, -2, and -3, contribute to this process (Fig. 4) (10). Substitution mutations in each of these clusters have weak but distinct effects on focal adhesion formation. In contrast, substitution mutations depressing bacterial internalization are centered in cyto-2. Such mutations have very strong effects on bacterial internalization (Table I) and weak effects on focal contact formation (10).

**Discussion**

The results presented here clearly indicate that integrin-mediated bacterial internalization and focal contact localization have different requirements. Previous studies demonstrated that the cytoplasmic domain of the β1 subunit allows association of integrins with the cytoskeleton as well as localization of integrins to focal contacts (9, 32). Three clusters of amino acids distributed along the cytoplasmic domain of the β1 subunit, denoted cyto-1, -2, and -3, contribute to this process (Fig. 4) (10). Substitution mutations in each of these clusters have weak but distinct effects on focal adhesion formation. In contrast, substitution mutations depressing bacterial internalization are centered in cyto-2. Such mutations have very strong effects on bacterial internalization (Table I) and weak effects on focal contact formation (10).
These results were unexpected because it had been thought that association of integrins with the cytoskeleton was required for bacterial uptake. This hypothesis was based on the fact that invasin-mediated uptake by cultured epithelial cells is accompanied by a reorganization of F-actin around the internalized bacteria (21) and is inhibited by cytochalasins (22). Also, the role of actin in phagocytosis has been well established as actin filaments as well as actin-binding proteins associate with nascent phagosomes in macrophages (47). Electron micrographs of HEp-2 cells, however, show that during the early phases of bacterial uptake, actin filaments, visualized by decoration with myosin S-1 pieces, associate mainly with cell protrusions at the tip of the nascent phagosome, with only a few nucleation sites associated with the host cell membrane. Clearly, actin is involved in bacterial uptake, but whether or not it directly binds to integrin receptors during uptake is questionable based on the results of this work.

In this study, we found that deletions removing the cyto-1 and cyto-2 regions as well as amino acid substitutions of residues 785-788 (NPIY) in cyto-2 abolished the ability of the integrin receptor to mediate bacterial uptake. Physical studies using short peptides similar to NPIY indicate that tyrosine-containing motifs have a high propensity to promote tight-turn configurations (36, 48). When these sequences are proximal to an α-helical segment, they have been shown to allow receptor localization to clathrin-coated pits (33), perhaps by interacting with adaptor molecules (34). Although the NPIY sequence corresponding to cyto-2 and the predicted α-helical cyto-1 appear to follow this paradigm closely, there has been little previous evidence that these determinants allow integrin association with clathrin-coated pits. For example, it has been reported that mutations in the NPYXY sequences do not prevent internalization of the α9β1 integrin in Chinese hamster ovary cells (49). On the other hand, immunofluorescence and immunocolloidal gold experiments have shown that a portion of the fibronectin receptor population localizes within clathrin-coated pits (50-52). It is possible that β1-integrins are internalized via multiple pathways, one of which requires the β1 chain cytoplasmic sequence NPIY and clathrin-coated pits.

In this study, large lattices of clathrin and AP2 adaptor complexes are formed beneath bound bacteria in the early stages of the internalization process. These structures were not detected in uninfected cells and appeared to be induced during the bacterial internalization process. Consistent with this observation, a redistribution of the clathrin coat from the basolateral to the apical surface of the cell, in association with focal adhesion contacts, was detected in cells infected with bacteria (53). This indicates that the proximal NPIY sequence is involved in bacterial uptake but that it does not eliminate a possible role for this region in the uptake process. It is possible that the presence of the amino acid changes used in this work may not disrupt signaling functions in cyto-3 that are important for uptake.

Previous studies on Fc receptors in the macrophage have suggested that phagocytosis and endocytosis of the receptor involve different processes (54). Mutational analysis of the cytoplasmic domain of the Fc receptor for IgE in macrophages indicates that residues involved in endocytosis of the receptor are different from residues involved in phagocytosis of IgE-coated erythrocytes (54). On the other hand, as pointed out above, coated pits have been shown to associate with early phagosomes during Fc receptor-mediated phagocytosis in macrophages (55). Perhaps with some receptors, clathrin associates with phagosomes mainly to ensure receptor recycling, rather than playing an active role during phagocytosis, as indicated by our results with integrin-mediated bacterial internalization. To illustrate that phagocytic internalization of different receptors may occur via different pathways, phagocytosis via the receptors for C3 derivatives is not accompanied by a strong release of H2O2, whereas Fc receptor-mediated uptake results in such a burst (56). It is possible that phagocytosis via Fc receptors in macrophages involves different processes than integrin-mediated bacterial internalization in normally nonphagocytic HEp-2 cells, and the results described here may not apply to all classes of receptors.

Phagocytosis via either Fc receptors or integrins seems to require tyrosine phosphorylation (57, 58) and formation of F-actin (21, 22). Although the integrin β2 chain presents a potential tyrosine phosphorylation site at Tyr800 (59), its substitution with an alanine residue did not influence bacterial uptake. It is likely, however, that tyrosine phosphokinases play an active role in transmitting signals necessary for bacterial internalization steps, and a number of studies indicate that a variety of cytoplasmic components are phosphorylated in response to adhesion of integrins to substrates (4, 13, 60).
ther studies aimed at identifying these signals should allow a better understanding of the internalization process.

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