Interaction of Ipa Proteins of *Shigella flexneri* with $\alpha_5\beta_1$ Integrin Promotes Entry of the Bacteria into Mammalian Cells

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Summary

Shigella is a genus of highly adapted bacterial pathogens that cause bacillary dysentery in humans. Bacteria reaching the colon invade intestinal epithelial cells by a process of bacterial-directed endocytosis mediated by the Ipa proteins: IpaB, IpaC, and IpaD of *Shigella*. The invasion of epithelial cells is thought to be a receptor-mediated phenomenon, although the cellular components of the host that interact with the Ipa proteins have not yet been identified. We report here that in a *Shigella flexneri* invasive system and Chinese hamster ovary (CHO) cell monolayers, the Ipa proteins were capable of interacting directly with $\alpha_5\beta_1$ integrin. The invasive capacity of *S. flexneri* for CHO cells increased as levels of $\alpha_5\beta_1$ integrin were elevated. When CHO cells were infected with *S. flexneri*, the tyrosine phosphorylation both of pp 125^{FAK}, an integrin-regulated 125 K focal adhesion kinase, and of paxillin was stimulated. In contrast, an isogenic strain of *S. flexneri* that was defective in invasion owing to a mutation in its *spa32* gene failed to induce such phosphorylation. Under in vitro and in vivo conditions, the released IpaB, IpaC, and IpaD proteins bound to $\alpha_5\beta_1$ integrin in a manner different from that of soluble fibronectin but similar to that of the tissue form of fibronectin. At the site of attachment of *S. flexneri* to CHO cells, $\alpha_5\beta_1$ integrin converged with polymerization of actin. These data thus suggest that the capacity of Ipa proteins to interact with $\alpha_5\beta_1$ integrin may be an important *Shigella* factor in triggering the reorganization of actin cytoskeletons.

Shigella are the causative agents of bacillary dysentery (shigellosis), a disease that provokes severe, bloody diarrhea in humans and other primates. An early essential step leading to shigellosis is the invasion of colonic epithelial cells; this is followed by bacterial multiplication and spread into adjacent cells. The invasive capacity of *Shigella* depends on proteins encoded by a subset of three contiguous operons (*ipa, mxi,* and *spa*) in a 31-kb DNA region on the large 230-kb plasmid (1, 2–4). The three invasins IpaB, IpaC, and IpaD, encoded by the *ipaBCD* genes in the *ipa* operon, have been shown to play a crucial role in the invasion of epithelial cells by *Shigella* (5), in which they could be directly involved in eliciting rearrangement of host actin cytoskeletons at the site of bacterial attachment to the epithelial cells (6) and disrupting the phagocytic vacuoles surrounding the invading bacterium (3, 5). Although Ipa sequences do not contain classical signal peptide sequences (4, 7), the IpaB, IpaC, and IpaD proteins can be secreted onto the bacterial surface and released into the external medium, but only when the Mxi and Spa functions are expressed (1, 2, 6, 8, 9). Release of Ipa proteins into the external medium is triggered upon contact of the bacterium with the epithelial cells (10–12), and the released Ipa proteins form high molecular matrix structures (13).

When *Shigella* infecting tissue-cultured cell islets are observed by Giemsa stain, the bacteria tend to enter through the edges of peripheral cells, which correspond to the basolateral surface of polarized epithelial cells. Indeed, when polarized colonic epithelial cells such as Caco-2 cells grown on a permeable filter were infected with *Shigella flexneri,* the bacteria entered from the basolateral surface much more efficiently than from the apical surface (14). In agreement with this, it has been shown that the release of Ipa proteins from *S. flexneri* is triggered upon contact with polarized epithelial cells, and that the amounts of protein released from bacteria attached to the basolateral side are much higher than from those attached to the apical surface (11). Furthermore, we previously observed that contact with extracellular matrix, such as fibronectin (Fn)[1] laminin, or collagen type IV, can also trigger the release of Ipa proteins from *S. flexneri*

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; Fn, fibronectin; RGDS, Arg-Gly-Asp-Ser; S-Fn, superfibronectin.

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(11). Finally, Vasselon et al. (15) have reported that when chicken embryo fibroblasts are infected with *S. flexneri*, the bacteria invaded cells at focal adhesions, and then started to move along stress fibers that radiated from the focal adhesions toward the nucleus. Taken together, these studies strongly suggested to us that Ipa proteins released into the environmental medium could interact with host receptor(s), such as $\alpha_5\beta_1$ integrin, which are predominately expressed on the basolateral side of the polarized epithelial cells and are capable of mediating actin cytoskeleton rearrangement (16).

We thus attempted to test the above hypothesis using Chinese hamster ovary (CHO) cells. We found that released Ipa proteins can interact with human $\alpha_5\beta_1$ integrin. In this study, we show that the levels of $\alpha_5\beta_1$ integrin expressed in CHO cells correlated with the invasive capacity of *S. flexneri* for these cells. Infection of CHO cells with *S. flexneri* elicited protein tyrosine phosphorylation of integrin-directed focal adhesion kinase $pp125^{FAK}$ (17) or paxillin (18). The sites of bacterial attachment to the cells were associated with enhanced aggregation of $\alpha_5\beta_1$ integrin and polymerization of actin. Our data suggest that the released Ipa proteins mimic in part the function of the tissue form of Fn, superfibronectin (S-Fn) (19), as the multiple ligands interact with $\alpha_5\beta_1$ integrin.

Materials and Methods

Bacterial Strains and Cell Culture. *S. flexneri* 2a YSH6000T, a spontaneous tetracycline-, chloramphenicol-, ampicillin-, and streptomycin-sensitive derivative of YSH6000, has been previously described (20). Strain CS2585 contained an in-frame deletion in the *spa32* gene on the large plasmid derived from YSH6000T (11). *Escherichia coli* K-12 HB101 carrying pINV (*Yersinia pseudotuberculosis inv* gene) was a generous gift from T.F. Meyer (Max-Planck-Institut, Tübingen, Germany). Unless otherwise indicated, the bacteria were routinely grown in brain-heart infusion (BHI) broth (Difco, Detroit, MI) at 37°C. CHO cells were maintained in MEM (Nissui, Tokyo, Japan) containing 10% FCS (Nichirei, Tokyo, Japan) in a 5% CO_2 atmosphere.

Invasion Assay. The invasive capacity of bacteria was measured by counting the number of viable bacteria internalized into the epithelial cytoplasm; a gentamicin-protection assay (11) was used for this purpose.

Transfection of CHO Cells and Analysis of a Clone Overexpressing the $\alpha_5\beta_1$ Integrin. The $\alpha_5\beta_1$ integrin–transfected CHO cells were constructed as follows: a 3.8-kb full-length cDNA clone encoding the human α_5 integrin subunit obtained from pECE-FNRα_5 (21) and a 3.6-kb full-length cDNA clone encoding the human β_1 integrin subunit obtained from pECE-FNRβ_1 (21) were separately cloned into pcDNA1neo (Invitrogen, San Diego, CA). Both plasmids were transfected together into CHO cells, which were then selected for geneticin-resistant colonies. To select the transfectants expressing high levels of $\alpha_5\beta_1$ integrin, each of the transfectants was grown to confluence in a 24-well plate and assayed for the formation of Fn fibrils using FITC-labeled anti-Fn antibody (21). The amounts of $\alpha_5\beta_1$ integrin expressed from the two transfectants, CHO-HFR4 and CHO-HFR5 cells, were quantified by a previously described method (22).

Interaction of Released Ipa Proteins with $\alpha_5\beta_1$ Integrin: ELISA. The ability of $\alpha_5\beta_1$ integrin to bind to bacteria or Ipa proteins was measured as follows: A 50-μl aliquot of MEM containing either $\sim10^8$ bacteria or IpaB, IpaC, and IpaD released from $\sim10^8$ bacteria of wild-type *S. flexneri* YSH6000T (11, 12) was placed into 96-well microtiter plates and incubated at room temperature for 2 h. The MEM was then removed, and the wells were washed twice with PBS-0.05% Tween 20. 50 μl of PBS containing $\alpha_5\beta_1$ integrin (10 μg/ml) prepared with a 120 K–Fn column (21) was added, and the plate was incubated at 37°C for 1 h. The amount of bound $\alpha_5\beta_1$ integrin was determined by ELISA with anti-α_5(mAb KH/33) and β_1(mAb SG/19) integrin antibody.

Immunoprecipitation Assay. $\alpha_5\beta_1$ integrin added to 1 ml of MEM containing Ipa proteins released from $\sim10^8$ cells of *S. flexneri* YSH6000T ($\alpha_5\beta_1$ integrin concentration, 20 μg/ml) was incubated at 37°C for 20 min in an incubator containing 5% CO_2. The sample was then immunoprecipitated with the appropriate antibody and incubated at 4°C overnight; this step was followed by precipitation using protein A–Sepharose beads for 1 h at room temperature. The precipitates were washed with PBS and analyzed by immunoblotting with either anti-$\alpha_5\beta_1$ integrin antibody or anti-IpaB, IpaC, and IpaD antibody. $\alpha_5\beta_1$ integrin was detected by 7% SDS-PAGE under nonreducing conditions, whereas IpaB, IpaC and IpaD were detected by 10% SDS-PAGE under reducing conditions.

Immunostaining Assay. CHO-HFR5 cells ($\sim2 \times 10^5$ cells/ml) were suspended in 2 ml of MEM containing IpaB, IpaC, and IpaD released from $\sim10^8$ bacteria of YSH6000T (wild-type) or CS2585 (*Δspa32*) and then cultured for 24 h at 37°C. IpaC or $\alpha_5\beta_1$ integrin were labeled by indirect immunofluorescence with rabbit antiserum raised against IpaC or mouse monoclonal antibody against $\alpha_5\beta_1$ integrin plus either a goat anti–rabbit rhodamine-conjugated IgG or an anti-mouse FITC-conjugated IgG as the second antibody.

S-Fn Production. S-Fn was produced as follows (19). Fn was incubated with III_1-C, which was generated by a cDNA encoding the COOH-terminal two-thirds of the first type III repeat of Fn (19), at 37°C for 24 h. As a negative control for III_1-C, III_{11}, which is an analogous region from the 11th type III repeat (19), was used instead of III_1-C. The recombinant III_1-C or III_{11} proteins were produced by the QIA express system with prokaryotic expression plasmid pQE-12 (Qiagen, Chatsworth, CA). Recombinant proteins were purified on Ni-NTA chromatography (Qiagen).

Integrin-Matrix Complex Invasion Inhibition Assay. $\alpha_5\beta_1$ integrin was mixed with an equal concentration of Arg-Gly-Asp-Ser (RGDS) peptides, Fn, Fn and III_1-C (S-Fn), or Fn and III_{11} in Tris-saline (pH 7.5) containing 3 mM $MnCl_2$ buffer (21) and incubated for 3 h at 4°C. The $\alpha_5\beta_1$ integrin–matrix complex thus produced was added to MEM in which YSH6000T was suspended at a multiplicity of infection of 100. This suspension was added onto the CHO-HFR5 cell monolayers, centrifuged at 700 *g* for 10 min at room temperature, and incubated for 20 min at 37°C. The number of internalized bacteria was measured by the gentamicin-protection assay.

Analysis of Tyrosine Phosphorylation. CHO cells were cultured in six-well plates to 80% confluence and infected with either wild-type *S. flexneri* strain YSH6000T or with the *spa32* mutant CS2585 at a multiplicity of infection of 100 in MEM. Infection was carried out by centrifugation at 700 *g* for 10 min at room temperature followed by incubation for 10–30 min at 37°C. Cells were washed with HBSS and lysed in 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 1% Triton X-100, 100 μM Na_3VO_4, 1 mM PMSF) per well. Immunoprecipitation and immunoblotting were

performed as described previously (11, 12); mouse monoclonal antiphosphotyrosine antibody PT-66, anti-pp125^{FAK}, antibody 2A7, and antipaxillin antibody 349 were purchased from Sigma Chemical Co. (St. Louis, MO), Upstate Biotechnology Inc. (Lake Placid, NY), and Affiniti Research Products Ltd. (Exeter, United Kingdom), respectively.

Confocal Laser Scanning Microscopy. Cells were examined on a confocal laser scanning microscope equipped with dual detectors and an argon-krypton (Ar/Kr) laser for simultaneous scanning of three different fluorochromes (MRC-1000; Bio-Rad Laboratories, Richmond, CA).

Results

Effect of Levels of $\alpha_5\beta_1$ Integrin on the Invasion Capacity of S. flexneri. To assess whether the level of $\alpha_5\beta_1$ integrin expressed in mammalian cells affected the invasion efficiency of *S. flexneri*, we used CHO cells since these cells have been extensively used to characterize functions of $\alpha_5\beta_1$ integrin (21). To obtain CHO cell lines capable of expressing $\alpha_5\beta_1$ integrin at a high level, pcDNA1neo vectors containing cDNA encoding human α_5 or β_1 integrin (21) were cotransfected into the CHO cells. Transfectants capable of expressing high levels of $\alpha_5\beta_1$ integrin were chosen to test the relationship between levels of $\alpha_5\beta_1$ integrin and the invasion efficiency of *S. flexneri*. Two transfectants, CHO-HFR5 and CHO-HFR4, expressed $\alpha_5\beta_1$ integrin levels that were 7.4- and 3.1-fold higher, respectively, than those of the parent CHO cells. The invasion efficiencies of the two transfectants by the wild-type strain of *S. flexneri* YSH6000T were 15.4-fold (CHO-HFR5) and 7.6-fold (CHO-HFR4), respectively, higher than that for the CHO cells (Fig. 1), as measured by the gentamicin-protection assay. The invasion of the two transfectants and the parental CHO cells by CS2585 (a noninvasive *spa32* mutant) (11) was always low (<1%) compared with that of CHO cells by YSH6000T (100%). Strain CS2585 carries an in-frame deletion mutation in *spa32*, a gene in the *spa* operon required for secretion of the IpaB, IpaC, and IpaD proteins into the external medium. This mutation renders the bacteria deficient in their ability to enter the host cell (11, 12). An *E. coli* K-12 strain (HB101 harboring pINV) that expresses *Yersinia* invasin, an outer membrane protein required for the bacteria to bind to $\alpha_5\beta_1$ integrin (23), entered the transfectants with higher efficiencies (15.5-fold for CHO-HFR5 and 7.0-fold for CHO-HFR4, respectively) than did the parental CHO cells (Fig. 1).

Binding of Ipa Proteins to $\alpha_5\beta_1$ Integrin. Since the IpaB, IpaC, and IpaD proteins released from *Shigella* were most likely interacting with the target cell (10, 11), we tested $\alpha_5\beta_1$ integrin for its ability to bind to Ipa proteins released from YSH6000T into the tissue-culture medium (MEM). IpaB, IpaC, and IpaD proteins present in MEM were coated onto the wells of a microtiter plate, to which $\alpha_5\beta_1$ integrin was added; the plate was incubated at 37°C for 1 h. As shown in Fig. 2 *A*, a significant amount of bound $\alpha_5\beta_1$ integrin was detected. We then seeded YSH6000T or HB101 carrying pINV grown in brain-heart infusion broth at 37°C into the wells of a microtiter plate, added $\alpha_5\beta_1$ integrin, and incubated the plate at 37°C for 1 h. However, the binding of $\alpha_5\beta_1$ integrin to whole YSH6000T bacteria was barely detectable (Fig. 2 *A*), even though the IpaB, IpaC, and IpaD proteins were presented on the surface (11, 24). As has been previously shown (23), a significant amount of $\alpha_5\beta_1$ integrin bound to HB101 harboring pINV.

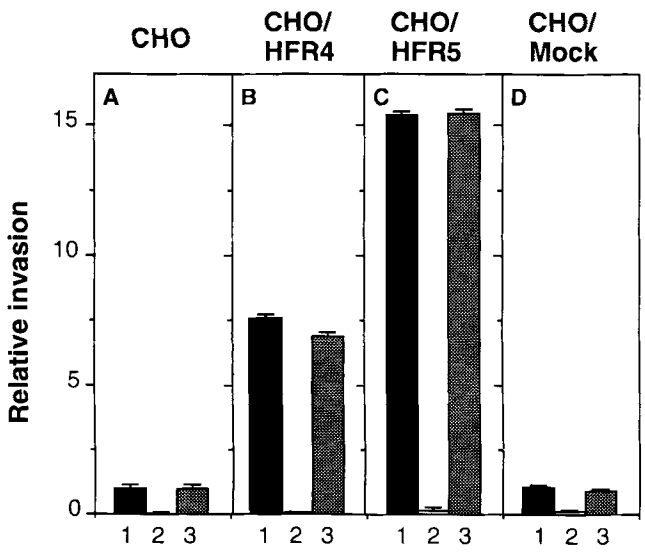

Figure 1. Quantification of bacterial invasion of $\alpha_5\beta_1$ integrin–transfected CHO cells. The number of internalized bacteria were measured by gentamicin-protection assay. Figures represent relative numbers of internalized bacteria compared with CHO cells. (*A*) CHO cells; (*B*) CHO-HFR4 cells; (*C*) CHO-HFR5 cells; (*D*) CHO-Mock (vector). Lanes *1*, YSH6000T (wild-type *S. flexneri*); lanes *2*, CS2585 (*Δspa32*); lanes *3*, *E. coli* K-12 HB101 carrying pINV. The data shown are the means of triplicate experiments; the top bar shows the SEM.

To further demonstrate the ability of $\alpha_5\beta_1$ integrin to bind to the released Ipa proteins, $\alpha_5\beta_1$ integrin was added to MEM containing released IpaB, IpaC, and IpaD proteins, and the mixture was incubated at 37°C for 20 min. The proteins in the MEM were then immunoprecipitated with anti-α_5 or anti-β_1 antibody, or with anti-IpaB, anti-IpaC, or anti-IpaD antibody; the precipitated proteins were separated on SDS-PAGE and analyzed by immunoblotting with antibody specific for IpaB, IpaC, and IpaD proteins. As shown in Fig. 2 *B*, all the precipitates contained similar levels of Ipa proteins. Each of the precipitates separated by SDS-PAGE were also immunoblotted with anti-α_5 or anti-β_1 integrin antibody. Both the α_5 and β_1 integrins were precipitated by the antibodies specific for either IpaB, IpaC, or IpaD (Fig. 2 *C*); this observation suggests that the Ipa proteins bound to the integrin to form a complex.

To visualize directly the specific interaction of $\alpha_5\beta_1$ integrin with the Ipa proteins, CHO-HFR5 cells cultivated in the presence of IpaB, IpaC, and IpaD proteins in MEM at 37°C for 1 d were stained with FITC-labeled anti-$\alpha_5\beta_1$ integrin monoclonal antibody or rhodamine-labeled anti-IpaC antibody and were examined for the localization of the integrin and Ipa proteins by use of confocal laser scanning microscopy. As seen in Fig. 2 *D*, sites at the periphery of CHO-HFR5 cell islets or spots beneath the cells rich in

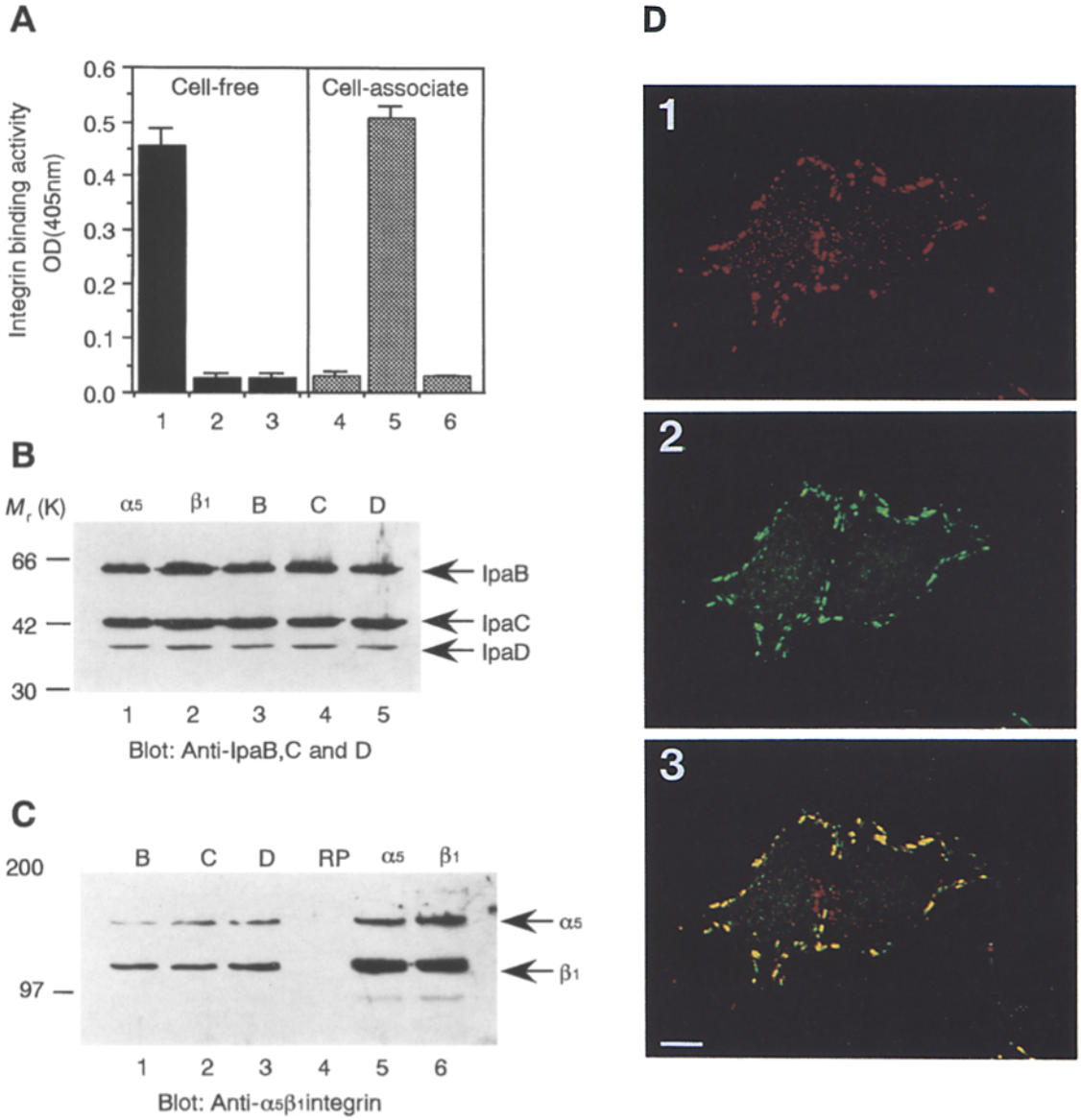

Figure 2. Binding of IpaB, IpaC, and IpaD to $\alpha_5\beta_1$ integrin. (*A*) Integrin-binding activity. The left and right panels indicate binding activity of $\alpha_5\beta_1$ integrin to Ipa proteins released into the external medium or whole bacterial cells, respectively. Lanes *1*, culture supernatant (MEM) containing IpaB, IpaC, and IpaD released from YSH6000T (wild-type *S. flexneri*); lane *2*, same as 1 but with IpaB, IpaC, and IpaD removed by immunoprecipitation with anti-IpaB, IpaC, and IpaD antibodies; lane *3*, MEM from the *spa32* mutant of *S. flexneri* (no Ipa proteins); lane *4*, YSH6000T; lane *5*, *E. coli* K-12 HB101 carrying pINV; lane *6*, no bacteria (bovine serum albumin). (*B*) Demonstration of affinity of Ipa proteins for $\alpha_5\beta_1$ integrin by immunoprecipitation with anti-IpaB, anti-IpaC, or anti-IpaD antibody and anti-α_5 or anti-β_1 integrin antibody. Immunoprecipitates with anti-α_5 integrin (lane *1*), anti-β_1 integrin (lane *2*), anti-IpaB (lane *3*), anti-IpaC (lane *4*), or anti-IpaD (lane *5*) were analyzed by immunoblotting with anti-IpaB, IpaC, and IpaD. (*C*) Immunoprecipitates with anti-IpaB (lane *1*), anti-IpaC (lane *2*), anti-IpaD (lane *3*), rabbit preimmune serum (lane *4*), anti-α_5 integrin (lane *5*), or anti-β_1 integrin (lane *6*) were analyzed by immunoblotting with anti-$\alpha_5\beta_1$ integrin. (*D*) Interaction of Ipa proteins with $\alpha_5\beta_1$ integrin. Confocal microscopic images of CHO-HFR5 cells cultivated with released Ipa proteins (panels *1–3*). Panels *1*, immunostaining with rhodamine–anti-IpaC (*red*); panel *2*, FITC–anti-$\alpha_5\beta_1$ integrin (*green*). The red and green fluorescence images were recorded separately (panels *1* and *2*) and merged (panel *3*). Co-localization is in yellow. Bar, 10 μm.

$\alpha_5\beta_1$ integrin also showed accumulation of IpaC protein. Similar results were also obtained for IpaB and IpaD proteins (data not shown). From these findings we concluded that Ipa proteins released into the external medium can interact directly with $\alpha_5\beta_1$ integrin.

Interference of $\alpha_5\beta_1$ Integrin with the Invasion of CHO Cells by S. flexneri. To confirm that the binding of Ipa proteins to the integrin was indeed involved in the invasion of CHO cells by *Shigella*, we investigated whether the addition of $\alpha_5\beta_1$ integrin into the MEM affected the invasive capacity of YSH6000T for CHO-HFR5 cells. We found that as the concentration of added $\alpha_5\beta_1$ integrin in MEM increased, the invasive capacity of YSH6000T decreased, and at a concentration of 20 μg/ml, inhibition of the inva-

sive capacity of YSH6000T for CHO-HFR5 reached the maximum of 64% of the inhibition (zero) seen without $\alpha_5\beta_1$ integrin (Fig. 3 *A*). Since $\alpha_5\beta_1$ integrin is the receptor for Fn, and the RGDS peptides are responsible for binding to $\alpha_5\beta_1$ integrin (25), Fn or RGDS peptides mixed with $\alpha_5\beta_1$ integrin were added to the MEM to see whether the invasive capacity of YSH6000T was affected. The results showed that both $\alpha_5\beta_1$ integrin–Fn and –RGDS peptides complexes added to the MEM-inhibited invasive capacity (Fig. 3 *A*), suggesting that the interaction of Ipa proteins with $\alpha_5\beta_1$ integrin is apparently different from that with Fn or RGDS peptides. A recent study indicated that the tissue form of Fn exhibits disulfide cross-linked structures with multimers of high relative molecular mass that resemble matrix fibrils; this structure is S-Fn (19). In that study, the interaction of $\alpha_5\beta_1$ integrin with S-Fn was differentiated from that with Fn (19), thus prompting us to test whether the $\alpha_5\beta_1$ integrin–S-Fn complex added to the MEM interfered with efficient CHO cells invasion by YSH6000T. By incubating Fn with III_1–C fragment (COOH-terminal two-thirds of the III_1 repeat of Fn molecular) (19), the resulting S-Fn was checked by the methods described previously (19) and incubated with $\alpha_5\beta_1$ integrin before addition of the MEM. The addition of $\alpha_5\beta_1$ integrin–S-Fn complex did restore the invasive capacity to YSH6000T at a level similar to that observed without $\alpha_5\beta_1$ integrin (Fig. 3 *A*). However, the addition of Fn incubated with III_{11} fragment (negative control) (19) to the MEM failed to restore the invasive capacity (Fig. 3 *A*), suggesting that the interaction of Ipa proteins with $\alpha_5\beta_1$ integrin was distinct from that with soluble Fn, but rather resembles that with S-Fn. To confirm this, the capacity of S-Fn to interfere with the binding of Ipa proteins to $\alpha_5\beta_1$ integrin was further investigated.

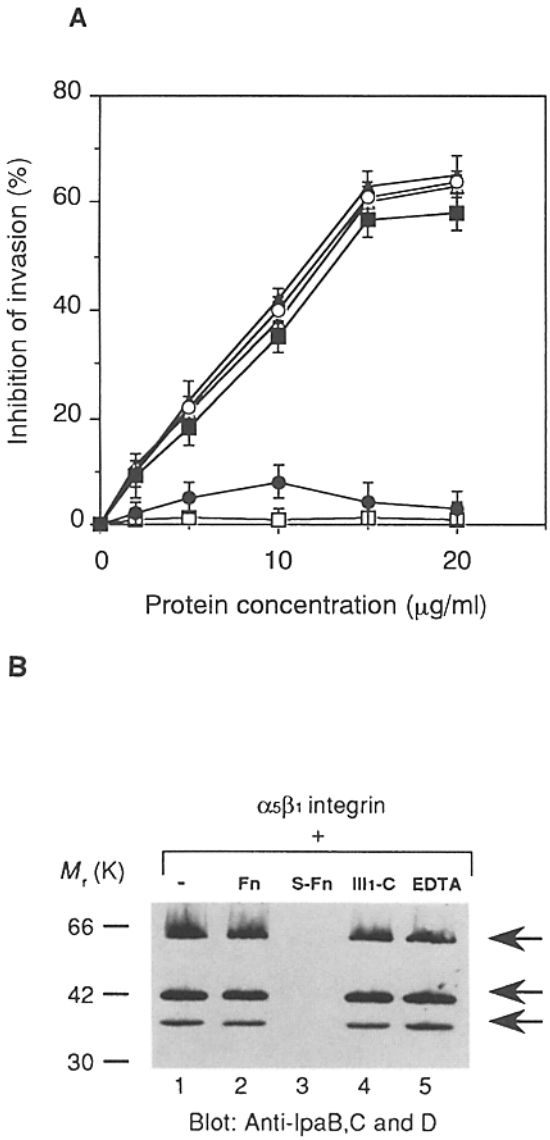

Figure 3. Effect of S-Fn on binding of IpaB, IpaC, and IpaD to $\alpha_5\beta_1$ integrin. (*A*) Inhibition of YSH6000T (wild-type *S. flexneri*) invasion of CHO-HFR5 cells. Various proteins were added to MEM at various concentrations. Bovine serum albumin (□); $\alpha_5\beta_1$ integrin (○); $\alpha_5\beta_1$ integrin and RGDS peptides (△); $\alpha_5\beta_1$ integrin and Fn (■); $\alpha_5\beta_1$ integrin and S-Fn (produced by 1 μM III_1-C per μg/ml Fn) (●); $\alpha_5\beta_1$ integrin and Fn/III_{11} (negative control; produced by 1 μM III_{11} per μg/ml Fn) (▲). Results are shown as a percentage of the number of invaded bacteria that was reduced by addition of the indicated protein–protein complexes to MEM. Data shown represent the mean of triplicate experiments, and the bars show the SE of the mean. (*B*) Inhibition of binding of IpaB, IpaC, and IpaD to $\alpha_5\beta_1$ integrin. The $\alpha_5\beta_1$ integrin (20 μg/ml)–matrix complex (lane *1*, no addition; lane *2*, Fn [20 μg/ml]; lane *3*, S-Fn [Fn, 20 μg/ml; III_1-C, 20 μM]; lane *4*, III_1-C [20 μM]) or 10 mM EDTA (lane *5*) was added to MEM containing released Ipa proteins. Ipa proteins and $\alpha_5\beta_1$ integrin were immunoprecipitated with anti-$\alpha_5\beta_1$ integrin antibody and then probed with anti-IpaB, IpaC, and IpaD antibodies.

Preincubation of $\alpha_5\beta_1$ integrin with S-Fn or Fn was added to the MEM containing IpaB, IpaC, and IpaD proteins, and the mixture was incubated at 37°C for 20 min. Proteins immunoprecipitated by anti-$\alpha_5\beta_1$ integrin antibody were then analyzed by immunoblots using anti-IpaB, IpaC, and IpaD antibodies. The data showed that $\alpha_5\beta_1$ integrin preincubated with S-Fn, but not with Fn, failed to bind all three Ipa proteins (Fig. 3 *B*). In this assay, we also tested the effect of EDTA (10 mM) on the binding of the Ipa proteins in MEM to added $\alpha_5\beta_1$ integrin, since the binding of Fn, but not S-Fn, to $\alpha_5\beta_1$ integrin was previously shown to be completely blocked in the presence of EDTA (26). As expected, the presence of 10 mM EDTA had no effect on the binding of Ipa proteins to the $\alpha_5\beta_1$ integrin; this finding further supports the concept that the interaction is similar to S-Fn binding (Fig. 3 *B*). These results thus clearly demonstrate that the interaction of Ipa proteins with $\alpha_5\beta_1$ integrin is involved in the invasion of CHO cells by *Shigella*.

Induction of Tyrosine Phosphorylation of pp125^{FAK} and Paxillin upon Infection of CHO cells by S. flexneri. Since protein tyrosine phosphorylation is one of the earliest events detected in response to integrin stimulation (16), we tested whether infection of CHO cells by *Shigella* induced tyrosine phosphorylation of proteins. Accordingly, CHO cells were infected with YSH6000T for 10, 20, and 30 min, and tyrosine-phosphorylated proteins were analyzed by immunoblotting with an antiphosphotyrosine monoclonal antibody. *S. flexneri* infection increased the tyrosine phosphorylation of at least two prominent species of relative molecular

mass 125,000 (125 kD) and 70,000 (70 kD) (data not shown), Cultured CHO cells infected using CS2585 (*spa32* mutant) failed to show an increased tyrosine phosphorylation of these proteins (data not shown), strongly suggesting that entry of *Shigella* into CHO cells was responsible for the induced tyrosine phosphorylation of the 125- and 70-kD proteins that was observed. These proteins were tentatively identified as integrin-regulated focal adhesion kinase pp125^{FAK} (17) and paxillin (18). Hence, the phosphorylated 125- and 70-kD proteins appearing in the CHO cells infected with *S. flexneri* were examined using anti-pp125^{FAK} or anti-paxillin mAbs. At 10, 20, and 30 min after infection of CHO cells with *S. flexneri* or with the noninvasive *spa32* mutant CS2585, whole CHO cell lysates were immunoprecipitated with the anti-pp125^{FAK} or anti-paxillin antibody. The proteins in the precipitates were separated by SDS-PAGE and immunoblotted with the anti-phosphotyrosine antibody. Phosphorylated 125- and 70-kD proteins were detected in CHO cells infected with wild-type *S. flexneri* (Fig. 4, *A* and *B*, lanes *3* and *4*) that were not observed in CHO cells infected with the noninvasive mutant CS2585 (*spa32* mutant) (Fig. 4, *A* and *B*, lanes *5*–*7*). In agreement with this finding, when CHO cells were pretreated with genistein (125 μM), a tyrosine kinase inhibitor, the levels of phosphorylation of pp125^{FAK} and paxillin at 20 or 30 min after infection with YSH6000T were substantially lower than those without genistein (data not shown). Appearance of tyrosine-phosphorylated pp125^{FAK} and paxillin were also detected in human colonic Caco-2 cells when infected with YSH6000T, but not with CS2585 (data not shown). Indeed, the capacity of YSH6000T to invade CHO cells declined to 7.9% of the initial invasive capacity (100%) when the CHO cells were pretreated with genistein (125 μM). This observation demonstrates that infection of CHO cells by *S. flexneri* involves the tyrosine phosphorylation of proteins, including pp125^{FAK} and paxillin.

Accumulation of $\alpha_5\beta_1$ Integrin upon Attachment of S. flexneri to CHO Cells. Enhancement of the tyrosine phosphorylation of pp125^{FAK} and paxillin in CHO cells has been

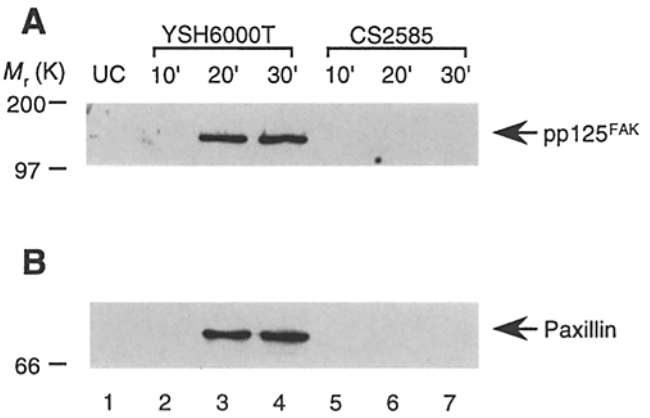

Figure 4. Tyrosine phosphorylation of CHO cell proteins upon *S. flexneri* invasion. Samples are from uninfected cells (lane *1*), from cells infected with wild-type *S. flexneri* for 10 min (lane *2*), 20 min (lane *3*), 30 min (lane *4*), and from cells infected with the *S. flexneri spa32* mutant for 10 min (lane *5*), 20 min (lane *6*), and 30 min (lane *7*). Numbers to the left indicate the positions of molecular weight standards. Cell lysates were immunoprecipitated with anti-pp125^{FAK} mAb 2A7 (*A*) or anti-paxillin mAb 347 (*B*); the precipitates were separated by 10% SDS-PAGE, transferred to nitrocellulose, and probed with the antiphosphotyrosine mAb PT-66.

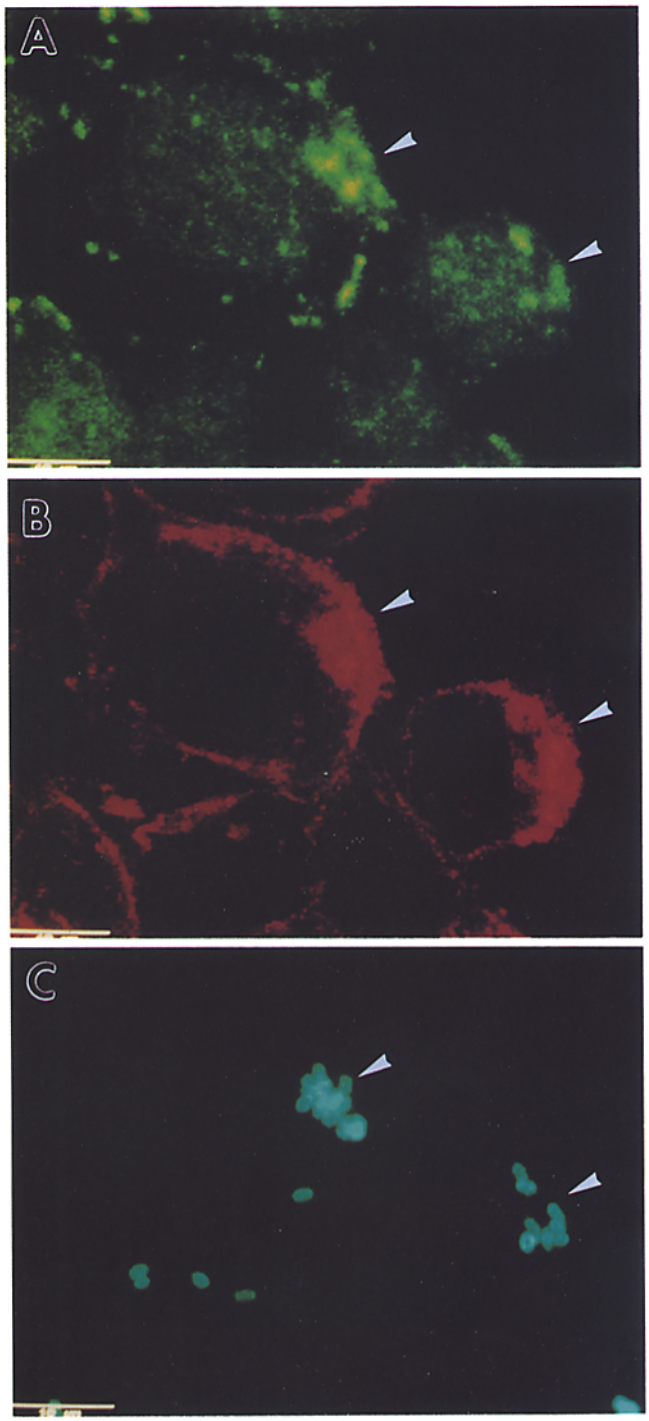

Figure 5. Localized rearrangement of $\alpha_5\beta_1$ integrin and F-actin at the site of *S. flexneri* attachment to CHO-HFR5 cells. (*A*) Mouse FITC-labeled anti-$\alpha_5\beta_1$ integrin. (*B*) Rhodamine-labeled phalloidin. (*C*) Cy5-labeled anti-*S. flexneri* 2a LPS. The arrowheads mark $\alpha_5\beta_1$ integrin clustering sites (*A*), actin polymerization (*B*), and attached bacteria (*C*).

shown to stimulate a rapid reorganization of the actin cytoskeleton at the focal point of adhesion, a consequence of the clustering of integrins (27). We examined whether clustering of $\alpha_5\beta_1$ integrin was induced upon invasion of epithelial cells by *Shigella*. 20 min after infection of CHO-HFR5 cells with the wild-type strain of *S. flexneri*, the CHO cells were immunostained with FITC-labeled anti-$\alpha_5\beta_1$ integrin mAb (Fig. 5 *A*), rhodamine-labeled phalloidin (Fig. 5 *B*), and Cy5-labeled anti-*S. flexneri* 2a LPS antibody (Fig. 5 *C*); the stained cells were analyzed by confocal laser scanning microscopy. Accumulation of $\alpha_5\beta_1$ integrin appeared at the site of bacterial attachment, where actin polymerization occurred (Fig. 5). The accumulated $\alpha_5\beta_1$ integrin at the site of attachment of YSH6000T resulted in the convergence of vinculin and talin (data not shown). As expected, attachment of CS2585 (*spa32* mutant) induced neither actin polymerization nor accumulation of $\alpha_5\beta_1$ integrin (11).

Discussion

The data provided in this study indicate that Ipa proteins released from *S. flexneri* are capable of direct binding to $\alpha_5\beta_1$ integrin—a reaction that may mediate the signal transduction required for activating focal adhesion functions such as tyrosine phosphorylation of $pp125^{FAK}$ and paxillin or reorganization of actin cytoskeletons. The evidence for this was deduced from five independent approaches: *(i)* the effect of different levels of $\alpha_5\beta_1$ integrin expressed from CHO cells on the invasive capacity of *S. flexneri*; *(ii)* the binding capacity of $\alpha_5\beta_1$ integrin to IpaB, IpaC, and IpaD proteins; *(iii)* competitive inhibition of *Shigella* invasion with $\alpha_5\beta_1$ integrin; *(iv)* protein tyrosine phosphorylation of $pp125^{FAK}$ and paxillin by infecting *S. flexneri*; and *(v)* the convergence of $\alpha_5\beta_1$ integrin, vinculin, and talin at the site of bacterial attachment to CHO cells. These results agree with the observed properties of invasion by *Shigella*. Indeed, the bacterial entry into the polarized epithelial cells takes place much more efficiently through the basolateral side than from the apical surface, in that $\alpha_5\beta_1$ integrin is predominantly expressed on the basolateral surface (14). The release of Ipa proteins from *Shigella* is triggered upon contact with extracellular matrix such as Fn, Laminin, or collagen type IV (11). *S. flexneri* infecting chicken embryo fibroblasts were shown to enter from the focal adhesions, and the invading bacterium move along stress fibers toward the nuclei (15).

The IpaB, IpaC, and IpaD proteins bind to $\alpha_5\beta_1$ integrin in MEM, as shown by immunoprecipitation methods with either anti-IpaB, -IpaC, or-IpaD antibody, or with anti-α_5 or $-\beta_1$ integrin antibody (Fig. 2). Although $\alpha_5\beta_1$ integrin added to microtiter plate wells coated with Ipa proteins exhibited binding ability, the integrin failed to bind to *S. flexneri* cell-coated wells, even though the bacteria expressed Ipa proteins on the cell surface. This observation indicates that only released Ipa proteins, and not the cell-bound form, can interact with the host receptor, $\alpha_5\beta_1$ integrin. Since Ipa proteins released into the external medium from *Shigella* exist as a complex (28) or as high molecular matrices (13) and the ability of *Shigella* to release Ipa proteins is essential for invasiveness (11, 12), it is likely that only the Ipa proteins released into the medium are able to interact with host receptors, such as $\alpha_5\beta_1$ integrin. However, this possibility remains to be proved. If our notion is true that the Ipa proteins could be released to allow invasion by *S. flexneri*, then we need to address the question of how this process leads to bacterial entry into cells. Although no data are now available, it is possible that Ipa proteins detached from the cell surface upon contact with extracellular matrix may still be loosely associated with the bacterial surface and may form some matrix-like structures (13) through which the released Ipa proteins could be associated with other Ipa proteins still bound to the bacterial surface. This process may lead to uptake of bacteria by the host cells at actin and integrin foci.

The Ipa protein interaction with $\alpha_5\beta_1$ integrin seemed different from that between soluble Fn and $\alpha_5\beta_1$ integrin; rather, this interaction resembled that between S-Fn and $\alpha_5\beta_1$ integrin. As shown in Fig. 3, neither the invasive efficiency of *Shigella* for CHO cells nor the binding capacity of the Ipa proteins to $\alpha_5\beta_1$ integrin was inhibited by the presence of Fn or its RGDS peptides, but was competitively inhibited by S-Fn. It has been shown that S-Fn binds more efficiently to $\alpha_5\beta_1$ integrin than does soluble Fn, and that the binding is less dependent on the presence of divalent-cations than that of Fn (19). Indeed, the binding of $\alpha_5\beta_1$ integrin to the Ipa proteins was not inhibited even in the presence of 10 mM EDTA. Since released Ipa proteins can form high molecular matrix forms (13) (Watarai, M., unpublished results), it is likely that they mimic, at least in part, the multivalent ligand-binding property of S-Fn to $\alpha_5\beta_1$ integrin. In addition, we showed that attachment of YSH6000T (wild type *S. flexneri*), but not CS2585 (*spa32* mutant), to CHO cells elicits convergence of $\alpha_5\beta_1$ integrin and F-actin (Fig. 5), that is concomitant with accumulation of vinculin or talin (Watarai, M., unpublished results). These cellular events, including the tyrosine phosphorylation of $pp125^{FAK}$ and paxillin, have been demonstrated to be induced in response to the clustering of integrin caused by multiple ligand interactions (16, 29).

Although the detailed mechanisms underlying the interaction of the Ipa protein complex with $\alpha_5\beta_1$ integrin remain to be elucidated, it is worth noting that some properties of the binding of Ipa proteins to $\alpha_5\beta_1$ integrin are apparently different from the characteristics reported for *Yersinia* invasin (Inv), a protein able to bind to $\alpha_5\beta_1$ integrin and to promote uptake of bacteria by epithelial cells (23). *Yersinia* Inv can interact with $\alpha_5\beta_1$ integrin as an outer membrane-bound form (23), whereas the Ipa proteins of *Shigella* can interact only after they are released from the bacterial surface. The binding of Inv protein to $\alpha_5\beta_1$ integrin can be competitively inhibited by Fn or its RGDS peptides, but Fn interaction requires divalent-cation such as Mg^{2+} (30), whereas the binding capacity of Ipa proteins to $\alpha_5\beta_1$ integrin can be competitively inhibited by S-Fn (Fig. 3). Thus, it would be of great interest to distinguish further

the functional difference in the interaction of the released Ipa proteins with $\alpha_5\beta_1$ integrin from that of *Yersina* Inv protein at the molecular level.

Entry of pathogenic bacteria into the intracellular compartment of host cells or intimate attachment to the host cells is involved in eliciting protein tyrosine phosphorylation, although the pathogenic life cycles of these bacteria are quite divergent. Uptake of *Y. pseudotuberculosis* into epithelial cells is triggered by the binding of Inv protein to β_1 integrins of the host cells (23). Uptake of the bacteria can be specifically blocked by inhibitors of protein tyrosine kinase (31). *Salmonella typhimurium* can efficiently enter polarized epithelial cells such as MDCK and Henle-407 cells from the apical surface (32), and evokes host-cell protein tyrosine phosphorylation, which is required for membrane ruffling at the site of bacterial attachment to the epithelial cells (33). Intimate contact of enteropathogenic *E. coli* (EPEC) with epithelial cells elicits tyosine phosphorylation of a yet unidentified host protein called Hp90, which seems to be involved in assembly of the host actin cytoskeleton and, possibly, in the uptake of bacteria by the epithelial cells (34). *S. flexneri* entry into HeLa or Caco-2 cells has recently been observed to induce tyrosine phosphorylation of cortactin, a cytoskeleton-associated protein tyrosine dinase substrate, through overexpression of proto-oncoprotein $pp60^{c\text{-}src}$ (35). Furthermore, we have observed that the entry of YSH6000T (wild type of *S. flexneri*) into CHO cells (Fig. 4) or Caco-2 cells (Watarai, M., unpublished results) can elicit tyrosine phosphorylation of $pp125^{FAK}$ and paxillin proteins, and that the invasive capacity of YSH6000T for CHO cells declined to 7.9% of the initial invasive capacity (100%) when the CHO cells were pretreated with genistein (125 μM). Thus, these results suggest that protein phosphorylation associated with the reorganization of the actin cytoskeleton is involved in the invasion of epithelial cells by *Shigella*, although it remains to be elucidated what kind of cellular signal transduction pathway(s) are used in the invasion of epithelial cells by *Shigella*. Further studies are needed to understand the mechanisms underlying the process by which *Shigella* enters eukaryotic cells that are non-professional phagocytes.

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References

1. Andrews, G.P., and A.T. Maurelli. 1992. *mxiA* of *Shigella flexneri* 2a, which facilitates export of invasion plasmid antigens, encodes a homolog of the low-calcium response protein, LcrD, of *Yersinia pestis*. *Infect. Immunol.* 60:3287–3295
2. Venkatesan, M.M., J.M. Buysse, and E.V. Oaks. 1992. Surface presentation of *Shigella flexneri* invasion plasmid antigens requires the products of the *spa* locus. *J. Bacteriol.* 174:1990–2001.
3. Ménard, R., P.J. Sansonetti, and C. Parsot. 1993. Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. *J. Bacteriol.* 175:5899–5906.
4. Sasakawa, C., B. Adler, T. Tobe, N. Okada, S. Nagai, K. Komatsu, and M. Yoshikawa. 1989. Functional organization and nucleotide sequence of virulence Region-2 on the large virulence plasmid in *Shigella flexneri* 2a. *Mol. Microbiol.* 3: 1191–1201.
5. High, N., J. Mounier, M.-C. Prévost, and P.J. Sansonetti. 1992. IpaB of *Shigella flexneri* causes entry into epithelial cells and escape from the phagocytic vacuole. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:1991–1999.
6. Clerc, P., and P.J. Sansonetti. 1987. Entry of *Shigella flexneri* into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. *Infect. Immunol.* 55:2681–2688.
7. Venkatesan, M.M., J.M. Buysse, and D.J. Kopecko. 1988. Characterization of invasion plasmid antigen genes (*ipaBCD*) from *Shigella flexneri*. *Proc. Natl. Acad. Sci. USA.* 85:9317–9321.
8. Andrews, G.P., A.E. Hromockyj, C. Coker, and A.T. Maurelli. 1991. Two novel virulence loci, *mxiA* and *mxiB*, in *Shigella flexneri* 2a facilitate excretion of invasion plasmid antigens. *Infect. Immunol.* 59:1997–2005.
9. Allaoui, A., P.J. Sansonetti, and C. Parsot. 1992. MxiJ, a lipoprotein involved in secretion of *Shigella* Ipa invasins, is homologous to YscJ, a secretion factor of the *Yersinia* Yop proteins. *J. Bacteriol.* 174:7661–7669.
10. Ménard, R., P.J. Sansonetti, and C. Parsot. 1994. The secretion of the *Shigella flexneri* Ipa invasins is activated by epithelial cells and controlled by IpaB and IpaC. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:5293-5302.
11. Watarai, M., T. Tobe, M. Yoshikawa, and C. Sasakawa. 1995. Contact of *Shigella* with host cells triggers release of Ipa invasins and is an essential function of invasiveness. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:2461–2470.
12. Watarai, M., T. Tobe, M. Yoshikawa, and C. Sasakawa.

1995. Disulfide oxidoreductase activity of *Shigella flexneri* is required for release of Ipa proteins and invasion of epithelial cells. *Proc. Natl. Acad. Sci. USA.* 92:4927–4931.

13. Parsot, C., R. Ménard, P. Gounon, and P.J. Sansonetti. 1995. Enhanced secretion through the *Shigella flexneri* Mxi-Spa translocon leads to assembly of extracellular proteins into macromolecular structures. *Mol. Microbiol.* 16:291–300.
14. Mounier, J., T. Vasselon, R. Hellio, M. Lesourd, and P.J. Sansonetti. 1992. *Shigella flexneri* enters human colonic Caco-2 epithelial cells through the basolateral pole. *Infect. Immunol.* 60:237–248.
15. Vasselon, T., J. Mounier, M.-C. Prévost, R. Hellio, and P.J. Sansonetti. 1991. Stress fiber-based movement of *Shigella flexneri* within cells. *Infect. Immunol.* 59:1723–1732.
16. Clark, E.A., and J.S. Brugge. 1995. Integrin and signal transduction pathways: the road taken. *Science (Wash. DC).* 268: 233–239.
17. Schaller, M.D., C.A. Borgman, B.S. Cobb, R.R. Vines, A.B. Reynolds, and J.T. Parsons. 1992. pp125^{FAK}, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. USA.* 89:5192–5196.
18. Burridge, K., C.E. Turner, and L.H. Romer. 1992. Tyrosine phosphorylation of paxillin and pp125^{FAK} accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J. Cell Biol.* 119:893–903.
19. Morla, A., Z. Zhang, and E. Ruoslahti. 1994. Superfibronectin is a functionally distinct form of fibronectin. *Nature (Lond.).* 367:193–196.
20. Nakata, N., C. Sasakawa, N. Okada, T. Tobe, I. Fukuda, T. Suzuki, K. Komatsu, and M. Yoshikawa. 1992. Identification and characterization of *virK*, a virulence-associated large plasmid gene essential for intercellular spreading of *Shigella flexneri*. *Mol. Microbiol.* 2:2387–2395.
21. Giancotti, F.G., and E. Ruoslahti. 1990. Elevated levels of the $\alpha_5\beta_1$ fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell.* 60:849–859.
22. Schreiner, C.L., J.S. Bauer, Y.N. Danilov, S. Hussein, M.M. Sczekan, and R.L. Juliano. 1989. Isolation and characterization of Chinese hamster ovary cell variants deficient in the expression of fibronectin receptor. *J. Cell Biol.* 109:3157–3167.
23. Isberg, R.R., and J.M. Leong. 1990. Multiple β_1 chain integrins are receptor for invasin, a protein that promotes bacterial penetration into mammalian cells. *Cell.* 60:861–871.
24. Sasakawa, C., K. Komatsu, T. Tobe, T. Suzuki, and M. Yoshikawa. 1993. Eitht genes in region 5 that form an operon are essential for invasion of epithelial cells by *Shigella flexneri* 2a. *J. Bacteriol.* 175:2334–2346.
25. Pierschbacher, M.D., and E. Ruoslahti. 1984. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature (Lond.).* 309:30–33.
26. Ruoslahti, E., and M.D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. *Science (Wash. DC).* 238:491–497.
27. Hynes, R.O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell.* 69:11–25.
28. Ménard, R., P. Sansonetti, C. Parsot, and T. Vasselon. 1994. Extracellular association and cytoplasmic partitioning of the IpaB and IpaC invasins of *S. flexneri*. *Cell.* 79:515–525.
29. Miyamoto, S., S.K. Akiyama, and K.M. Yamada. 1995. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science (Wash. DC).* 267:883–885.
30. 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.
31. Rosenshine, I., V. Duronio, and B.B. Finlay. 1992. Tyrosine protein kinase inhibitors block invasin-promoted bacterial uptake by epithelial cells. *Infect. Immunol.* 60:2211–2217.
32. Ginocchio, C., J. Pace, and J.E. Galán. 1992. Identification and molecular characterization of a *Salmonella typhimurium* gene involved in triggering the internalization of *Salmonellae* into cultured epithelial cells. *Proc. Natl. Acad. Sci. USA.* 89:5976–5980.
33. Francis, C.L., T.A. Ryan, B.D. Jones, S.J. Smith, and S. Falkow. 1993. Ruffles induced by *Salmonella* and other stimuli direct macropinocytosis of bacteria. *Nature (Lond.).* 364: 639–642.
34. Rosenshine, I., M.S. Donnenberg, J.B. Kaper, and B.B. Finlay. 1992. Signal transduction between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells: EPEC induces tyrosine phosphorylation of host cell proteins to initiate cytoskeletal rearrangement and bacterial uptake. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:3551–3560.
35. Dehio, C., M.-C. Prévost, and P.J. Sansonetti. 1995. Invasion of epithelial cells by *Shigella flexneri* induces tyrosine phosphorylation of cortactin by a pp60$^{c\text{-}src}$-mediated signaling pathway. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:2471–2482.