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 F\(~\)~\(\gamma\), 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), laminin, or collagen type IV, can also trigger the release of Ipa proteins from *S. flexneri*

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**Abbreviations used in this paper:** CHO, Chinese hamster ovary; Fn, fibronectin; RGDS, Arg-Gly-Asp-Ser; S-Fn, superfibronectin.
polymerization of actin. Our data suggest that the released blebs of mediating actin cytoskeleton rearrangement (16). Mental medium could interact with host receptor(s), such as αβi 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 αβ3 integrin. In this study, we show that the levels of αβ3 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 pp125FAK (17) or paxillin (18). The sites of bacterial attachment to the cells were associated with enhanced aggregation of αβ3 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 αβ3 integrin.

Materials and Methods

Bacterial Strains and Cell Culture. S. flexneri 2a YSH6000T, a spontaneous tetracycline-, chloramphenicol-, ampicillin-, and streptomycin-sensitive mutant 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 for this purpose.

S. flexneri 2a YSH6000T (ci5~31 integrin concentration, 20 μg/ml) was incubated at 37°C for 20 min in an incubator containing 5% CO2. 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-αβ3 integrin antibody or anti-IpaB, IpaC, and IpaD antibody. αβ3 integrin was detected by 7% SDS-PAGE under nonreducing conditions, whereas IpaB, IpaC and IpaD were detected by 10% SDS-PAGE under reducing conditions.

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 these cells. Infection of CHO cells with S. flexneri YSH6000T (ci5~31 integrin concentration, 20 μg/ml) was incubated at 37°C for 20 min in an incubator containing 5% CO2.

S-Fn Production. S-Fn was produced as follows (19). Fn was incubated with III1-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 III1-C, III11, which is an analogous region from the 11th type III repeat (19), was used instead of III1-C. The recombinant III1-C or III11 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).

Interaction of Released Ipa Proteins with αβ3 Integrin: ELISA. The ability of αβ3 integrin to bind to bacteria or Ipa proteins was measured as follows: A 50-μl aliquot of MEM containing either ~108 bacteria or IpaB, IpaC, and IpaD released from ~108 bacteria of wild-type S. flexneri YSH6000T (11, 12) was placed into 96-well microtiter plates and incubated at room temperature for 2 h. This was then removed, and the wells were washed twice with PBS-0.05% Tween 20. 50 μl of PBS containing αβ3 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 αβ3 integrin was determined by ELISA with anti-αβ3 (mAb KH/33) and β3 (mAb SG/19) integrin antibody.

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performed as described previously (11, 12); mouse monoclonal antiphosphotyrosine antibody PT-66, 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 αsβ1 Integrin on the Invasion Capacity of S. flexneri. To assess whether the level of αsβ1 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 αsβ1 integrin (21). To obtain CHO cell lines capable of expressing αsβ1 integrin at a high level, pcDNA1neo vectors containing cDNA encoding human αs or β1 integrin (21) were cotransfected into the CHO cells. Transfectants capable of expressing high levels of αsβ1 integrin were chosen to test the relationship between levels of αsβ1 integrin and the invasion efficiency of S. flexneri. 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 parental 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 αsβ1 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 αsβ1 Integrin. Since the IpaB, IpaC, and IpaD proteins released from Shigella were most likely interacting with the target cell (10, 11), we tested αsβ1 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 αsβ1 integrin was added; the plate was incubated at 37°C for 1 h. As shown in Fig. 2 A, a significant amount of bound αsβ1 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 αsβ1 integrin, and incubated the plate at 37°C for 1 h. However, the binding of αsβ1 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 αsβ1 integrin bound to HB101 harboring pINV.

To further demonstrate the ability of αsβ1 integrin to bind to the released Ipa proteins, αsβ1 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-α5 or anti-β1 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 antibody specific for IpaB, IpaC, and IpaD proteins. To visualize directly the specific interaction of αsβ1 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-αsβ1 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 pNV; 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-\( \alpha_5 \) or anti-\( \beta_1 \) integrin antibody. Immunoprecipitates with anti-\( \alpha_5 \) integrin (lane 1), anti-\( \beta_1 \) 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-\( \alpha_5 \) integrin (lane 5), or anti-\( \beta_1 \) 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 \( \mu\)m.

\( \alpha_5\beta_1 \) 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 \( S.\) flexneri, we investigated whether the addition of \( \alpha_5\beta_1 \) integrin into the MEM affected the invasive capacity of \( S.\) flexneri for CHO-HFR5 cells. We found that as the concentration of added \( \alpha_5\beta_1 \) integrin in MEM increased, the invasive capacity of \( S.\) flexneri decreased, and at a concentration of 20 \( \mu\)g/ml, inhibition of the inva-
The invasive capacity of YSH6000T for CHO-HFR5 reached the maximum of 64% of the inhibition (zero) seen without α5β1 integrin (Fig. 3A). Since α5β1 integrin is the receptor for Fn, and the RGD peptides are responsible for binding to α5β1 integrin (25), Fn or RGD peptides mixed with α5β1 integrin were added to the MEM to see whether the invasive capacity of YSH6000T was affected. The results showed that both α5β1 integrin–Fn and RGD peptides complexes added to the MEM-inhibited invasive capacity (Fig. 3A), suggesting that the interaction of Ipa proteins with α5β1 integrin is apparently different from that with Fn or RGD 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 α5β1 integrin with S-Fn was differentiated from that with Fn (19), thus prompting us to test whether the α5β1 integrin–S-Fn complex added to the MEM interfered with efficient CHO cells invasion by YSH6000T. By incubating Fn with III1–C fragment (COOH-terminal two-thirds of the III1 repeat of Fn molecular) (19), the resulting S-Fn was checked by the methods described previously (19) and incubated with α5β1 integrin before addition of the MEM. The addition of α5β1 integrin–S-Fn complex did restore the invasive capacity to YSH6000T (Fig. 3A). However, the addition of Fn incubated with III1–C fragment (negative control) (19) to the MEM failed to restore the invasive capacity (Fig. 3A), suggesting that the interaction of Ipa proteins with α5β1 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 α5β1 integrin was further investigated.

Preincubation of α5β1 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-α5β1 integrin antibody were then analyzed by immunoblots using anti-IpaB, IpaC, and IpaD antibodies. The data showed that α5β1 integrin preincubated with S-Fn, but not with Fn, failed to bind all three Ipa proteins (Fig. 3B). In this assay, we also tested the effect of EDTA (10 mM) on the binding of the Ipa proteins in MEM to added α5β1 integrin, since the binding of Fn, but not S-Fn, to α5β1 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 α5β1 integrin; this finding further supports the concept that the interaction is similar to S-Fn binding (Fig. 3B). These results thus clearly demonstrate that the interaction of Ipa proteins with α5β1 integrin is involved in the invasion of CHO cells by Shigella.

Induction of Tyrosine Phosphorylation of p125FAK 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.
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 pp125FAK (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-pp125FAK 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-pp125FAK 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 pp125FAK 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 pp125FAK 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 pp125FAK and paxillin.

**Accumulation of αβ1 Integrin upon Attachment of S. flexneri to CHO Cells.** Enhancement of the tyrosine phosphorylation of pp125FAK and paxillin in CHO cells has been

![Figure 4](image-url)

**Figure 4.** Tyrosine phosphorylation of CHO cell proteins upon S. flexneri invasion. Samples are from uninfected cells (lane 0), 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-pp125FAK 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](image-url)

**Figure 5.** Localized rearrangement of αβ1 integrin and F-actin at the site of S. flexneri attachment to CHO-HFR5 cells. (A) Mouse FITC-labeled anti-αβ1 integrin. (B) Rhodamine-labeled phalloidin. (C) Cy5-labeled anti-S. flexneri 2a LPS. The arrowheads mark αβ1 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 α5β1 integrin was induced upon invasion of epithelial cells by Shigella. 20 min after infection of CHO-HF5 cells with the wild-type strain of S. flexneri, the CHO cells were immunostained with FITC-labeled anti-α5β1 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 α5β1 integrin appeared at the site of bacterial attachment, where actin polymerization occurred (Fig. 5). The accumulated α5β1 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 α5β1 integrin (11).

Discussion

The data provided in this study indicate that Ipa proteins released from S. flexneri are capable of direct binding to α5β1 integrin—a reaction that may mediate the signal transduction required for activating focal adhesion functions such as tyrosine phosphorylation of pp125FAK and paxillin or reorganization of actin cytoskeletons. The evidence for this was derived from five independent approaches: (i) the effect of different levels of α5β1 integrin expressed from CHO cells on the invasive capacity of S. flexneri; (ii) the binding capacity of α5β1 integrin to IpaB, IpaC, and IpaD proteins; (iii) competitive inhibition of Shigella invasion with α5β1 integrin; (iv) protein tyrosine phosphorylation of pp125FAK and paxillin by infecting S. flexneri; and (v) the convergence of α5β1 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 α5β1 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 α5β1 integrin in MEM, as shown by immunoprecipitation methods with either anti-IpaB, -IpaC, or -IpaD antibody, or with anti-α5 or -β1 integrin antibody (Fig. 2). Although α5β1 integrin added to microtiter plates 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, α5β1 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 matrices 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 α5β1 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 α5β1 integrin seemed different from that between soluble Fn and α5β1 integrin; rather, this interaction resembled that between S-Fn and α5β1 integrin. As shown in Fig. 3, neither the invasive efficiency of Shigella for CHO cells nor the binding capacity of the Ipa proteins to α5β1 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 α5β1 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 α5β1 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 α5β1 integrin. In addition, we showed that attachment of YSH6000T (wild type S. flexneri), but not CS2585 (spa32 mutant), to CHO cells elicits convergence of α5β1 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 pp125FAK 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 α5β1 integrin remain to be elucidated, it is worth noting that some properties of the binding of Ipa proteins to α5β1 integrin are apparently different from the characteristics reported for Yersinia invasin (Inv), a protein able to bind to α5β1 integrin and to promote uptake of bacteria by epithelial cells (23). Yersinia Inv can interact with α5β1 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 α5β1 integrin can be competitively inhibited by Fn or its RGDS peptides, but Fn interaction requires divalent-cation such as Mg2+ (30), whereas the binding capacity of Ipa proteins to α5β1 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 αβ2 integrin from that of Yersinia 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 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 tyrosine 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 kinase substrate, through overexpression of proto-oncoprotein pp60v-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 pp125Fak 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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