

Invasion of Host Cells by *Salmonella typhimurium* Requires Focal Adhesion Kinase and p130Cas^D

Jing Shi and James E. Casanova

Department of Cell Biology, University of Virginia Health Sciences Center, Charlottesville, VA 22908-0732

Submitted June 5, 2006; Revised July 18, 2006; Accepted August 9, 2006

Monitoring Editor: Ralph Isberg

Salmonella typhimurium colonizes the intestinal epithelium by injecting an array of effector proteins into host cells that induces phagocytic uptake of attached bacteria. However, the host molecules targeted by these effectors remain poorly defined. Here, we demonstrate that *S. typhimurium* induces formation of focal adhesion-like complexes at sites of bacterial attachment and that both focal adhesion kinase (FAK) and the scaffolding protein p130Cas are required for *Salmonella* uptake. Entry of *Salmonella* into FAK^{-/-} cells is dramatically impaired and can be restored to control levels by expression of wild-type FAK. Surprisingly, reconstitution of bacterial internalization requires neither the kinase domain of FAK nor activation of c-Src, but does require a C-terminal PXXP motif through which FAK interacts with Cas. Infection of Cas^{-/-} cells is also impaired, and reconstitution of invasiveness requires the central Cas YXXP repeat domain. The invasion defect in Cas^{-/-} cells can be suppressed by overexpression of FAK, suggesting a functional link between FAK and Cas in the regulation of *Salmonella* invasion. Together, these findings reveal a novel role for focal adhesion proteins in the invasion of host cells by *Salmonella*.

INTRODUCTION

Bacteria of the genus *Salmonella* are the causative agents of diseases ranging from gastroenteritis to typhoid fever. A key feature of *Salmonella* pathogenesis in humans and other animals is the ability of the bacteria to enter and penetrate the intestinal epithelium. Several other enteric pathogens, such as *Yersinia* and *Listeria*, utilize a “zipper” mechanism to enter host cells, in which a bacterial surface ligand is used to engage host cell surface receptors. For example, the invasin protein of *Yersinia* binds β 1 integrin, whereas the internalin proteins of *Listeria* bind to both E-cadherin and the receptor tyrosine kinase c-Met. In contrast, both *Salmonella* and *Shigella* enter cells by a “trigger” mechanism characterized by massive membrane ruffling and actin rearrangements at sites of invasion (Cossart and Sansonetti, 2004). Using a type III secretion system encoded by the *Salmonella* pathogenicity island-1 (SPI-1) chromosomal locus (Collazo and Galan, 1997; Darwin and Miller, 1999), a set of bacterial effector proteins are translocated into host cells, where they manipulate host actin dynamics and signaling pathways to promote extensive reorganization of the actin cytoskeleton that culminates in bacterial entry (Patel and Galan, 2005).

Focal adhesions are a complex assembly of proteins that provide a physical linkage between integrins and the actin cytoskeleton (Zamir and Geiger, 2001). Proteins enriched at focal adhesions include cytoskeletal components such as talin, vinculin, and α -actinin, scaffolding proteins such as paxillin and p130Cas, and signaling molecules such as ty-

rosine kinases (e.g., focal adhesion kinase [FAK], Src), serine/threonine kinases (PAK, Akt), phosphatases (e.g., PTEN, SHP-2), and GTPase modulators (e.g., ASAP, GRAF). Thus focal adhesion proteins not only physically link integrins to the cytoskeleton but also transmit adhesion-dependent signals to the cell interior (Zamir and Geiger, 2001).

A number of bacterial pathogens have been shown to employ focal adhesion proteins to facilitate their internalization into host cells. The *Shigella* effector protein IpaA binds to vinculin, inducing local actin depolymerization that is essential for formation of the phagocytic apparatus (Finlay *et al.*, 1991; Tran Van Nhieu *et al.*, 1997; Bourdet-Sicard *et al.*, 1999). Similarly, FAK has been shown to promote invasion of host cells by *Yersinia* and *Staphylococcus* (Alruz and Isberg, 1998; Bruce-Staskal *et al.*, 2002; Agerer *et al.*, 2005). Interestingly, a number of focal adhesion proteins, such as talin and vinculin, have been observed to accumulate at *Salmonella*-induced membrane ruffles despite a lack of evidence showing involvement of integrins (Finlay *et al.*, 1991). However, whether focal adhesion proteins play a role in *Salmonella* invasion remains largely unknown.

Here, we show that *Salmonella* recruit focal adhesion proteins including FAK, Cas, and paxillin, but not β 1 integrin, to sites of invasion at the apical surface of epithelial cells and demonstrate a requirement for both FAK and p130Cas, but not paxillin, in the invasion of host cells by *Salmonella*. In contrast to the integrin-mediated internalization of *Staphylococcus*, which requires FAK kinase activity, the catalytic domain of FAK is not necessary for invasion. Although phosphorylation of p130Cas is induced during *Salmonella* invasion, this is also not required for bacterial internalization. Instead, Cas appears to be necessary for the proper assembly of actin into a productive phagocytic apparatus. Finally, we show that overexpression of FAK in Cas^{-/-} cells completely restores *Salmonella* internalization, suggesting that FAK and Cas may act in concert to promote bacterial invasion. Together, these results identify a role for focal

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06-06-0492>) on August 16, 2006.

^D The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

Address correspondence to: James E. Casanova (jec9e@virginia.edu).

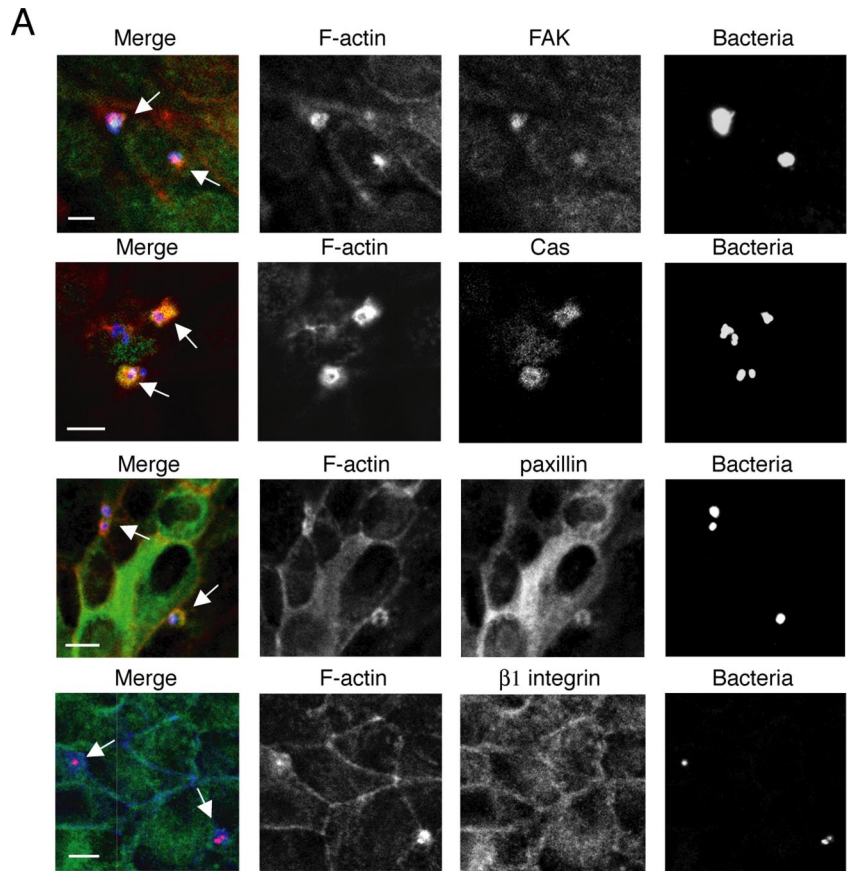
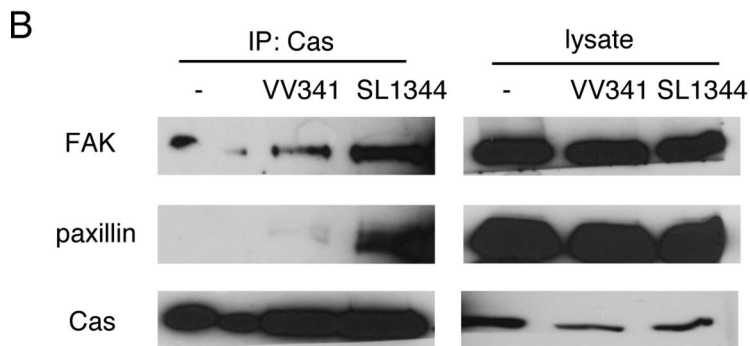


Figure 1. Focal adhesion-like complexes form at sites of *Salmonella* invasion. (A) MDCK cells were infected apically with *S. typhimurium* strain SL1344 for 20 min. For FAK, Cas, and paxillin staining, F-actin was labeled with rhodamine-conjugated phalloidin (red); endogenous FAK, Cas, or paxillin were detected with specific monoclonal antibodies, followed by a Cy2-conjugated secondary antibody (green); *Salmonella* were stained with a polyclonal antibody against bacterial LPS followed by a Cy5-conjugated secondary (blue). Merged images show colocalization of FAK, Cas, or paxillin with F-actin at sites of bacterial entry. For $\beta 1$ integrin staining, F-actin was labeled with Alexa 647-conjugated phalloidin (blue); endogenous $\beta 1$ integrins were stained with a polyclonal antibody E63E followed by a Cy2 secondary (green); *Salmonella* were labeled with a mouse anti-LPS IgA followed by a TR-conjugated secondary (red). Arrows indicate the bacterially induced membrane ruffles. Images were taken at 100 \times magnification on a Nikon C1 confocal microscope. Bars, 10 μ m. (B) MDCK cells were infected apically with SL1344 or the invasion-defective mutant VV341 at a MOI = 100 for 30 min. Cas was immunoprecipitated with the polyclonal antibody Cas3B, and the precipitates were probed for associated FAK and paxillin. Blots were then stripped and reprobed for Cas.



adhesion components in the *Salmonella* invasion process and provide new insight into the host signaling pathways utilized by the bacteria to facilitate their cellular invasion.

MATERIALS AND METHODS

Bacterial Strains

The wild-type *S. typhimurium* strain SL1344 and its isogenic derivative VV341, which is rendered entry-deficient by deletion of the *hilA* locus, have been previously described (Hueck *et al.*, 1995). Invasive bacteria were generated by growth under O₂-limited conditions as originally described (Lee and Falkow, 1990). Under these conditions, bacteria were in a late logarithmic phase of growth, corresponding regularly to 5–7 $\times 10^8$ colony forming units (CFU)/ml.

Cell Culture

MDCK cells were grown in DMEM with 4.5 g/l glucose, 10% FBS, 1 mM sodium pyruvate, and antibiotics in a 37°C CO₂ incubator. FAK^{+/+} and FAK^{-/-} mouse embryonic fibroblasts (MEFs) were kindly provided by J. T. Parsons (University of Virginia, Charlottesville, VA). Cas^{+/+} and Cas^{-/-} MEFs were provided by A. H. Bouton (University of Virginia). Paxillin^{+/+}

and paxillin^{-/-} MEFs were provided by A. R. Horwitz (University of Virginia). MEFs were grown in DMEM with 4.5 g/l glucose, 10% FBS, and antibiotics in a 37°C CO₂ incubator. HeLa cells were grown in DMEM with 4.5 g/l glucose, 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, and antibiotics.

DNA Constructs and Antibodies

All FAK constructs were provided by J. T. Parsons (University of Virginia), all Cas constructs were provided by A. H. Bouton (University of Virginia), and the paxillin construct was provided by C. E. Turner (State University of New York, Syracuse).

The mouse monoclonal anti-FAK antibody clone 447 and anti-phosphotyrosine antibody 4G10 were obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-FAK antibody A-17 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-Cas antibody 8G4 and the rabbit polyclonal anti-Cas antibody Cas3B were kindly provided by A. H. Bouton. Rabbit polyclonal anti- $\beta 1$ integrin antibody E63E was kindly provided by D. W. DeSimone (University of Virginia). Mouse monoclonal anti-paxillin, anti-Rac1, and anti-Cdc42 antibodies were obtained from BD Biosciences (San Jose, CA). Rhodamine-phalloidin, FITC-phalloidin, and Alex 647-phalloidin were obtained from Molecular Probes (Eugene, OR).

Invasion Assays

The gentamicin resistance invasion assay was essentially as described (Criss *et al.*, 2001). Approximately 2×10^5 cells per well were plated in a 24-well plate. Cells were infected at an MOI = 30 for 1 h at 37°C. Internalization is expressed as a percentage of the initial bacterial inoculum. Data represent the means of three independent wells per experiment, and each experiment was performed at least three times.

The intracellular versus extracellular immunofluorescence invasion assay was essentially as described (Bruce-Staskal *et al.*, 2002). Briefly, cells were infected at an MOI of 30 for 1 h at 37°C. Then, cells were fixed in 2% paraformaldehyde, and extracellular *Salmonella* were stained using a rabbit polyclonal antibody against *Salmonella* LPS (1:500), followed by a Cy2-conjugated goat anti-rabbit antibody (1:400). Cells were then permeabilized by incubation with PBS-NGS (10% normal goat serum) containing 0.2% saponin. Staining for total (intracellular and extracellular) *Salmonella* was then performed by an additional incubation with the same rabbit anti-LPS antibody (1:500) followed by a Texas red (TR)-conjugated goat anti-rabbit antibody (1:400). To detect cells expressing myc-tagged FAK or Cas constructs, a mouse anti-myc antibody 9E10 (1:1000) was included in the second incubation with anti-LPS antibody, followed by a Cy2-conjugated goat anti-mouse antibody (1:500). Under these conditions both extracellular bacteria and intracellular myc-tagged proteins are labeled green. However, the bacterial staining is uniformly brighter and more focal than the transfected protein, making it possible to clearly identify both. Care was taken to ensure that cells used for quantitation expressed similar levels of exogenous protein. Bacterial internalization was determined as number of intracellular bacteria per cell. An index of internalization is defined relative to the number of intracellular bacteria per cell in wild-type MEFs (this value is set equal to 1). Data represent the means of at least three independent experiments.

Immunoprecipitation and Western Blotting

MDCK cells were infected at an MOI = 100 with *S. typhimurium* strain SL1344 or VV341 for 30 min. Cells were washed with cold HBSS⁺ and lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 10% glycerol, 0.1% SDS, 2 mM EDTA supplemented with protease inhibitors and 1 mM Na₃VO₄). Lysates were immunoprecipitated with a rabbit polyclonal anti-Cas antibody Cas3B or A-17 anti-FAK for 30 min, and the immunoprecipitates were probed by immunoblotting with mouse monoclonal antibodies: 8G4 anti-Cas (1:1000), anti-FAK clone 4.47 (1:1000), and anti-paxillin (1:1000), and 4G10 anti-phospho-tyrosine (1:500).

GTPase Activation Assays

Levels of GTP-bound Rac1 and Cdc42 were measured essentially as previously described (Criss *et al.*, 2001). Briefly, FAK^{+/+} and FAK^{-/-} cells were infected with SL1344 or VV341 at an MOI = 100 or maintained in HBSS⁺ for 30 min and then lysed in 50 mM Tris-HCl, pH 7.5, 2 mM MgCl₂, 0.1 M NaCl, 1% NP-40, and 10% glycerol with protease inhibitors. Lysates were incubated for 45 min at 4°C with 40 μg GST (control) or GST-PBD coupled to glutathione-Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ). Proteins were separated on 13% acrylamide gels and transferred to nitrocellulose (Rac1) or polyvinylidene difluoride membrane (Cdc42). Rac1 and Cdc42 were detected using monoclonal antibodies (BD Transduction Laboratories, Lexington, KY; Rac1, 1:1000; Cdc42, 1:250).

Immunofluorescence Microscopy and Quantification of Actin Foci

Cells were infected with wild-type *S. typhimurium* SL1344 for 20 min, washed with PBS, and fixed in 4% paraformaldehyde. Samples were incubated with antibodies to FAK, Cas, or paxillin in combination with antibodies to *Salmonella* LPS and fluorescently tagged phalloidin to detect filamentous actin. Images were acquired at 60× magnification on a Nikon Eclipse E800 microscope (Melville, NY) using a QImaging Retiga cooled CCD camera (Burnaby, BC, Canada), or at 60× or 100× magnification on a Nikon C1 confocal microscope. Frequency of focus formation was quantified as the number of foci per cell. At least 100 cells were examined for each cell line. The area of the actin foci was measured from images and calculated using Image J. At least 40 foci were measured.

RNA Interference

SIRNA duplex against the sequence 5'-GUGUGGAGCCUUCUUGGU-3' of human paxillin has been described previously (Sanders and Basson, 2005). Knockdown of paxillin in HeLa cells was performed by transient transfection using Oligofectamine (Invitrogen, Carlsbad, CA). Invasion assays and Western blotting were performed 72 h after transfection.

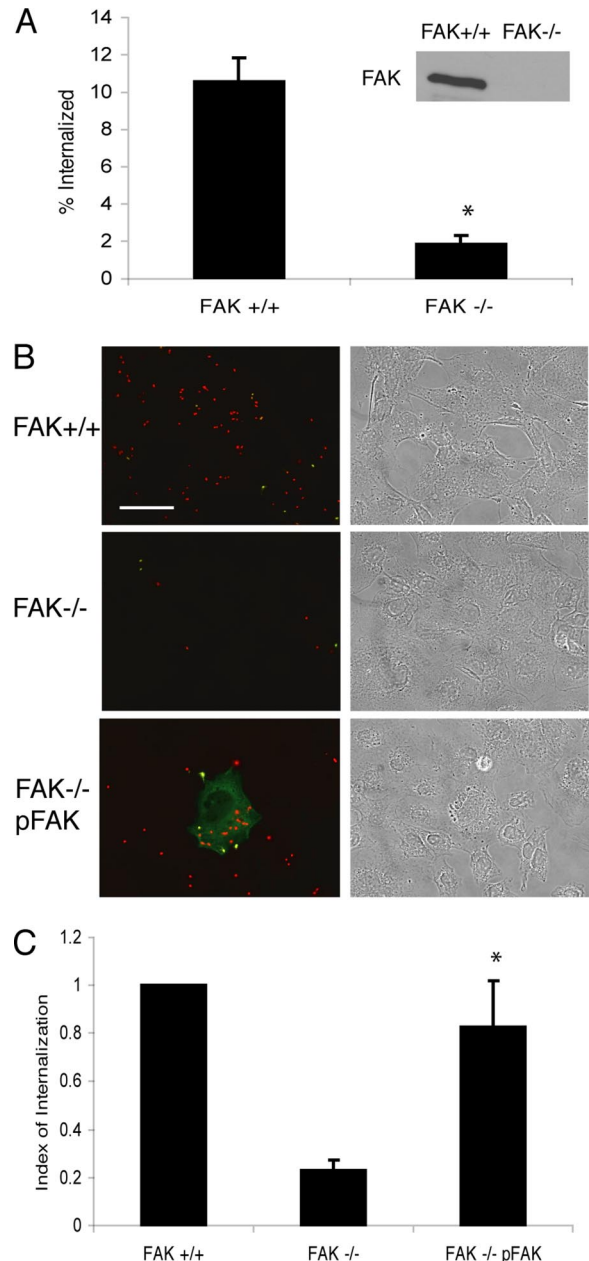


Figure 2. FAK is required for *Salmonella* internalization. (A) FAK^{+/+} and FAK^{-/-} MEFs were infected with wild-type *S. typhimurium* (SL1344) at a MOI = 30 for 1 h. *Salmonella* internalization was assayed by a standard gentamicin resistance assay as described in *Materials and Methods*. Expression levels of FAK in these cells were detected using the FAK mAb 4.47. **p* < 0.001 (Student's *t* test). (B and C) FAK^{+/+}, FAK^{-/-} (mock transfected) and FAK^{-/-} cells transfected with myc-FAK were infected with SL1344 at a MOI = 30 for 1 h before a two-color immunofluorescence invasion assay as described in *Materials and Methods*. (B) Transfected cells were labeled with an anti-myc antibody 9E10 followed by a Cy2-conjugated donkey anti-mouse secondary antibody. Phase-contrast images of the same field are shown on the right. Intracellular bacteria appear red and extracellular bacteria appear yellow. The bottom panel shows a FAK-reconstituted cell with internalized (red) and adhered bacteria (yellow). Bar, 50 μm. (C) Quantification of *Salmonella* internalization as measured using the two-color immunofluorescence invasion assay. **p* < 0.01 compared with mock-transfected FAK^{-/-} cells (Student's *t* test).

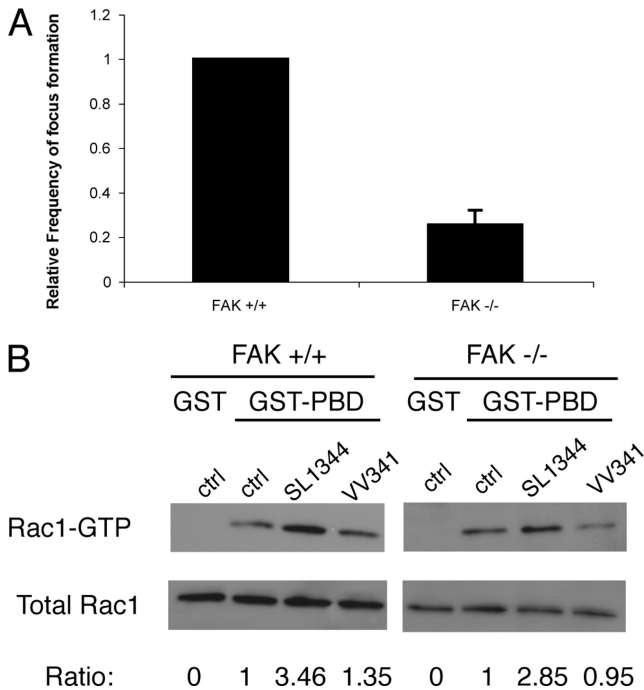


Figure 3. Rac1 activation is not impaired in FAK^{-/-} MEFs. (A) FAK^{+/+} and FAK^{-/-} cells were infected with wild-type *S. typhimurium* (SL1344) for 20 min before fixation. F-actin and bacteria were stained using rhodamine-conjugated phalloidin and rabbit anti-LPS, respectively. Frequency of focus formation was quantified as described in *Materials and Methods*. (B) FAK^{+/+} and FAK^{-/-} cells were infected with SL1344 (lane 3 and 7) or the entry-deficient strain VV341 (lane 4 and 8), or maintained in HBSS⁺ (lane 1–2 and 5–6). GTP-bound Rac1 was isolated from cell lysates by affinity precipitation with GST-PBD (lane 2–4 and 6–8) as described in *Materials and Methods*. GST alone was used as negative control (lane 1 and 5). Pulldowns and a fraction of each lysate were simultaneously blotted for Rac1. The level of GTP-bound Rac is expressed relative to the basal level in uninfected cells (lane 2 and 6).

RESULTS

Salmonella Recruit Focal Adhesion Proteins to Sites of Entry

Although *Salmonella* are not thought to use integrins to facilitate their entry into host cells, invasion of cultured MDCK and HeLa epithelial cells has been shown to be accompanied by recruitment of two focal adhesion components, talin and vinculin, to sites of invasion (Finlay *et al.*, 1991). Because *Salmonella* target polarized intestinal epithelial cells in vivo, we used confocal immunofluorescence microscopy to localize endogenous focal adhesion components in polarized MDCK cells infected apically with *Salmonella*. As shown in Figure 1A, FAK, p130Cas, and paxillin were all observed to be strongly concentrated in the membrane protrusions surrounding the invading bacteria, colocalizing with filamentous actin at sites of *Salmonella* entry. Other focal adhesion components, including vinculin, α -actinin, and VASP also accumulated at bacterial invasion foci (unpublished data). However, we did not observe a corresponding accumulation of β 1 integrin at *Salmonella* invasion sites (Figure 1A), suggesting that β 1 integrins are not clustered at sites of *Salmonella* invasion.

These observations suggested that *Salmonella* infection induces the assembly of focal adhesion-like structures at the apical plasma membrane. To test this hypothesis, we exam-

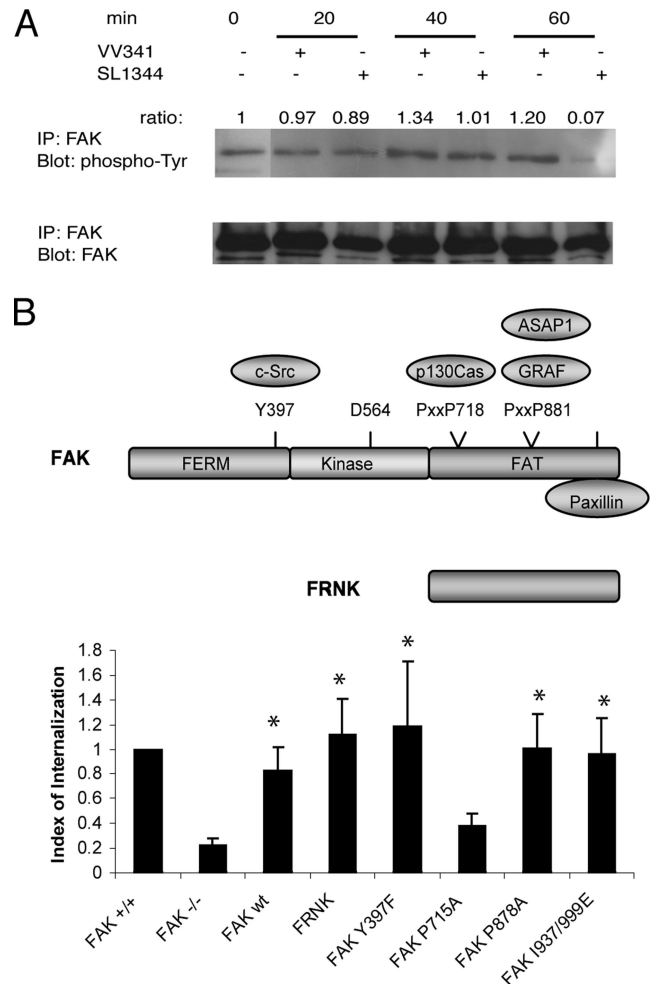


Figure 4. FAK kinase activity is not required for *Salmonella* entry. (A) MDCK cells were infected apically with wild-type *S. typhimurium* (SL1344) or the invasion-defective mutant VV341 (Δ HilA) at a MOI = 20 for 20, 40, or 60 min. Cells maintained in the absence of bacteria were used as controls. FAK was immunoprecipitated from the lysates using polyclonal antibody A-17. The precipitates were divided into two sets and probed with either anti-phospho-tyrosine antibody 4G10 or a mAb clone 4.47 against total FAK. (B) FAK^{+/+}, FAK^{-/-} (mock transfected), and FAK^{-/-} cells transfected with wild-type FAK, FRNK, FAK Y397F, FAK P715A, FAK P878A, or FAK I937/999E mutant were subjected to the two-color immunofluorescence invasion assay. Bacterial internalization was normalized to control FAK^{+/+} cells. **p* < 0.05 compared with mock-transfected FAK^{-/-} cells (Student's *t* test).

ined the effects of *Salmonella* infection on the association of endogenous p130Cas, FAK, and paxillin in a coprecipitation assay. MDCK cells infected apically with either the wild-type *Salmonella* strain SL1344 or the syngeneic entry-deficient mutant VV341(Δ hilA) were lysed and immunoprecipitated with antibody to p130Cas, and the levels of associated FAK or paxillin were determined by immunoblotting. Figure 1B shows that a small amount of FAK and no detectable paxillin coprecipitated with Cas in uninfected cells. Although only a small enhancement of coprecipitation was observed in cells infected with the noninvasive VV341 strain, infection with SL1344 robustly enhanced the interaction of p130Cas with both FAK and paxillin. Together, these data indicate that apical infection of epithelial cells with

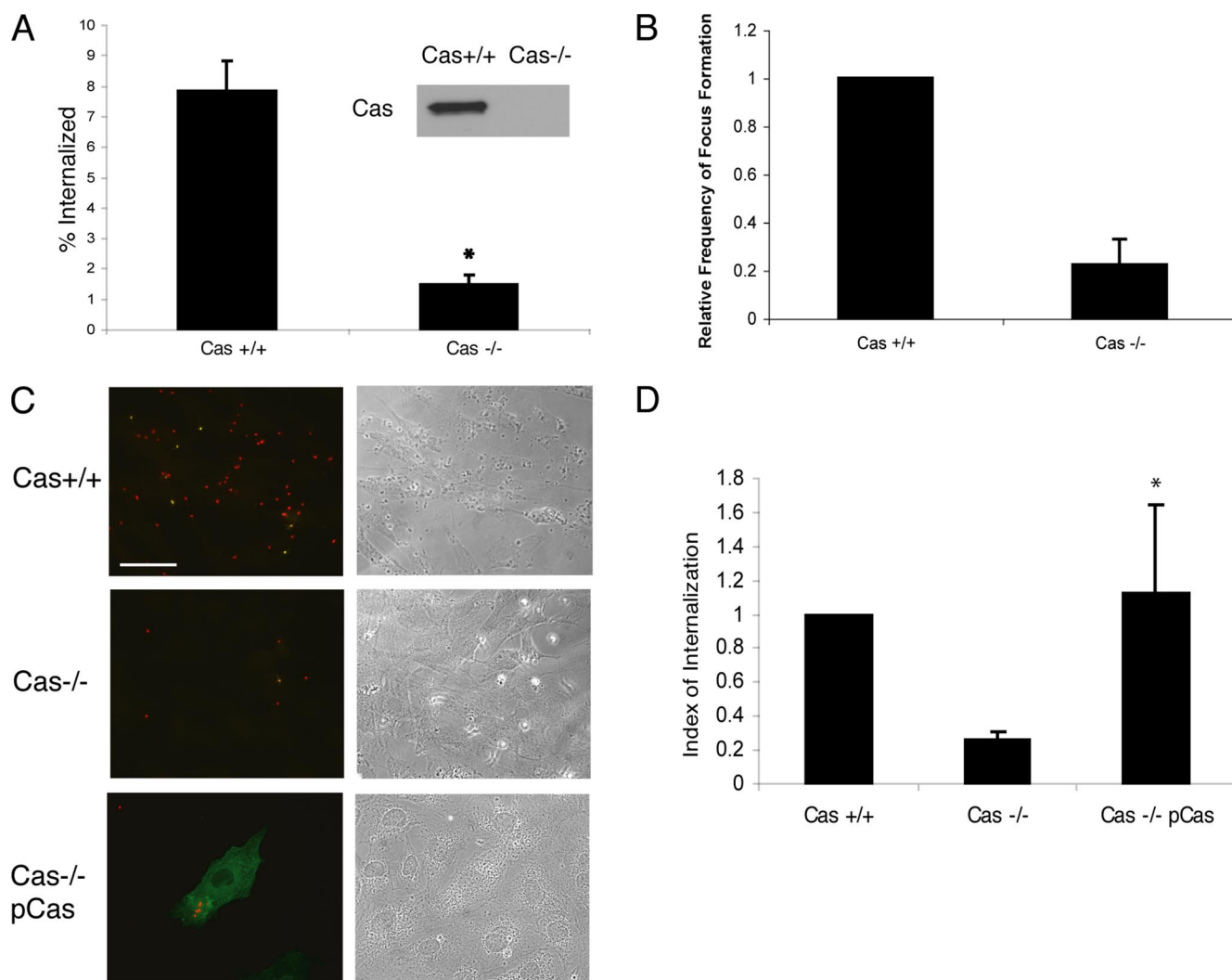


Figure 5. p130Cas is necessary for *Salmonella* invasion. (A) Cas^{+/+} and Cas^{-/-} MEFs were infected with wild-type *S. typhimurium* (SL1344) and subjected to the gentamicin resistant assay. *p < 0.001 (Student's *t* test). Expression levels of Cas in these cells were detected using a Cas mAb 8G4. (B) Frequency of focus formation in Cas^{+/+} and Cas^{-/-} cells was determined as described Figure 3A. (C and D) Cas^{+/+}, Cas^{-/-} (mock transfected), and Cas^{-/-} cells transfected with myc-Cas were subjected to the two-color immunofluorescence invasion assay. Transfected cells were labeled with 9E10 followed by a Cy2-conjugated donkey anti-mouse secondary antibody. (C) The bottom panel shows a Cas reconstituted cell with internalized (red) and adhered bacteria (yellow). Bar, 50 μ m. (D) Quantification of *Salmonella* internalization using the two-color invasion assay. *p < 0.05 compared with mock-transfected Cas^{-/-} cells (Student's *t* test).

Salmonella stimulates the formation of a Cas-FAK-paxillin complex.

FAK Is Required for *Salmonella* Invasion of Host Cells

To determine whether focal adhesion components have an important function in *Salmonella* entry, we examined bacterial invasion in MEFs derived from either FAK knockout mice or their wild-type littermates. As observed in MDCK cells, endogenous FAK colocalized extensively with filamentous actin at sites of bacterial attachment in wild-type MEFs (see Supplementary Material, Figure S1A). In contrast, and in agreement with our observations in MDCK cells, β 1 integrin did not accumulate around invading bacteria (unpublished data). To determine if FAK is necessary for bacterial internalization, FAK^{+/+} and FAK^{-/-} MEFs were infected for 1 h at 37°C, and *Salmonella* invasion was analyzed using a standard gentamicin resistance assay (see *Materials and Methods*). Under these conditions, the number of bacteria

internalized by FAK^{-/-} cells was reduced by 83% (\pm 4%) relative to FAK^{+/+} cells (Figure 2A). This result was confirmed using a previously described two-color immunofluorescence invasion assay, which distinguishes intracellular versus extracellular bacteria (see *Materials and Methods*). In agreement with the biochemical assay, the fluorescence assay revealed that invasion efficiency was reduced by 77% (\pm 5%) in FAK^{-/-} cells relative to controls (Figure 2, B and C). Importantly, reconstitution of FAK^{-/-} cells with a plasmid encoding myc-tagged wild-type FAK restored invasion to near wild-type levels (Figure 2, B and C), demonstrating that FAK is necessary for efficient *Salmonella* internalization into host cells.

Rho GTPase activation Is Not Impaired in FAK^{-/-} Cells

To investigate the mechanisms that account for the impairment of *Salmonella* uptake in FAK^{-/-} MEFs, we visualized the actin-rich foci induced by invading bacteria in FAK^{+/+} and FAK^{-/-} cells. Cells were infected at 37°C for 20 min,

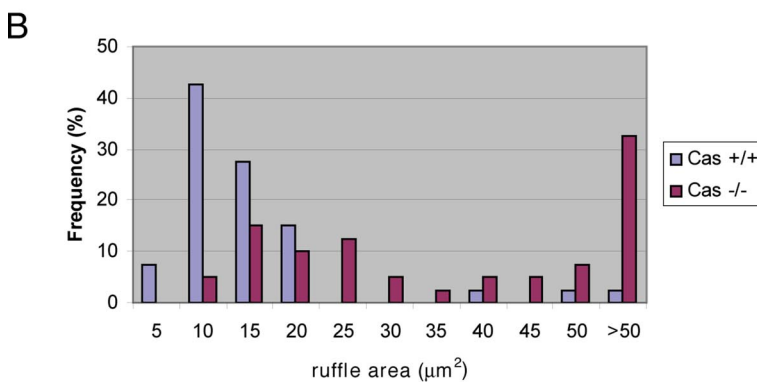
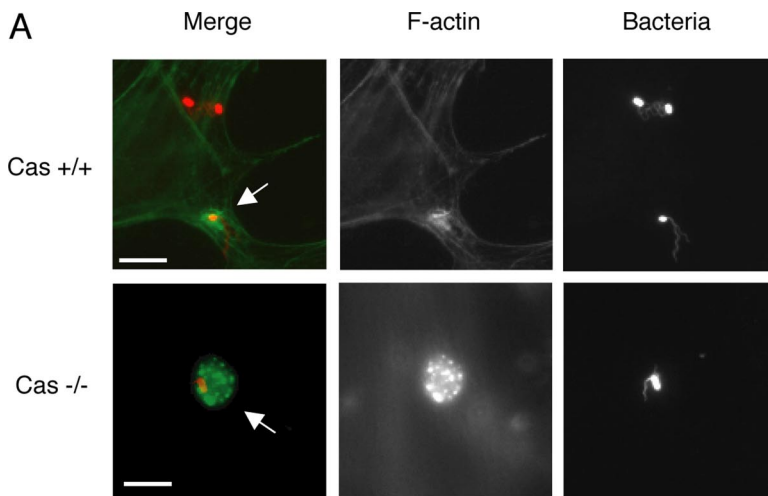


Figure 7. Aberrant phagocytic cups are induced in Cas^{-/-} fibroblasts upon *Salmonella* infection. (A) Cas^{+/+} and Cas^{-/-} cells were infected with wild-type *S. typhimurium* (SL1344) for 20 min before fixation, and F-actin was stained using FITC-conjugated phalloidin (green). *Salmonella* were labeled with polyclonal anti-LPS antibody followed by a TR-conjugated anti-rabbit IgG (red). Arrows indicate the bacterially induced phagocytic cups. Note the bright actin puncta within the enlarged phagocytic structure. Bar, 10 μm. (B) Quantification of ruffle size in Cas^{+/+} and Cas^{-/-} cells; n = 40.

tyrosine antibody, 4G10. Surprisingly, we found that FAK tyrosine phosphorylation levels remained essentially unchanged in cells infected by either invasive or noninvasive bacteria for 20 or 40 min, although a slight increase was observed in VV341 infected cells at later time points (40 and 60 min; Figure 4A). These observations suggest that *Salmonella* invasion does not induce FAK activation. Interestingly, a significant decrease in FAK phosphorylation was apparent after 60 min of infection with strain SL1344 that did not occur with the invasion-deficient strain VV341. This decrease is likely due to the tyrosine phosphatase activity of the *Salmonella* effector protein SptP, because it was not observed in cells infected with an SptP-deficient strain (unpublished data).

To determine if the kinase activity of FAK is required for its function in *Salmonella* internalization, we reconstituted FAK^{-/-} cells with either FRNK, the autonomously expressed C-terminus of FAK that lacks the catalytic domain (Figure 4B), or FAK Y397F, a mutant lacking the autophosphorylation site, and quantitated bacterial internalization using the immunofluorescence-based invasion assay. Interestingly, both FRNK and FAKY397F were able to restore *Salmonella* invasion as well as wild-type FAK (Figure 4B). Because FRNK does not contain the kinase domain and FAK Y397F has only low kinase activity (Calalb *et al.*, 1995), these results indicate that the catalytic activity of FAK is not necessary for *Salmonella* uptake. Consistent with this notion, overexpression of FRNK in HeLa cells, which has been shown to displace endogenous FAK from focal adhesions, did not impair *Salmonella* internalization (see Supplementary Material Figure S2). Together, these observations sup-

port the view that the C-terminal noncatalytic domain of FAK is sufficient for bacterial uptake.

The p130Cas Binding Site on FAK Is Necessary for Salmonella Internalization

The FAK C-terminal nonkinase domain contains two proline-rich motifs (Figure 4B), one of which (PXXP718) binds to Cas. The other (PXXP881) is a binding site for two different GTPase regulators, the Arf GAP ASAP1 and the Rho GAP GRAF. The C terminus also contains a paxillin-binding site (Parsons, 2003; Mitra *et al.*, 2005). To determine if any of these binding partners is necessary for *Salmonella* invasion, we analyzed bacterial internalization in FAK^{-/-} cells transfected with a panel of FAK mutants: FAK P715A, which largely attenuates binding to Cas (Polte and Hanks, 1995; Harte *et al.*, 1996); FAK P878A, which impairs binding to ASAP1 and GRAF1 (Taylor *et al.*, 1998; Randazzo *et al.*, 2000; Liu *et al.*, 2002); or FAK I937/999E, which reduces binding to paxillin (K. H. Martin and J. T. Parsons, unpublished observation). Figure 4B shows that in contrast to the other two mutants, which completely restored *Salmonella* uptake, FAK P715A failed to rescue bacterial internalization. These data suggest that the interaction between FAK and Cas is necessary for *Salmonella* entry.

Cas Is Required for Salmonella Invasion

As observed in apically infected MDCK cells, Cas was strongly concentrated at sites of bacterial internalization in mouse fibroblasts (see Supplementary Material, Figure S1B). To determine if Cas is required for bacterial entry, we compared *Salmonella* invasion in Cas^{+/+} and Cas^{-/-} MEFs.

Both the gentamicin-resistance assay (Figure 5A) and the fluorescence-invasion assay (Figure 5, C and D) revealed that invasion efficiency was reduced by ~80% ($\pm 5\%$; $p < 0.001$) in *Cas*^{-/-} cells compared with *Cas*^{+/+} controls. Moreover, reconstitution of *Cas*^{-/-} cells with wild-type p130Cas was sufficient to restore invasiveness (Figure 5, C and D), indicating that Cas is also required for *Salmonella* entry. As we observed in the *FAK*^{-/-} cells, bacterially induced actin focus formation was also dramatically reduced in *Cas*^{-/-} cells (Figure 5B).

Previous work has reported that tyrosine phosphorylation of Cas in response to cell adhesion or receptor activation serves important regulatory functions and certainly modulates its interactions with SH2 domain-containing proteins (Bouton *et al.*, 2001). To determine whether tyrosine phosphorylation of Cas is altered in response to *Salmonella* infection, we immunoprecipitated Cas from MDCK cells infected for 20, 40, or 60 min and then blotted with the anti-phosphotyrosine antibody, 4G10. Relative to the basal level, infection with wild-type *Salmonella* enhanced tyrosine phosphorylation of Cas at 20 min of infection, peaked at 40 min (~8-fold increase over control), and then decreased at 60 min of infection (Figure 6A). In contrast, this pattern did not occur in cells infected with the noninvasive strain VV341, suggesting that the observed changes were due to secreted effector proteins. As described above for FAK, the decrease in tyrosine phosphorylation at 60 min after infection is likely to be due to dephosphorylation of Cas by the bacterial phosphatase SptP.

Because it has been shown that c-Src is the major tyrosine kinase that phosphorylates Cas (Astier *et al.*, 1997; Ruest *et al.*, 2001; Agerer *et al.*, 2005), we assayed *Salmonella* internalization in cells treated with a Src inhibitor, PP2. Although PP2 treatment efficiently inhibited tyrosine phosphorylation of Cas during *Salmonella* invasion (see Supplementary Material, Figure S3), bacterial internalization remained unaffected under these conditions (Figure 6B). In agreement with these observations, *Salmonella* internalization in SYF (*Src/Yes/Fyn*)^{-/-} MEFs was found to be only slightly reduced compared with wild-type MEFs (unpublished data). Together, these data indicate that although tyrosine phosphorylation of Cas is substantially elevated during *Salmonella* invasion, this modification appears to be dispensable for bacterial entry.

We then used the reconstitution approach to determine which domain(s) of Cas are necessary for its function in *Salmonella* invasion. Wild-type p130Cas and two Cas mutants: Δ SH3 (which lacks the N-terminal FAK/Pyk2 binding domain), and Δ YXXP (which lacks the central substrate binding domain) (Figure 6C), were reintroduced into *Cas*^{-/-} MEFs, and bacterial internalization was assayed using the two-color fluorescence assay. We found that reconstitution with the Cas Δ SH3 mutant was able to restore *Salmonella* internalization, whereas the Cas Δ YXXP mutant could not (Figure 6C), suggesting that the substrate-binding YXXP repeats, rather than the FAK-binding SH3 domain, is essential for bacterial uptake. This result was surprising, given our earlier finding that the Cas-binding PXXP718 domain on FAK is necessary for *Salmonella* internalization. One possible explanation for this observation is that overexpression of Cas may reduce the need for a direct interaction with FAK. Alternatively, the PXXP718 motif on FAK could bind some other unknown SH3-containing protein that is required for invasion, and that Cas, although necessary, may not need to interact directly with FAK.

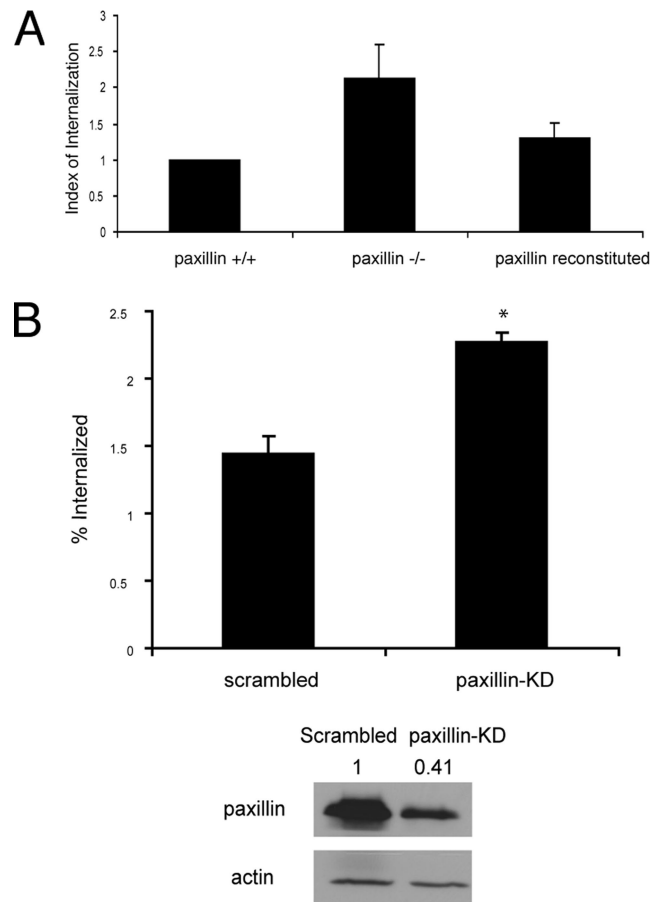


Figure 8. Paxillin is not necessary for *Salmonella* invasion. (A) Paxillin^{+/+}, paxillin^{-/-} (mock transfected), or paxillin^{-/-} MEFs transfected with GFP-paxillin were infected with SL1344 at a MOI = 30 for 1 h and were subjected to the two-color immunofluorescence assay. (B) HeLa cells were transfected with scrambled siRNA or anti-paxillin siRNA 72 h before the gentamicin resistance invasion assay. The efficiency of paxillin knockdown is shown in the top panel. A corresponding actin immunoblot (bottom) is shown to demonstrate equal loading. * $p < 0.001$ compared with control cells transfected with scrambled siRNA (Student's *t* test).

Aberrant Morphology of *Salmonella*-induced Phagocytic Cups in *Cas*^{-/-} Fibroblasts

As described above, the number of bacterially induced actin foci was substantially reduced in both *FAK*^{-/-} and *Cas*^{-/-} MEFs upon *Salmonella* invasion; however, the ones formed in *FAK*^{-/-} cells appeared essentially normal. In contrast, foci induced in *Cas*^{-/-} cells were abnormally large and diffuse and displayed bright, condensed actin patches (Figure 7A). Quantification of the size of these invasion foci in wild-type MEFs showed that they were relatively small (10–20 μm^2), and only a small fraction of foci (<10%) were larger than 20 μm^2 (Figure 7B). However, in *Cas*^{-/-} MEFs, 70% of the foci were larger than 20 μm^2 and more than 40% were larger than 40 μm^2 . These results suggest that loss of Cas leads to a dysregulation of actin assembly at sites of bacterial attachment and that this aberrant actin organization may impair internalization.

Paxillin Is Not Functionally Required for *Salmonella* Invasion

As shown in Figure 1, paxillin is also recruited to the *Salmonella*-induced focal adhesion-like complex together with

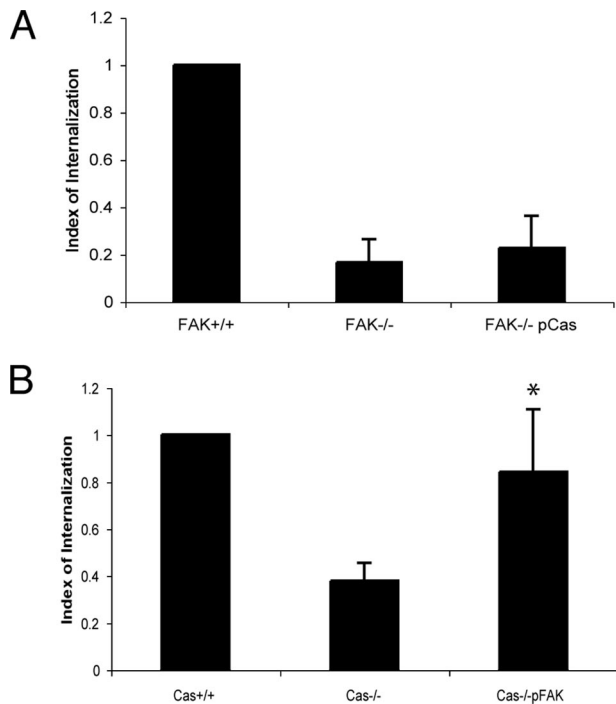


Figure 9. Overexpression of FAK rescues *Salmonella* invasion in Cas^{-/-} MEFs. (A) FAK^{-/-} cells were either mock-transfected (FAK^{-/-}) or transfected with an expression plasmid encoding p130Cas (FAK^{-/-} pCas). FAK^{+/+} cells were used as a positive control. Cells were infected with SL1344 at a MOI = 30 for 1 h, and bacterial invasion was quantified using the two-color immunofluorescence assay. (B) Cas^{-/-} cells were either mock-transfected (Cas^{-/-}) or transfected with an expression plasmid encoding FAK (Cas^{-/-} pFAK). Cells were then infected using wild-type MEFs (Cas^{+/+}) as a positive control and invasion efficiency quantified as in A. *p < 0.05 compared with mock-transfected Cas^{-/-} cells (Student's *t* test).

FAK and Cas and coprecipitates with these proteins in infected cells. To determine whether *Salmonella* internalization requires paxillin, the gentamicin resistance invasion assay and the two-color immunofluorescence assay were performed in wild-type and paxillin^{-/-} MEFs. Surprisingly, bacterial invasion efficiency was actually enhanced in paxillin^{-/-} MEFs compared with wild-type cells (Figure 8A). Moreover, reconstitution of paxillin^{-/-} MEFs with wild-type paxillin resulted in bacterial invasion efficiency comparable with wild-type cells (Figure 8A). In agreement with this observation, small interfering RNA (siRNA)-mediated knockdown of paxillin in HeLa cells also resulted in enhanced invasion efficiency (Figure 8B). Together, these results suggest that although paxillin is also recruited to sites of *Salmonella* invasion, it is not necessary for bacterial internalization; instead, it may play a negative role in this process.

Overexpression of FAK Rescues *Salmonella* Internalization in Cas^{-/-} Cells

Emerging studies have shown that FAK/Cas interaction is essential for mediating signaling to downstream effectors and that a FAK mutant lacking the Cas binding site compromises cell migration (Parsons, 2003). Therefore, we sought to examine whether FAK and Cas function independently or if they cooperate to promote *Salmonella* invasion. To examine the relationship between FAK and Cas in the

context of bacterial invasion, we overexpressed wild-type Cas in FAK^{-/-} cells or FAK in Cas^{-/-} cells and measured internalization efficiency. Interestingly, as shown in Figure 9, we found that overexpression of Cas was not able to rescue bacterial invasion in FAK^{-/-} cells. In contrast, expression of FAK in Cas^{-/-} cells completely restored *Salmonella* internalization. One interpretation of these results is that FAK and Cas act in the same signaling cascade, where FAK functions downstream of Cas. Alternatively, FAK and Cas may function in distinct pathways, and the FAK-dependent pathway may be sufficient to compensate for the lack of Cas when FAK is overexpressed.

DISCUSSION

Integrins have been exploited by a number of different microbial pathogens as a means of entering host cells. *Yersinia*, *Staphylococcus*, and *Streptococcus* species have all been reported to enter host cells by integrin-dependent mechanisms, either directly (*Yersinia*; Isberg and Leong, 1990; Rankin *et al.*, 1992) or indirectly through interaction with components of the extracellular matrix (*Staphylococcus* and *Streptococcus*; Ozeri *et al.*, 1996, 1998; Okada *et al.*, 1997; Sinha *et al.*, 1999; Fowler *et al.*, 2000). In each case, integrin ligation results in the assembly of focal adhesion complexes at sites of bacterial attachment and the activation of integrin-dependent signaling cascades. Here we show that *S. typhimurium* can also promote assembly of focal adhesion complexes in response to bacterial attachment. However, assembly of these structures is unlikely to be nucleated by integrins, which are generally absent from the apical plasma membrane of epithelial cells. Our results reveal that both FAK and p130Cas have essential, but novel functions in *Salmonella* internalization.

Apart from its well-characterized role in integrin-mediated cell adhesion and migration (Parsons, 2003; Mitra *et al.*, 2005), FAK has also been implicated in a number of different integrin-dependent phagocytic events. For instance, integrin $\alpha_v\beta_5$ -mediated internalization of spent photoreceptor outer segment fragments by retinal pigment epithelium requires FAK (Finnemann, 2003). Similarly, uptake of *Yersinia* upon invasin-mediated engagement of β_1 integrin and *Streptococcus* via fibronectin-binding integrins is dependent on FAK (Alrutz and Isberg, 1998; Ozeri *et al.*, 2001; Bruce-Staskal *et al.*, 2002). More recently, Agerer *et al.* (2005) showed that FAK plays an essential role in integrin $\alpha_1\beta_5$ -mediated invasion of *Staphylococcus*. However, each of these processes is accompanied by increased tyrosine phosphorylation of FAK, indicative of its activation during particle internalization. Moreover, the autophosphorylation site Y397 has been shown to be necessary for internalization of both *Yersinia* and *Staphylococcus*, as expression of FAK Y397F impairs bacterial uptake (Alrutz and Isberg, 1998; Agerer *et al.*, 2005). In each of these cases, it is likely that FAK activation results from integrin clustering.

In contrast, we found that *Salmonella* internalization does not appear to induce significant tyrosine phosphorylation of FAK. Because of the high basal level of FAK phosphorylation in polarized MDCK cells, we cannot exclude the possibility that a small fraction of the total FAK pool becomes phosphorylated as a result of infection. However, we did observe significant FAK dephosphorylation between 40 and 60 min after infection (Figure 4A), an event likely to be mediated by the secreted *Salmonella* tyrosine phosphatase SptP (Murli *et al.*, 2001). The C-terminus of SptP is structurally related to the secreted *Yersinia* effector protein YopH, which has been shown to dephosphorylate both FAK and

p130Cas. However, in the context of *Yersinia* infection YopH-dependent dephosphorylation actually inhibits bacterial internalization, whereas *Salmonella* strains that express SptP are efficiently internalized. Furthermore, our reconstitution experiments with a panel of mutants indicate that *Salmonella* entry requires neither the FAK autophosphorylation site (Y397) nor the central kinase domain (Figure 4B) but does require the noncatalytic C-terminal focal adhesion targeting domain. These observations support the notion that FAK plays a novel role in *Salmonella* invasion, serving as a scaffolding protein, but not as a protein tyrosine kinase.

We found that tyrosine phosphorylation of p130Cas, unlike that of FAK, is enhanced in response to *Salmonella* invasion (Figure 6A) and that this is primarily due to activation of Src family kinases. Although inhibition of c-Src with PP2 blocked the *Salmonella*-induced increase in Cas phosphorylation, it had no effect on internalization efficiency, suggesting that c-Src activation parallels but is not necessary for bacterial uptake. This finding is in agreement with an earlier observation that tyrosine kinase activity is not required for *Salmonella* entry (Rosenshine *et al.*, 1994).

Although Cas phosphorylation is not essential for *Salmonella* internalization, we found that cells lacking Cas were severely impaired in bacterial uptake. This is likely due to aberrant assembly of the phagocytic apparatus, which in Cas null cells was characterized by its large size and the presence of numerous distinct actin patches. Cas^{-/-} fibroblasts have been reported to lack actin stress fibers, and reconstitution experiments have mapped this defect to the central substrate-binding domain, specifically to YDxP motifs within this domain (Huang *et al.*, 2002). When phosphorylated, these pYDxP motifs bind two adaptor proteins, CrkII and Nck, which can link Cas to other regulators of the actin cytoskeleton. CrkII binding to Cas has been shown to be essential for *Yersinia* uptake (Weidow *et al.*, 2000; Bruce-Staskal *et al.*, 2002), most likely through its interaction with the Rac nucleotide exchange factor DOCK180 (Kiyokawa *et al.*, 1998; Weidow *et al.*, 2000). However, *Salmonella* activate Rac directly through the secreted effector protein SopE (Hardt *et al.*, 1998; Criss *et al.*, 2001) and presumably do not require host proteins to perform this function. Nevertheless, we have tested the role of both CrkII and Nck in *Salmonella* entry using dominant inhibitory mutants and found that neither protein is necessary for bacterial internalization (unpublished data). It is therefore likely that binding of some other, unidentified protein(s) to the substrate-binding domain mediates the effects of Cas on assembly of the actin cytoskeleton. Moreover, because inhibition of c-Src by PP2 did not impair internalization, it seems likely that this binding would not depend on Src-mediated phosphorylation of tyrosines within the substrate-binding domain. One possibility is that one or more of the secreted bacterial effector proteins binds Cas via this domain and that this interaction is required for efficient *Salmonella* uptake.

Cas binds to FAK through interaction of the N-terminal Cas SH3 domain with a proline-rich region (PXXP718) on FAK (Harte *et al.*, 1996). Surprisingly, our reconstitution experiments indicate that bacterial entry requires the Cas-binding domain of FAK, but not the SH3 domain of Cas, (Figures 4B and 6C). Although our data indicate that *Salmonella* infection does enhance the interaction between endogenous FAK and Cas, it remains unclear whether this interaction is required for efficient *Salmonella* entry. One possible explanation for this observation is that Cas can localize to focal adhesions independently of FAK, as has been previously shown (Harte *et al.*, 2000) and that direct interaction between FAK and Cas is not absolutely required. Addition-

ally, it is possible that the PXXP718 motif on FAK binds proteins other than Cas that are necessary for bacterial entry.

We also found that ectopic expression of FAK was able to rescue *Salmonella* invasion in Cas^{-/-} cells (Figure 9). One interpretation of this result is that endogenous levels of FAK are limiting and that interaction with Cas is necessary to concentrate it at sites of bacterial entry. When Cas is absent, local FAK levels may be insufficient to support internalization. However, when overexpressed, FAK may no longer require Cas as a scaffold and could concentrate at bacterial adhesion sites through weaker interactions with other focal adhesion proteins. An alternative interpretation is that FAK and Cas function independently of one another, but that FAK-dependent processes are sufficient to support bacterial internalization in the absence of Cas. Interestingly, Bruce-Staskal *et al.* (2002) showed that overexpression of Cas can rescue *Yersinia* uptake in FAK^{-/-} cells, suggesting that the relationship between the two proteins is reversed in the context of *Yersinia* infection.

As an important component of focal adhesions, paxillin interacts with several other focal adhesion proteins to coordinate adhesion-dependent signaling events. In the context of *Salmonella* invasion, paxillin is enriched at the membrane protrusions induced by bacteria. However, cells deficient in paxillin were actually infected more efficiently by *Salmonella*, suggesting that paxillin may play a negative role in this process. It has been shown that tyrosine phosphorylation of paxillin and Cas play opposing roles in cell migration and contact inhibition of cell growth, where phosphorylated paxillin reduces cell migration and Cas has the opposite effect (Yano *et al.*, 2000). Therefore, future experiments to examine the mechanisms by which paxillin may attenuate *Salmonella* invasion will provide more insight into the molecular regulation of this process.

Taken together, our results demonstrate a requirement for both FAK and Cas in *Salmonella* invasion. Although both proteins have been implicated in the integrin-mediated uptake of other bacterial pathogens, this is the first evidence that FAK can function in this process independently of its kinase activity. Furthermore, because pathogenic *Salmonella* strains can infect epithelial cells at the integrin-poor apical plasma membrane (Finlay *et al.*, 1988, 1989), it is apparent that the adherent bacteria can nucleate assembly of focal adhesion-like complexes through an alternative, integrin-independent mechanism. We are currently working to determine if this mechanism involves secreted bacterial effector proteins, other transmembrane host proteins, or both.

ACKNOWLEDGMENTS

We thank Tom Parsons (University of Virginia, Charlottesville, VA) for FAK^{-/-} cells and FAK constructs; Amy Bouton (University of Virginia) for Cas^{-/-} cells, Cas constructs, and Cas antibodies; Rick Horwitz (University of Virginia) for the paxillin^{-/-} cells; Chris Turner (SUNY, Syracuse) for paxillin constructs; and Doug DeSimone (University of Virginia) for the $\beta 1$ integrin antibody. We also thank Amy Bouton and David Castle for helpful comments on the manuscript. This work was supported by grants from the National Institutes of Health (DK58536) and the Crohn's and Colitis Foundation of America (to J.E.C.).

REFERENCES

- Agerer, F., Lux, S., Michel, A., Rohde, M., Ohlsen, K., and Hauck, C. R. (2005). Cellular invasion by *Staphylococcus aureus* reveals a functional link between focal adhesion kinase and cortactin in integrin-mediated internalisation. *J. Cell Sci.* 118, 2189–2200.
- Alrutz, M. A., and Isberg, R. R. (1998). Involvement of focal adhesion kinase in invasin-mediated uptake. *Proc. Natl. Acad. Sci. USA* 95, 13658–13663.

- Astier, A., Avraham, H., Manie, S. N., Groopman, J., Canty, T., Avraham, S., and Freedman, A. S. (1997). The related adhesion focal tyrosine kinase is tyrosine-phosphorylated after beta1-integrin stimulation in B cells and binds to p130cas. *J. Biol. Chem.* *272*, 228–232.
- Bourdet-Sicard, R., Rudiger, M., Jockusch, B. M., Gounon, P., Sansonetti, P. J., and Nhieu, G. T. (1999). Binding of the *Shigella* protein IpaA to vinculin induces F-actin depolymerization. *EMBO J.* *18*, 5853–5862.
- Bouton, A. H., Riggins, R. B., and Bruce-Staskal, P. J. (2001). Functions of the adapter protein Cas: signal convergence and the determination of cellular responses. *Oncogene* *20*, 6448–6458.
- Bruce-Staskal, P. J., Weidow, C. L., Gibson, J. J., and Bouton, A. H. (2002). Cas, Fak and Pyk2 function in diverse signaling cascades to promote *Yersinia* uptake. *J. Cell Sci.* *115*, 2689–2700.
- Calalb, M. B., Polte, T. R., and Hanks, S. K. (1995). Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Mol. Cell Biol.* *15*, 954–963.
- Chen, L. M., Hobbie, S., and Galan, J. E. (1996). Requirement of CDC42 for *Salmonella*-induced cytoskeletal and nuclear responses. *Science* *274*, 2115–2118.
- Collazo, C. M., and Galan, J. E. (1997). The invasion-associated type-III protein secretion system in *Salmonella*—a review. *Gene* *192*, 51–59.
- Cossart, P., and Sansonetti, P. J. (2004). Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science* *304*, 242–248.
- Criss, A. K., Ahlgren, D. M., Jou, T. S., McCormick, B. A., and Casanova, J. E. (2001). The GTPase Rac1 selectively regulates *Salmonella* invasion at the apical plasma membrane of polarized epithelial cells. *J. Cell Sci.* *114*, 1331–1341.
- Darwin, K. H., and Miller, V. L. (1999). Molecular basis of the interaction of *Salmonella* with the intestinal mucosa. *Clin. Microbiol. Rev.* *12*, 405–428.
- Finlay, B. B., Fry, J., Rock, E. P., and Falkow, S. (1989). Passage of *Salmonella* through polarized epithelial cells: role of the host and bacterium. *J. Cell Sci. Suppl.* *11*, 99–107.
- Finlay, B. B., Gumbiner, B., and Falkow, S. (1988). Penetration of *Salmonella* through a polarized Madin-Darby canine kidney epithelial cell monolayer. *J. Cell Biol.* *107*, 221–230.
- Finlay, B. B., Ruschkowski, S., and Dedhar, S. (1991). Cytoskeletal rearrangements accompanying *Salmonella* entry into epithelial cells. *J. Cell Sci.* *99*(Pt 2), 283–296.
- Finnemann, S. C. (2003). Focal adhesion kinase signaling promotes phagocytosis of integrin-bound photoreceptors. *EMBO J.* *22*, 4143–4154.
- Fowler, T., Wann, E. R., Joh, D., Johansson, S., Foster, T. J., and Hook, M. (2000). Cellular invasion by *Staphylococcus aureus* involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell beta1 integrins. *Eur. J. Cell Biol.* *79*, 672–679.
- Hardt, W. D., Chen, L. M., Schuebel, K. E., Bustelo, X. R., and Galan, J. E. (1998). *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell* *93*, 815–826.
- Harte, M. T., Hildebrand, J. D., Burnham, M. R., Bouton, A. H., and Parsons, J. T. (1996). p130Cas, a substrate associated with v-Src and v-Crk, localizes to focal adhesions and binds to focal adhesion kinase. *J. Biol. Chem.* *271*, 13649–13655.
- Harte, M. T., Macklem, M., Weidow, C. L., Parsons, J. T., and Bouton, A. H. (2000). Identification of two focal adhesion targeting sequences in the adapter molecule p130(Cas). *Biochim. Biophys. Acta* *1499*, 34–48.
- Huang, J., Hamasaki, H., Nakamoto, T., Honda, H., Hirai, H., Saito, M., Takato, T., and Sakai, R. (2002). Differential regulation of cell migration, actin stress fiber organization, and cell transformation by functional domains of Crk-associated substrate. *J. Biol. Chem.* *277*, 27265–27272.
- Hueck, C. J., Hantman, M. J., Bajaj, V., Johnston, C., Lee, C. A., and Miller, S. I. (1995). *Salmonella typhimurium* secreted invasion determinants are homologous to *Shigella* Ipa proteins. *Mol. Microbiol.* *18*, 479–490.
- Isberg, R. R., and Leong, J. M. (1990). Multiple beta 1 chain integrins are receptors for invasins, a protein that promotes bacterial penetration into mammalian cells. *Cell* *60*, 861–871.
- Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., and Matsuda, M. (1998). Activation of Rac1 by a Crk SH3-binding protein, DOCK180. *Genes Dev.* *12*, 3331–3336.
- Lee, C. A., and Falkow, S. (1990). The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. *Proc. Natl. Acad. Sci. USA* *87*, 4304–4308.
- Liu, Y., Loijens, J. C., Martin, K. H., Karginov, A. V., and Parsons, J. T. (2002). The association of ASAP1, an ADP ribosylation factor-GTPase activating protein, with focal adhesion kinase contributes to the process of focal adhesion assembly. *Mol. Biol. Cell* *13*, 2147–2156.
- Mitra, S. K., Hanson, D. A., and Schlaepfer, D. D. (2005). Focal adhesion kinase: in command and control of cell motility. *Nat. Rev. Mol. Cell Biol.* *6*, 56–68.
- Murli, S., Watson, R. O., and Galan, J. E. (2001). Role of tyrosine kinases and the tyrosine phosphatase SptP in the interaction of *Salmonella* with host cells. *Cell Microbiol.* *3*, 795–810.
- Okada, N., Watarai, M., Ozeri, V., Hanski, E., Caparon, M., and Sasakawa, C. (1997). A matrix form of fibronectin mediates enhanced binding of *Streptococcus pyogenes* to host tissue. *J. Biol. Chem.* *272*, 26978–26984.
- Ozeri, V., Rosenshine, I., Ben-Ze'ev, A., Bokoch, G. M., Jou, T. S., and Hanski, E. (2001). De novo formation of focal complex-like structures in host cells by invading *Streptococci*. *Mol. Microbiol.* *41*, 561–573.
- Ozeri, V., Rosenshine, I., Mosher, D. F., Fassler, R., and Hanski, E. (1998). Roles of integrins and fibronectin in the entry of *Streptococcus pyogenes* into cells via protein F1. *Mol. Microbiol.* *30*, 625–637.
- Ozeri, V., Tovi, A., Burstein, I., Natanson-Yaron, S., Caparon, M. G., Yamada, K. M., Akiyama, S. K., Vlodaysky, I., and Hanski, E. (1996). A two-domain mechanism for group A streptococcal adherence through protein F to the extracellular matrix. *EMBO J.* *15*, 989–998.
- Parsons, J. T. (2003). Focal adhesion kinase: the first ten years. *J. Cell Sci.* *116*, 1409–1416.
- Patel, J. C., and Galan, J. E. (2005). Manipulation of the host actin cytoskeleton by *Salmonella*—all in the name of entry. *Curr. Opin. Microbiol.* *8*, 10–15.
- Polte, T. R., and Hanks, S. K. (1995). Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. *Proc. Natl. Acad. Sci. USA* *92*, 10678–10682.
- Randazzo, P. A., Andrade, J., Miura, K., Brown, M. T., Long, Y. Q., Stauffer, S., Roller, P., and Cooper, J. A. (2000). The Arf GTPase-activating protein ASAP1 regulates the actin cytoskeleton. *Proc. Natl. Acad. Sci. USA* *97*, 4011–4016.
- Rankin, S., Isberg, R. R., and Leong, J. M. (1992). The integrin-binding domain of invasins is sufficient to allow bacterial entry into mammalian cells. *Infect. Immun.* *60*, 3909–3912.
- Rosenshine, I., Ruschkowski, S., Foubister, V., and Finlay, B. B. (1994). *Salmonella typhimurium* invasion of epithelial cells: role of induced host cell tyrosine protein phosphorylation. *Infect. Immun.* *62*, 4969–4974.
- Ruest, P. J., Shin, N. Y., Polte, T. R., Zhang, X., and Hanks, S. K. (2001). Mechanisms of CAS substrate domain tyrosine phosphorylation by FAK and Src. *Mol. Cell Biol.* *21*, 7641–7652.
- Sanders, M. A., and Basson, M. D. (2005). p130cas but not paxillin is essential for Caco-2 intestinal epithelial cell spreading and migration on collagen IV. *J. Biol. Chem.* *280*, 23516–23522.
- Sinha, B., Francois, P. P., Nusse, O., Foti, M., Hartford, O. M., Vaudaux, P., Foster, T. J., Lew, D. P., Herrmann, M., and Krause, K. H. (1999). Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin alpha5beta1. *Cell Microbiol.* *1*, 101–117.
- Taylor, J. M., Hildebrand, J. D., Mack, C. P., Cox, M. E., and Parsons, J. T. (1998). Characterization of graf, the GTPase-activating protein for rho associated with focal adhesion kinase. Phosphorylation and possible regulation by mitogen-activated protein kinase. *J. Biol. Chem.* *273*, 8063–8070.
- Tran Van Nhieu, G., Ben-Ze'ev, A., and Sansonetti, P. J. (1997). Modulation of bacterial entry into epithelial cells by association between vinculin and the *Shigella* IpaA invasin. *EMBO J.* *16*, 2717–2729.
- Weidow, C. L., Black, D. S., Bliska, J. B., and Bouton, A. H. (2000). CAS/Crk signalling mediates uptake of *Yersinia* into human epithelial cells. *Cell Microbiol.* *2*, 549–560.
- Yano, H., Uchida, H., Iwasaki, T., Mukai, M., Akedo, H., Nakamura, K., Hashimoto, S., and Sabe, H. (2000). Paxillin alpha and Crk-associated substrate exert opposing effects on cell migration and contact inhibition of growth through tyrosine phosphorylation. *Proc. Natl. Acad. Sci. USA* *97*, 9076–9081.
- Zamir, E., and Geiger, B. (2001). Components of cell-matrix adhesions. *J. Cell Sci.* *114*, 3577–3579.