Cas, Fak and Pyk2 function in diverse signaling cascades to promote Yersinia uptake

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Summary

The interplay between pathogen-encoded virulence factors and host cell signaling networks is critical for both the establishment and clearance of microbial infections. Yersinia uptake into host cells serves as an in vitro model for exploring how host cells respond to Yersinia adherence. In this study, we provide insight into the molecular nature and regulation of signaling networks that contribute to the uptake process. Using a reconstitution approach in Fak$^{-/-}$ fibroblasts, we have been able to specifically address the interplay between Fak, Cas and Pyk2 in this process. We show that both Fak and Cas play roles in the Yersinia uptake process and that Cas can function in a novel pathway that is independent of Fak. Fak-dependent Yersinia uptake does not appear to involve Cas-Crk signaling. By contrast, Cas-mediated uptake in the absence of Fak requires Crk as well as the protein tyrosine kinases Pyk2 and Src. In spite of these differences, the requirement for Rac1 activity is a common feature of both pathways. Furthermore, blocking the function of either Fak or Cas induces similar morphological defects in Yersinia internalization, which are manifested by incomplete membrane protrusive activity that is consistent with an inhibition of Rac1 activity. Pyk2 also functions in Yersinia uptake by macrophages, which are physiologically important for clearing Yersinia infections. Taken together, these data provide new insight into the host cellular signaling networks that are initiated upon infection with *Y. pseudotuberculosis*. Importantly, these findings also contribute to a better understanding of other cellular processes that involve actin remodeling, including the host response to other microbial pathogens, cell adhesion and migration.

Key words: Cas, Fak, Pyk2, Rac1, Yersinia

Introduction

The interplay between pathogen-encoded virulence factors and host cell signaling networks is critical for both the establishment and clearance of microbial infections. *Yersinia pseudotuberculosis* is an extracellular, Gram-negative bacteria that serves as an excellent model for studying host-pathogen interactions. This organism is generally acquired through ingestion. Upon reaching the intestine, the Yersinia surface protein invasin binds to β1 integrins located on the apical surface of M cells, facilitating translocation across the epithelium (Clark et al., 1998; Schulte, 2000). The pathogenicity of *Y. pseudotuberculosis* is determined to a large extent by plasmid-encoded cytotoxic and antiphagocytic virulence proteins (Yops) that are directly injected into host cells via a Type III secretion system (for a review, see Aepfelbacher et al., 1999) (Bliska, 2000). These molecules promote Yersinia growth and survival in the lymphoid follicles (Peyer's patches) underlying the intestinal epithelium by controlling the anti-bacterial activities of immune cells that reside at these sites.

One of the effector molecules encoded on the *Y. pseudotuberculosis* virulence plasmid is YopH, a protein tyrosine phosphatase (PTPase) that inhibits bacterial phagocytosis by host cells (Rosqvist et al., 1988a; Guan and Dixon, 1990). Substrates of YopH include host cell focal-adhesion-associated proteins such as Focal Adhesion Kinase (Fak), p130^{Cas} (Crk-associated substrate; Cas) and paxillin (Black and Bliska, 1997; Persson et al., 1997; Black et al., 1998). Focal adhesion targeting and phosphorylation of these proteins is critical for the activation of cellular signaling cascades that regulate the actin cytoskeleton (for reviews, see Brugge, 1998; Vuori, 1998; Critchley, 2000). YopH-mediated dephosphorylation of Fak, Cas and paxillin is believed to inhibit the actin cytoskeletal remodeling that is necessary for bacterial phagocytosis. The observation that YopH disrupts focal adhesions supports this model (Black and Bliska, 1997; Persson et al., 1997).

Focal adhesions are cellular structures that link transmembrane integrins to the actin cytoskeleton (Brugge, 1998; Critchley, 2000). The binding of integrins to extracellular matrix (ECM) ligands induces integrin clustering and recruitment of the non-receptor protein tyrosine kinase (PTK) Fak. This results in Fak autophosphorylation and the creation of a binding site for the Src-homology 2 (SH2) domain of Src (Schaller et al., 1994; Schaller, 1996). Fak-Src association increases Src PTK activity and promotes Src-dependent phosphorylation of additional tyrosine residues on Fak and Fak-associated proteins, such as Cas and paxillin (Schaller et al., 1994; Calalb et al., 1995; Thomas et al., 1998; Ruest et al., 2001). Integrin-dependent activation of Fak and Src has been implicated in numerous actin-based cellular processes, including cell cycle progression, adhesion and

migration (Ilic et al., 1997; Klemke et al., 1998; Cary and Guan, 1999; Oktay et al., 1999).

The Fak family member Pyk2/Cakβ/Raftk (Pyk2) shares a high degree of sequence identity with Fak, particularly in the N-terminus and kinase domain (Kanner et al., 1994; Avraham et al., 1995; Sasaki et al., 1995). Like Fak, Pyk2 contains binding sites for Cas and paxillin as well as an autophosphorylation site, which can serve as a Src SH2-domain binding site when it is phosphorylated (Dikic et al., 1996). Despite these similarities, Fak and Pyk2 have many distinct features. Although Fak is expressed fairly ubiquitously, Pyk2 shows a more restricted distribution, being highly expressed in the brain and hematopoietic cells (Avraham et al., 1995; Sasaki et al., 1995). Moreover, although both Fak and Pyk2 have been implicated in signaling to the actin cytoskeleton, only Fak routinely localizes to focal adhesions (Sasaki et al., 1995; Li et al., 1996; Astier et al., 1997a; Guan, 1997). Fak is activated primarily in response to integrin activation, whereas Pyk2 can be activated in response to stress, calcium flux or upon ligation of B and T cell receptors (for a review, see Avraham et al., 2000). Certain functions of Fak and Pyk2 may even be antagonistic, as Fak has been implicated in cell survival whereas Pyk2 signaling can result in the induction of apoptosis (Frisch et al., 1996; Xiong and Parsons, 1997).

Cas was first identified as a highly tyrosine-phosphorylated protein in v-Src-and v-Crk-transformed cells (Sakai et al., 1994). Cas functions as an adapter protein through its interactions with numerous cellular proteins (for reviews, see O'Neill et al., 2000; Bouton et al., 2001). It contains an N-terminal SH3 domain that binds to Fak and Pyk2, a substrate-binding domain that binds to the adapter molecules Crk and Nck, and a C-terminus that contains Src-binding sequences. The 15 YXXP motifs located in the substrate-binding domain serve as potential phosphorylation sites for cellular PTKs (Songyang et al., 1994; Songyang and Cantley, 1998). Recent work has shown that, although Fak and Pyk2 are both capable of phosphorylating Cas, Src appears to be responsible for the majority of Cas phosphorylation (Astier et al., 1997b; Ruest et al., 2001). Therefore, the role of Cas-Fak or Cas-Pyk2 interactions may be to bring Cas in close proximity to Src in order to promote its phosphorylation. Phosphorylation of the Cas substrate-binding domain can result in the recruitment of Crk and ultimately activation of Rac1, leading to cytoskeletal remodeling (for reviews, see Hall, 1998; Kiyokawa et al., 1998; Bishop and Hall, 2000; Ridley, 2000).

The interplay between Fak, Src, Cas, Crk and Rac1 has been extensively studied in the actin-dependent process of integrin-dependent migration. Fibroblasts from $Fak^{-/-}$, Src/Yes/Fyn $(SYF)^{-/-}$ and $Cas^{-/-}$ mouse embryos are all defective for migration (Ilic et al., 1995; Honda et al., 1999; Klinghoffer et al., 1999). The ability of ectopic Fak to rescue migration in $Fak^{-/-}$ cells requires its association with both Src and Cas (Cary et al., 1998; Sieg et al., 1999), providing evidence that the functions of these molecules are interconnected. Cell migration can also be enhanced in COS and FG-M pancreatic carcinoma cells by overexpression of wild-type Cas, but not by expression of a Cas mutant that is defective for Crk binding (Klemke et al., 1998; Cheresh et al., 1999; Cho and Klemke, 2000). Co-expression of Cas with dominant inhibitory Crk or Rac1 molecules blocks this Cas-dependent cell migration pathway, lending further support to the notion that Cas-Crk-Rac1 signaling plays a key role in this process.

The host cellular response to *Y. pseudotuberculosis* infection, which is largely dependent on invasin-β_1 integrin interactions (Rosqvist et al., 1988b; Marra and Isberg, 1997), involves many of the same molecules that function in cell migration. Like integrin binding to fibronectin (FN), uptake of *Y. pseudotuberculosis* is coincident with increased tyrosine phosphorylation of several host cell proteins, including Fak and Cas (Black and Bliska, 1997; Persson et al., 1997). This phosphorylation appears to be critical for uptake, since treatment of host cells with PTK inhibitors prevents bacterial uptake whereas treatment with PTPase inhibitors increases uptake (Andersson et al., 1996). Moreover, bacterial expression of the antiphagocytic PTPase YopH inhibits phosphorylation of Fak and Cas, and this correlates with an inhibition of bacterial uptake (Black and Bliska, 1997; Persson et al., 1997).

Previous studies have investigated whether the functions of Fak and Cas are required for Yersinia uptake. $Fak^{-/-}$ cells have been shown to be deficient in uptake of invasin-coated beads (Alrutz and Isberg, 1998) and HeLa cells expressing a dominant inhibitory variant of Cas were found to be defective in uptake of *Y. pseudotuberculosis* (Weidow et al., 2000). Nevertheless, important questions remain regarding the role of Cas and Fak in the process of Yersinia uptake. For example, although Fak has been implicated in invasin-mediated phagocytosis (Alrutz and Isberg, 1998), it has not yet been determined whether Fak and its downstream effectors are actually involved in uptake of live *Y. pseudotuberculosis*. Additionally, although Cas-Crk-Rac signaling has been shown to be important for Yersinia uptake in HeLa cells (Weidow et al., 2000), the events upstream of Cas phosphorylation have yet to be fully elucidated.

Since Fak and Cas function coordinately in the process of integrin-dependent migration, we hypothesized that Yersinia uptake may also require the concerted activities of both of these molecules. In this study, we shed light on the molecular pathways that are activated in response to Yersinia infection. First, we present evidence that both Fak and Cas play roles in the Yersinia uptake process and that Cas can in fact function in a novel pathway that is independent of Fak. Interestingly, in spite of this distinction, Fak and Cas appear to ultimately feed into a common Rac1-dependent signaling network. Blocking the function of either Fak or Cas induces similar morphological defects in Yersinia internalization, which are manifested by incomplete membrane protrusive activity that is consistent with an inhibition of Rac1 activity. We also provide evidence that Pyk2, which is expressed at high levels in $Fak^{-/-}$ mouse embryo fibroblasts (MEFs) relative to other fibroblasts (Sieg et al., 1998), plays a role in the Cas-dependent Yersinia uptake pathway that is active in these cells. We show that inhibition of Pyk2 blocks Cas-dependent Yersinia uptake, and that, while Pyk2 autophosphorylation is increased in response to infection with a Yersinia strain that does not express YopH, this response is inhibited in the presence of YopH. These data suggest that catalytic activity and autophosphorylation of Pyk2 play an important role in the Cas-dependent Yersinia uptake process in $Fak^{-/-}$ cells. Finally, we show that Pyk2 also functions in Yersinia uptake by macrophages, which express Pyk2 at levels comparable to those observed in $Fak^{-/-}$ cells. Macrophages are important for clearing *Y. pseudotuberculosis* infections in vivo.

Therefore, Pyk2 may be an integral part of the host response to Yersinia infections. Taken together, these data provide new insight into the host cellular signaling networks that are initiated upon infection with *Y. pseudotuberculosis*. Importantly, these findings also contribute to a better understanding of other cellular processes that involve actin remodeling, such as the host response to other microbial pathogens, cell adhesion and migration.

Materials and Methods

Cell culture

$Fak^{-/-}$ and $Fak^{+/+}$ MEF cells were generously provided by Dusko Ilic (UCSF, San Francisco, CA) and have been previously described (Ilic et al., 1995). HeLa cells stably expressing the vector pRK5 (HeLa/RK5) or Cas-ΔYXXP (HeLa/Cas-ΔYXXP) have also been described (Weidow et al., 2000). J774A.1 macrophages were purchased from American Type Culture Collection (Rockville, MD). All cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin, all of which were obtained from Gibco BRL Life Technologies (Rockville, MD). 1 mM sodium pyruvate was added to the MEF and HeLa cell media and 1 μM β-mercaptoethanol was included for MEFs. All cells were grown at 37ºC in 7.5% CO_2.

Antibodies and reagents

Cas monoclonal antibody (mAb) 8G4 has been described previously (Bouton and Burnham, 1997). Fak mAb 2A7 was kindly provided by J. T. Parsons (University of Virginia, Charlottesville, VA). Myc mAb 9E10 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). FLAG M5 mAb and FN were purchased from Sigma. A mAb recognizing Pyk2 was obtained from Transduction Laboratories (San Diego, CA) and phosphospecific Pyk2-Y^{402} was purchased from Biosource International (Camarillo, CA). Polyclonal Yersinia antibodies (SB349) were kindly provided by James Bliska (SUNY-Stony Brook, NY) and have been previously described (Black and Bliska, 2000). Fluoroscein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin (Ig), Texas Red (TR)-conjugated goat anti-rabbit Ig and Cy5-conjugated goat anti-rabbit Ig were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Cascade blue-conjugated goat anti-mouse Ig was purchased from Molecular Probes (Eugene, OR). ^{125}I-goat anti-mouse Ig and enhanced chemiluminescence (ECL) reagents were purchased from NEN Life Sciences (Boston, MA). Horseradish peroxidase (HRP)-conjugated sheep anti-mouse Ig, HRP-conjugated donkey anti-rabbit Ig and ^{125}I-conjugated protein A were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). FuGene 6 transfection reagent was obtained from Roche Molecular Biochemicals (Indianapolis, IN). PP2 was purchased from Calbiochem (LaJolla, CA).

Plasmids and transient transfections

The pRK5 plasmids encoding Myc-tagged full-length Cas, Cas-ΔYXXP or Cas-ΔSH3 have been previously described (Burnham et al., 2000; Harte et al., 2000). The pCAGGS expression vectors encoding Myc-tagged full-length Crk II (pCAGGS-Crk) or derivatives encoding point mutations (Crk-R38V and Crk-W169L) were generously provided by Michiyuki Matsuda (Tanaka et al., 1995). The pcDNA3-2AB plasmid encoding FLAG-tagged Fak were generously provided by J.T. Parsons (University of Virginia, Charlottesville, VA). The expression vector pCMV encoding Myc-tagged PRNK (residues 781-1009) was kindly provided by Wen-Chen Xiong (University of Alabama, Birmingham, AL). The pcDNA3-2AB plasmid encoding FLAG-tagged Rac-N17 and the pRK5 plasmid encoding Myc-tagged Rac-N17 were generously provided by Scott A. Weed (University of Colorado, Denver, CO). All constructs, except those encoding Rac1 derivatives, were transfected as per themanufacturer's instructions using FuGene6 transfection reagent (6 μl per 1 μg DNA for J774A.1 and 3 μl per 1 μg DNA for $Fak^{-/-}$ cells). The media used for transfection of macrophages did not contain antibiotics. Co-transfections were performed at an approximate molar equivalent ratio of 1:3 in the order listed. 24 hours post-transfection, 5×10^5 J774A.1 or 7×10^5 $Fak^{-/-}$ cells were plated onto FN-coated coverslips and incubated for an additional 18 hours before infection with Yersinia. For transfection with Rac1 constructs, 2×10^5 $Fak^{-/-}$ cells were plated directly onto FN-coated coverslips, transfected 24 hours later and incubated overnight. The media was changed, and the cells were incubated for an additional 18 hours. Expression of all ectopic proteins was confirmed by immunoblotting.

Bacterial uptake assay

The *Y. pseudotuberculosis* strains used for these studies have been described previously, as have bacterial growth conditions (Black and Bliska, 1997; Palmer et al., 1998). Multiplicities of infection (MOI) were determined by plating serial dilutions of final bacterial suspensions. Prior to infection, cells were washed twice with phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH=7.2), and antibiotic-free media was added. Cells were infected for 2 hours at an MOI of approximately 20, unless otherwise indicated, at 37ºC in 7.5% CO_2. Cells were washed three times with PBS between all subsequent steps. Cells were fixed in 3% paraformaldehyde for 20 minutes at room temperature (RT) before immunostaining [adapted from (Heesemann and Laufs, 1985)]. All antibodies were diluted in PBS containing 2% bovine serum albumin (BSA), and all incubations were at room temperature for 30 minutes. Extracellular Yersinia were stained by incubation of cells with SB349 (1:500) prior to incubation with FITC-conjugated goat anti-rabbit Ig (1:1000). Cells were permeabilized by incubation with 0.4% Triton X-100 in PBS for 2 minutes. Staining for total (extracellular and intracellular) Yersinia was then performed by an additional incubation with SB349 (1:500) followed by incubation with TR-conjugated goat anti-rabbit Ig (1:1000). To detect cells expressing ectopic proteins, mAbs directed against the ectopic protein were included in the second incubation with SB349 antibodies, followed by incubation with TR-conjugated goat anti-rabbit Ig and cascade blue-conjugated anti-mouse Ig at 1:150. To stain ectopic proteins, FLAG mAb M5 (1:150), Myc mAb 9E10 (1:300) and Cas 8G4 (1:100) were used. For co-transfections, cells were stained for the protein encoded by the plasmid that was transfected at the lower concentration (first construct listed in each figure). Cells were viewed with a Leica fluorescence microscope and photographed with a cooled charged-coupled device (CCD) camera controlled by Inovision Isee software. Bacterial uptake was determined for each experiment using the following calculation:% uptake=[total cell-associated Yersinia (stained with TR) – extracellular Yersinia (stained with FITC)] / total Yersinia (stained with TR)×100. For transient transfections, Yersinia were scored only if they were associated with cells positively stained for ectopic protein expression.

Transmission electron microscopy

For transmission electron microscopy (TEM) studies, 7×10^5 cells were plated onto FN-coated coverslips. The following day, cells were washed twice with PBS and DMEM containing 10% FCS, and 1 mM sodium pyruvate was added. Cells were infected at an MOI of approximately 50-500 for 15-60 minutes at 37ºC and 5% CO_2. Cells were washed three times with PBS and fixed by addition of 2.5% glutaraldehyde/4% paraformaldehyde for 30 minutes at room temperature. Cells were dehydrated, embedded and sectioned in the electron microscopy core facility at the University of Virginia. Samples were viewed by TEM and photographed in collaboration

with Jay Brown and William Newcomb (University of Virginia, Charlottesville, VA).

Immunoblotting

Cells were rinsed twice with PBS and lysed in modified RIPA (50 mM Tris, 150 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholate) containing protease and phosphatase inhibitors (100 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.15 unit/ml aprotinin, 1 mM vanadate) as previously described (Kanner et al., 1989). Protein concentrations were determined with the BCA Assay kit (Pierce, Rockford, IL). 40-50 μg of total cell lysate were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes and immunoblotted as previously described (Burnham et al., 2000; Weidow et al., 2000).

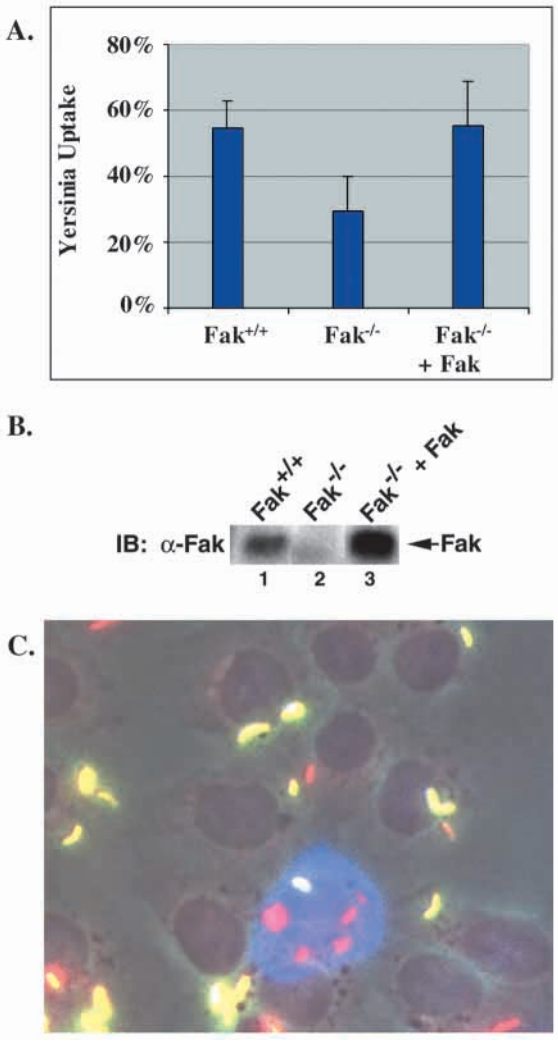

Fig. 1. Fak$^{-/-}$ MEFs are deficient for *Y. pseudotuberculosis* uptake. (A) The percentage of internalized *Y. pseudotuberculosis* observed in infected Fak$^{+/+}$, Fak$^{-/-}$ and Fak$^{-/-}$ MEFs expressing ectopic Fak is presented as the average of eight experiments. Error bars indicate standard deviation from the mean. (B) A representative immunoblot showing Fak expression from lysates derived from Fak$^{+/+}$, Fak$^{-/-}$, and Fak$^{-/-}$ MEFs reconstituted to express Fak. (C) Overlay of fluorescence images (400× magnification) of infected Fak$^{-/-}$ MEFs, including one Fak-transfected cell (blue). Intracellular *Y. pseudotuberculosis* appear red and extracellular *Y. pseudotuberculosis* appear yellow.

Statistical methods

In conjunction with the University of Virginia Division of Biostatistics and Epidemiology, a two-way analysis of variance (ANOVA) was performed on the log proportion of bacteria observed to be intracellular. After controlling for variability related to repetitions of experiments, contrasts between test condition means were used to determine statistical significance. A Bonferroni correction was then applied to the *P* values comparing test condition means to adjust for the number of comparisons (Miller, 1981; Rosner, 1995). Data obtained from experiments performed together were analyzed independently and maintained as separate data sets, as depicted in Tables 1-5.

Results

Delineation of a Fak-dependent *Y. pseudotuberculosis* uptake pathway in mouse embryo fibroblasts

Fak$^{-/-}$ cells are deficient for uptake of invasin-coated beads (Alrutz and Isberg, 1998). To determine whether Fak$^{-/-}$ cells are also deficient for uptake of live *Y. pseudotuberculosis*, Fak$^{+/+}$ and Fak$^{-/-}$ cells were infected for 2 hours with an avirulent strain of *Y. pseudotuberculosis* (YP17/pVector) that is efficiently internalized by HeLa cells (Black and Bliska, 1997; Weidow et al., 2000). Following infection, cells were immunostained to determine the level of Yersinia uptake. Whereas Fak$^{+/+}$ cells were observed to internalize an average of 54% of attached bacteria under these conditions, only 30% were internalized by Fak$^{-/-}$ cells (Fig. 1A). The analysis of variance (ANOVA; see Materials and Methods) performed on these data indicated that the impaired uptake exhibited by Fak$^{-/-}$ cells was significant at a greater than 95% confidence level ($P<0.0001$) (Table 1). To determine if transient re-expression of Fak in Fak$^{-/-}$ cells could rescue the defect in Yersinia uptake, Fak$^{-/-}$ cells were transfected with a construct encoding wild-type Fak (Fig. 1B, lane 3) and infected approximately 42 hours later with YP17/pVector. Invasion assays showed that reconstitution of Fak into Fak$^{-/-}$ cells promoted Yersinia uptake to a level comparable to that observed in Fak$^{+/+}$ cells (Fig. 1A,C; Table 1), indicating that the defect in Yersinia uptake exhibited by these cells was caused by a lack of Fak expression. It is important to note that Fak is not strictly required for this process, however, as Fak$^{-/-}$ cells do demonstrate low levels of Yersinia uptake. Regardless, these data confirm that Fak plays an important role in Yersinia uptake.

Yersinia uptake is also dependent on Cas-Crk-Rac1 signaling (Weidow et al., 2000). To determine whether Cas

Table 1. *Y. pseudotuberculosis* uptake by Fak$^{+/+}$ and Fak$^{-/-}$ cells

Test condition	Relative uptake*	*P* value vs Fak$^{+/+}$ †
Fak$^{+/+}$	1.00	
Fak$^{-/-}$	0.52	<0.0001‡
Fak$^{-/-}$+Fak	1.00	1.0000

*Relative uptake was calculated by ANOVA by comparing data from test conditions to Fak$^{+/+}$ cells. Average uptake of Fak$^{+/+}$ cells was 54%. Each condition was tested eight times.

†*P* value from analyses comparing data from test conditions with Fak$^{+/+}$ cells.

‡Statistically significant difference at a 95% confidence level.

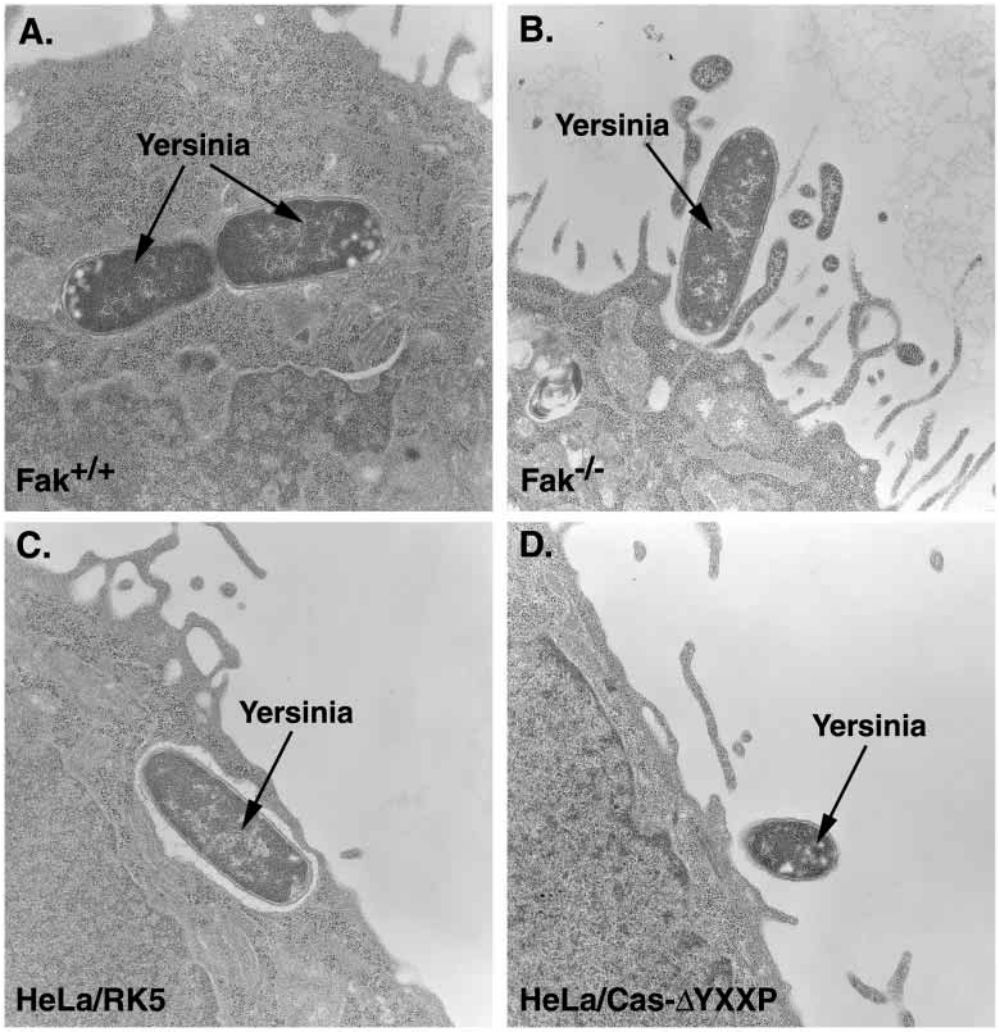

Fig. 2. The absence of functional Fak and Cas leads to similar morphological defects in Yersinia uptake. Representative transmission electron micrographs of $Fak^{+/+}$ MEFs (A), $Fak^{-/-}$ MEFs (B), HeLa/RK5 (C) or HeLa/Cas-ΔYXXP (D) cells infected with YP17/pVector.

function is specifically required for Fak-dependent Yersinia uptake, Fak was expressed in $Fak^{-/-}$ cells together with either wild-type Cas or a dominant-negative (DN) Cas molecule (Cas-ΔYXXP) that was shown to inhibit *Y. pseudotuberculosis* uptake by HeLa cells. Cells co-expressing Fak and wild-type Cas exhibited Yersinia uptake levels that were similar to those observed in Fak-expressing cells (Table 2; Group A). Statistical analysis of these data indicated that the level of uptake promoted by these cells was greater than mock-transfected cells (*P* value versus Mock; $P<0.0001$) but not different from Fak-transfected cells (*P* value versus Fak; $P=1.0000$). Co-expression of Fak and Cas-ΔYXXP also promoted Yersinia uptake, although the level of uptake was somewhat reduced compared with uptake levels observed in the presence of Fak or Fak plus wild-type Cas. This reduction may be due to the fact that ectopic Fak was expressed at slightly lower levels in cells co-transfected with Cas-ΔYXXP plasmids (data not shown). Regardless, the finding that Cas-ΔYXXP did not fully inhibit Fak-mediated uptake may suggest that functions that involve the Cas substrate-binding domain, such as Cas-Crk signaling, are not absolutely required for Yersinia uptake under these conditions.

To determine whether Fak-dependent Yersinia uptake was in fact independent of Crk, we asked whether expression

Table 2. Fak-mediated *Y. pseudotuberculosis* uptake by $Fak^{-/-}$ cells

Data group	Test condition	Relative uptake*	*P* value vs mock†	*P* value vs Fak‡
A	Mock	1.00		<0.0001§
	Fak	2.12	<0.0001§	
	Fak+Cas	2.07	<0.0001§	1.0000
	Fak+Cas-ΔYXXP	1.63	0.0006§	0.1219
B	Mock	1.00		0.0004§
	Fak	1.99	0.0004§	
	Fak+Crk	1.66	0.0073§	1.0000
	Fak+Crk-R38V	1.79	0.0044§	1.0000
	Fak+Crk-W169L	1.54	0.0426§	0.6588
C	Mock	1.00		0.0028§
	Fak	2.06	0.0028§	
	Fak+Rac-N17	0.80	1.0000	<0.0012§

*Relative uptake was calculated by ANOVA by comparing data from test conditions with mock-transfected $Fak^{-/-}$ cells. Average uptake of mock-transfected $Fak^{-/-}$ cells was 28% (A); 25% (B); 29% (C). Each condition was tested between four and 11 times.

†*P* value from analyses comparing data from test conditions with mock-transfected $Fak^{-/-}$ cells.

‡*P* value from analyses comparing data from test conditions with Fak-transfected $Fak^{-/-}$ cells.

§Statistically significant difference at a 95% confidence level.

of DN Crk molecules affected Fak-mediated uptake. Overexpression of Crk molecules containing point mutations that block the function of either the Crk SH2 (Crk-R38V) or the Crk SH3 (Crk-W169L) domain have previously been shown to inhibit uptake of *Y. pseudotuberculosis* by HeLa cells (Weidow et al., 2000). To determine whether expression of these molecules also affects Fak-mediated Yersinia uptake, invasion assays were performed in $Fak^{-/-}$ cells co-transfected with constructs encoding Fak and wild-type Crk, Crk-R38V or Crk-W169L. Fak-mediated uptake was slightly reduced in the presence of wild-type Crk compared with cells expressing Fak alone, but this decrease was not found to be statistically significant (Table 2, Group B). Similar levels of uptake were observed in cells coexpressing Fak and the mutant Crk molecules, indicating that there was no functional difference between expression of these molecules and expression of wild-type Crk. It is possible that this failure to effectively inhibit Fak-mediated uptake may have resulted from insufficient expression of the DN constructs. This does not appear to be the case, however, since similar expression levels of both Crk-R38V and Crk-W169L (data not shown) were able to effectively inhibit a second Yersinia uptake pathway in these cells (see below, Table 4). Taken together, these data suggest that Crk is not required for Fak-dependent Yersinia uptake by $Fak^{-/-}$ MEFs.

Rac1 has also been implicated in Yersinia uptake, and our previous study indicated that it functioned downstream of Cas in this process (Weidow et al., 2000; Alrutz et al., 2001; Werner et al., 2001; Wiedemann et al., 2001). To determine if Rac1 is necessary for efficient Fak-mediated uptake, $Fak^{-/-}$ cells were co-transfected with constructs encoding Fak and DN Rac1 (Rac1-N17). Expression of DN Rac1 was found to effectively block Fak-mediated Yersinia uptake (Table 2, Group C), indicating that uptake of Yersinia into Fak-expressing $Fak^{-/-}$ MEFs requires Rac1. Collectively, these data suggest that Rac1 activity is critical for Fak-dependent Yersinia uptake, but Cas-Crk interactions do not appear to play a major role in this process.

Membrane protrusive activity coincident with Yersinia uptake requires Fak and Cas

Y. pseudotuberculosis is internalized by host cells through a process that involves extension of membrane protrusions around the bacteria (Fallman et al., 1997; Isberg et al., 2000). To determine whether Fak expression is required for these cytoskeletal changes, $Fak^{+/+}$ and $Fak^{-/-}$ cells were infected with YP17/pVector and processed for electron microscopy. The majority of cell-associated Yersinia were observed to be fully engulfed by $Fak^{+/+}$ cells under the conditions of this assay (Fig. 2A). In contrast, $Fak^{-/-}$ cells exhibited short membrane protrusions near the bacteria, but full bacterial internalization was rarely observed (Fig. 2B). These data suggest that the defect in Y. *pseudotuberculosis* uptake exhibited by $Fak^{-/-}$ cells may arise from an inability to fully extend membrane protrusions at the site of bacterial adherence. Interestingly, macrophages expressing DN Rac1 exhibited a similar morphological defect in Fc-mediated phagocytosis (Massol et al., 1998), providing support for the theory that $Fak^{-/-}$ cells may be deficient for Yersinia uptake as a consequence of an inability to fully activate Rac1.

Rac1 has also been implicated in Cas-mediated uptake of *Y. pseudotuberculosis* in HeLa cells (Weidow et al., 2000). To determine whether the Cas-dependent mechanisms of Yersinia uptake might be morphologically similar to those involving Fak, HeLa cells stably expressing either vector (HeLa/RK5) or Cas-ΔYXXP (HeLa/Cas-ΔYXXP) were examined by electron microscopy following infection with YP17/pVector. Whereas numerous Yersinia were observed to be fully engulfed by HeLa/RK5 cells (Fig. 2C), cells that expressed DN Cas-ΔYXXP were rarely observed to contain fully internalized bacteria (Fig. 2D). Instead, short membrane protrusions in the vicinity of the bacteria were evident. This defect was strikingly similar to the phenotype exhibited by Fak-deficient cells, suggesting that Fak and Cas may feed into common Rac1-dependent signaling networks that regulate membrane protrusions during the course of Yersinia uptake.

Cas promotes *Y. pseudotuberculosis* uptake independently of Fak

Both Fak and Cas have the ability to independently signal to Rac1. Since Cas-Crk signaling did not appear to function downstream of Fak in $Fak^{-/-}$ fibroblasts to promote Yersinia uptake, we hypothesized that Cas might have the ability to function independently of Fak during this process. To address this hypothesis, $Fak^{-/-}$ cells were transfected with a construct encoding wild-type Cas in the absence of ectopic Fak. Interestingly, overexpression of Cas in $Fak^{-/-}$ cells reproducibly promoted Yersinia uptake to a level that was statistically greater than the level exhibited by mock-transfected cells (Table 3). Moreover, Cas-mediated uptake in $Fak^{-/-}$ cells was found to be dose-dependent (Fig. 3), suggesting that Cas may be a limiting factor for Yersinia uptake under conditions in which Fak is not expressed.

To determine whether Cas-Crk-Rac1 signaling plays a role in Fak-independent, Cas-mediated Yersinia uptake, we first asked whether phosphorylation of Cas is required for Yersinia uptake. It has been previously shown that Src kinases are primarily responsible for phosphorylation of Cas in its substrate-binding domain (Ruest et al., 2001) and that phosphorylation of this domain promotes Cas-Crk association (Sakai et al., 1994; Burnham et al., 1996). To determine whether Src kinase activity is necessary for Cas-mediated Yersinia uptake, $Fak^{-/-}$ cells were first transfected with a construct encoding wild-type Cas and then pretreated for 10

Table 3. Cas-dependent *Y. pseudotuberculosis* uptake by $Fak^{-/-}$ cells

Test condition	Relative uptake*	*P* value vs mock†
Mock	1.00	
Fak	2.05	<0.0001‡
Cas	1.79	<0.0001‡

*Relative uptake was calculated by ANOVA by comparing data from test conditions with mock-transfected $Fak^{-/-}$ cells. Average uptake of mock-transfected $Fak^{-/-}$cells was 30%. Each condition was tested 18 times.

†*P* value from analyses comparing data from test conditions with mock-transfected $Fak^{-/-}$ cells.

‡Statistically significant difference at a 95% confidence level.

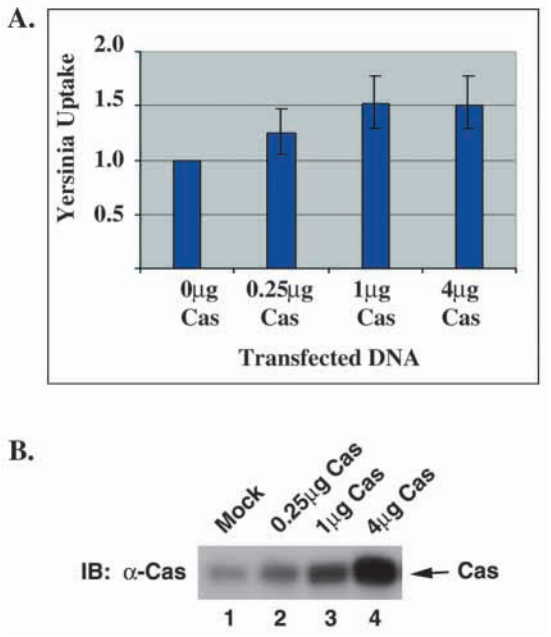

Fig. 3. Cas promotes Fak-independent *Y. pseudotuberculosis* uptake. (A) Fak$^{-/-}$ MEFs were transfected with the designated amounts of a plasmid encoding Cas. Uptake levels for each condition were compared by ANOVA to the level observed for mock-transfected cells. The data presented were obtained from 11 independent experiments. Uptake of YP17/pVector into mock-transfected cells averaged 35%. Error bars represent limits at a 95% confidence level. (B) Representative Cas immunoblot showing Cas expression in lysates from Fak$^{-/-}$ MEFs transfected with the designated amounts of DNA Cas.

Table 4. The role of Cas in Fak-independent *Y. pseudotuberculosis* uptake

Data group	Test condition	Relative uptake*	*P* value vs mock†	*P* value vs Cas‡
A	Mock	1.00		0.0113§
	Cas	2.33	0.0113§	
	Cas+10 μm PP2	0.85	1.0000	0.0027§
B	Mock	1.00		<0.0001§
	Cas	1.76	<0.0001§	
	Cas-ΔYXXP	1.41	0.0082§	0.0840
C	Mock	1.00		0.0006§
	Cas	1.64	0.0006§	
	Cas+Crk	1.65	0.0044§	1.0000
	Cas+Crk-R38V	1.14	1.0000	0.0195§
	Cas+Crk-W169L	1.05	1.0000	0.0017§
D	Mock	1.00		0.0298§
	Cas	1.57	0.0298§	
	Cas+Rac1-N17	0.69	0.0737	0.0003§
E	Mock	1.00		<0.0001§
	Cas	1.66	<0.0001§	
	Cas-ΔSH3	1.19	1.0000	0.0205§
F	Mock	1.00		0.0009§
	Cas	1.55	0.0009§	
	Cas+PRNK	1.11	1.0000	0.0163§

*Relative uptake was calculated by ANOVA by comparing data from test conditions with mock-transfected Fak$^{-/-}$. Average uptake of mock-transfected Fak$^{-/-}$ cells 26% (A); 33% (B); 37% (C); 31% (D); 26% (E); and 28% (F). Each condition was tested four to eight times.

†*P* value from analyses comparing data from test conditions with mock-transfected Fak$^{-/-}$ cells.

‡*P* value from analyses comparing data from test conditions with Cas-transfected Fak$^{-/-}$ cells.

§Statistically significant difference at a 95% confidence level.

minutes with a Src-family kinase inhibitor (PP2) (Hanke et al., 1996; Zhu et al., 1999) prior to infection with YP17/pVector. PP2 pretreatment of Cas-transfected cells resulted in a complete inhibition of uptake relative to the uptake levels exhibited by untreated, Cas-transfected, cells (Table 4, Group A). These data suggest that the activity of a PP2-sensitive kinase, most probably a Src-family kinase, is necessary for Cas to promote Yersinia uptake.

Next we investigated whether the substrate-binding domain was necessary for Cas-mediated uptake in the absence of Fak. Fak$^{-/-}$ cells were transfected with a plasmid encoding the Cas derivative that is deleted for this domain, Cas-ΔYXXP. Invasion assays performed on these cells showed that expression of Cas-ΔYXXP promoted uptake above the level observed in mock-transfected cells (Table 4, Group B; P=0.0082), although not to the level of cells expressing wild-type Cas. These data indicate that signaling via the substrate-binding domain of Cas is not absolutely required for Fak-independent, Cas-dependent Yersinia uptake.

Two observations led us to investigate whether Crk functioned in this Cas-dependent process. First, expression of Cas-ΔYXXP in Fak$^{-/-}$ MEFs did not promote Yersinia uptake to the same extent as wild-type Cas (see above). Second, expression of DN Crk molecules inhibits Yersinia uptake by HeLa cells (Weidow et al., 2000). Fak$^{-/-}$ cells were co-transfected with constructs encoding Cas and either wild-type or DN variants of Crk. Co-expression of Cas and Crk promoted uptake to a level that was similar to the level of uptake observed when Cas alone was expressed (Table 4, Group C). In contrast, co-expression of Cas with either Crk-R38V or Crk-W169L resulted in a significant decrease in Cas-dependent Yersinia uptake. In fact, the level of uptake observed in these cells was no different from that observed in mock-transfected cells (P=1.0000). The inhibitory effect of these DN Crk molecules appeared to be specific for Cas because expression of these same molecules at equivalent levels (data not shown) failed to significantly inhibit Fak-dependent Yersinia uptake (see Table 2, Group B). These data suggest that at least one component of the Cas-dependent Yersinia uptake pathway in Fak$^{-/-}$ cells may involve Crk.

As Rac1 functions downstream of Cas-Crk in HeLa cells during the process of Yersinia uptake (Weidow et al., 2000), we also investigated whether DN Rac1 could inhibit Cas-dependent Yersinia uptake in Fak$^{-/-}$ cells. Yersinia uptake was significantly impaired in cells co-transfected with plasmids encoding Cas and Rac1-N17 (Table 4, Group D). Collectively, these data indicate that the Cas-Crk-Rac1 pathway, which has previously been shown to function in HeLa cells to promote Yersinia uptake, can also function independently of Fak in MEFs to promote uptake of *Y. pseudotuberculosis*. However, because inhibition of Crk or Rac1, but not Cas-ΔYXXP, completely blocked Cas-mediated uptake, these data suggest that Crk and Rac1 may also play other roles in this process that are independent of the substrate-binding domain of Cas.

Cas and Pyk2 coordinately function to promote Fak-independent *Y. pseudotuberculosis* uptake

Cas can signal to Rac1 through mechanisms that are independent of Crk binding to the substrate-binding domain. Many of these involve the Cas SH3 domain. To determine whether this domain is required for Cas-dependent *Y. pseudotuberculosis* uptake, Fak$^{-/-}$ cells were transfected with a Cas construct in which the SH3 domain was deleted (Cas-ΔSH3). Overexpression of Cas-ΔSH3 failed to promote uptake above the level exhibited by mock-transfected cells (Table 4, Group E), suggesting that functions of Cas that involve its SH3 domain play a critical role in Cas-mediated Yersinia uptake in the absence of Fak.

One protein that binds to the Cas SH3 domain is Pyk2, and Cas/Pyk2 complexes have been detected in Fak$^{-/-}$ cells (Ueki et al., 1998) (data not shown). Consequently, we investigated whether the ability of Cas to promote *Y. pseudotuberculosis* uptake in the absence of Fak requires Pyk2 function. Fak$^{-/-}$ cells were co-transfected with constructs encoding Cas and a C-terminal derivative of Pyk2, termed PRNK, that can potentially serve as an inhibitor of Pyk2 (Xiong et al., 1998). PRNK expression did in fact reduce Cas-mediated uptake to a level that was similar to that exhibited by mock-transfected cells (Table 4, Group F). These data suggest that Pyk2 function may be required for Cas to promote *Y. pseudotuberculosis* in the absence of Fak.

Pyk2 functions in Yersinia uptake by macrophages

Although Fak$^{-/-}$ cells are a useful tool to investigate Fak-independent signaling pathways, most other fibroblasts do not express significant levels of Pyk2 (Sieg et al., 1998). By contrast, macrophages naturally express Pyk2 at levels comparable to Fak$^{-/-}$ MEFs (data not shown), and these cells play an important role in clearing *Y. pseudotuberculosis* infections in vivo. Therefore, we investigated whether Pyk2 also plays a role in Yersinia uptake in macrophages. J774A.1 macrophages were first transfected with a construct encoding PRNK and then infected with YP17/pVector to measure Yersinia uptake. Although mock-transfected macrophages internalized approximately 53% of attached Yersinia, cells expressing PRNK only internalized an average of 37%. This decrease in Yersinia uptake was shown to be statistically significant (Table 5; P=0.0012). These data suggest that, in addition to playing a role in Cas-dependent Yersinia uptake in Fak$^{-/-}$ MEFs, Pyk2 function may also be required to promote *Y. pseudotuberculosis* uptake by macrophages.

Table 5. The role of Pyk2 in *Y. pseudotuberculosis* uptake in J774A.1 macrophages

Test condition	Relative uptake*	*P* value vs mock†
Mock	1.00	
PRNK	0.69	0.0012‡

*Relative uptake was calculated by ANOVA by comparing data from test conditions to mock-transfected J774A.1 cells. Average uptake of mock-transfected J774A.1 cells was 53%. Each condition was tested eight times.

†*P* value from analyses comparing data from test conditions with mock-transfected J774A.1 cells.

‡Statistically significant difference at a 95% confidence level.

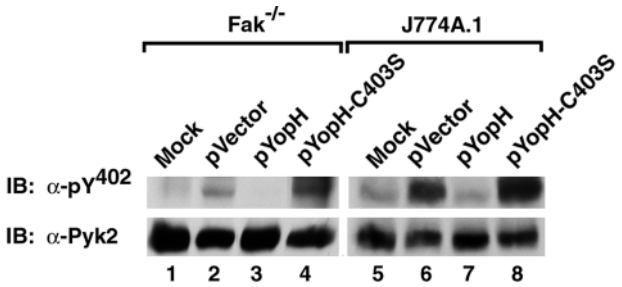

Fig. 4. *Y. pseudotuberculosis* induces Pyk2 autophosphorylation. Representative immunoblots containing lysates from Fak$^{-/-}$ MEFs (lanes 1-4) or J774A.1 macrophages (lanes 5-8) following infection with the indicated strains of *Y. pseudotuberculosis*. Lysates were immunoblotted for phospho-Y^{402} (upper panel) or Pyk2 (lower panel).

To explore the mechanism of Pyk2 function in Yersinia uptake, we investigated whether Pyk2 underwent changes in autophosphorylation in response to infection. Fak$^{-/-}$ cells (Fig. 4, lanes 1-4) and macrophages (lanes 5-8) were infected with various Yersinia strains, and lysates were immunoblotted with activation-specific antibodies directed against the autophosphorylation site of Pyk2, tyrosine 402 (Y^{402}). Infection with YP17/pVector resulted in increased levels of Y^{402} phosphorylation in both cell types, although the increase observed in Fak$^{-/-}$ cells was reproducibly lower than that observed in macrophages (upper panels). Infection with YP17/pYopH resulted in decreased levels of Y^{402} phosphorylation, whereas infection with YP17/pYopH-C403S (a *Y. pseudotuberculosis* strain expressing a catalytically-inactive, substrate-trapping mutant of YopH) augmented Y^{402} phosphorylation. Immunoblotting for Pyk2 indicated that equivalent levels of Pyk2 were present in each case (lower panels). Thus, infection of both Fak$^{-/-}$ MEFs and macrophages with *Y. pseudotuberculosis* was found to induce Pyk2 autophosphorylation. The finding that this site can also serve as a substrate for the antiphagocytic effector YopH further supports a role for Pyk2 activation in the process of Yersinia uptake.

Discussion

The establishment of a productive *Y. pseudotuberculosis* infection in humans relies on its ability to translocate across the intestinal epithelium and evade phagocytosis by immune cells that reside in the sub-epithelial lymphoid tissue. Yersinia uptake by host cells serves as an in vitro model for exploring how host cells respond to Yersinia adherence. In this study, we provide insight into the molecular nature and regulation of signaling networks that contribute to the uptake process. Using a reconstitution approach in Fak$^{-/-}$ fibroblasts, we have been able to specifically address the interplay between Fak, Cas and Pyk2 in this process.

Earlier studies measuring uptake of invasin-coated beads into Fak$^{-/-}$ MEFs and *Y. pseudotuberculosis* into HeLa cells indicated that both Fak and Cas may play a role in Yersinia uptake (Alrutz and Isberg, 1998; Weidow et al., 2000). Our data extend these initial reports by providing evidence that Fak$^{-/-}$ MEFs are defective in uptake of live *Y. pseudotuberculosis* and that this defect can be rescued by ectopic expression of Fak.

This Fak-dependent uptake pathway requires Rac1, but Cas-Crk signaling appears to be dispensable.

Surprisingly, ectopic expression of Cas was also found to rescue the defect in Yersinia uptake exhibited by $Fak^{-/-}$ cells. This is one of the first examples of Cas functioning independently of Fak in a biological process. Although the mechanism through which ectopic Cas promotes uptake is not known, endogenous levels of Cas are clearly not sufficient to promote Yersinia uptake in the absence of Fak, since $Fak^{-/-}$ cells are defective in this process even though they express the same amount of Cas as do $Fak^{+/+}$ MEFs (data not shown). This suggests that ectopic Cas drives Yersinia uptake either by supplementing the function of limiting amounts of endogenous Cas or by activating novel pathway(s) that are independent of the function of endogenous Cas. Although it is difficult to distinguish between these two possibilities, the finding that ectopic Cas promoted uptake in a dose-dependent fashion lends support for the theory that Cas may be a limiting factor in this Fak-independent Yersinia uptake pathway.

Investigation of the molecular requirements of Cas-dependent Yersinia uptake in $Fak^{-/-}$ cells showed that multiple Cas-binding proteins play a role in this process. The first of these molecules, Src, is implicated through studies using the PTK inhibitor PP2. Pretreatment of cells with PP2 abolished Cas-dependent Yersinia uptake, indicating that Src family kinases are required. The second Cas-binding protein that is implicated in this pathway is the small adapter molecule Crk, which binds to sites within the substrate-binding domain of Cas that become phosphorylated by Src (Sakai et al., 1994; Ruest et al., 2001). We have previously shown that Yersinia infection induces Cas-Crk complexes in cells that express endogenous Fak and that signaling downstream of Crk is critical for Yersinia uptake by these cells (Weidow et al., 2000). The finding that expression of DN Crk molecules blocked Cas-dependent Yersinia uptake in $Fak^{-/-}$ MEFs indicates that Cas-Crk signaling may also play a role in this process when Fak is not expressed.

Cas-Crk signaling has been implicated in a number of integrin-dependent processes, including cell migration and Yersinia uptake (Klemke et al., 1998; Cheresh et al., 1999; Cho and Klemke, 2000; Weidow et al., 2000). In these cases, the binding of Crk to Cas is thought to result in activation of the small GTPase Rac1 through recruitment of Crk-binding proteins such as DOCK180 and C3G (for review, see Feller, 2001). Our data are consistent with the involvement of Rac1 in Cas-dependent Yersinia uptake since expression of a DN Rac1 mutant effectively blocked this process. Thus, the Cas-Crk-Rac1 cascade, which has previously been shown to promote Yersinia uptake in cells that express Fak, appears to also function in the absence of Fak.

The role of Crk in Fak-dependent Yersinia uptake pathways is less clear, as expression of DN Crk constructs at levels equivalent to those that blocked Cas-dependent uptake failed to inhibit Fak-mediated uptake by $Fak^{-/-}$ MEFs. In spite of the apparent divergence in Fak- and Cas-dependent Yersinia uptake processes with respect to the requirement for Crk, however, both pathways appear to ultimately lead to Rac1 activation. Visualization of the Yersinia uptake process by electron microscopy in cells either lacking Fak or expressing a DN inhibitor of Cas indicated that both cell types exhibit a common morphological defect in uptake. We propose that it is the activation of Rac1, through distinct Fak- and Cas-dependent pathways, that leads to the establishment of membrane protrusions involved in bacterial internalization. Reports showing that Fak can activate Rac1 independently of Cas-Crk are consistent with this hypothesis (Cary and Guan, 1999; Schlaepfer et al., 1999; Hauck et al., 2000). In those cases where Fak or Cas signaling is compromised, the resultant decrease in Rac1 activation is likely to lead to decreased membrane protrusive activity and inhibition of Yersinia uptake. In this regard, Yersinia uptake appears to be similar to the process of Fc-mediated phagocytosis in macrophages, where expression of DN Rac1 correlates with a failure to fully extend and fuse membrane protrusions as well as an inhibition of particle uptake (Massol et al., 1998).

In addition to Cas-Crk-Rac signaling networks, we present evidence that Pyk2 plays a role in Yersinia uptake in both $Fak^{-/-}$ cells that express aberrantly high levels of Pyk2 and in macrophages that express both Fak and Pyk2. The full-length C-terminus of Pyk2, PRNK, has been shown to inhibit various functions of Pyk2 (Ivankovic-Dikic et al., 2000; Watson et al., 2001). In this study, we show that a shorter variant of PRNK (residues 680-1009) has inhibitory activity with respect to Cas-dependent Yersinia uptake in $Fak^{-/-}$ cells. This inhibition by PRNK may occur via several distinct mechanisms. For example, PRNK may compete with Pyk2 for localization to critical subcellular compartments or it may sequester Pyk2 binding partners. However, direct interactions between PRNK and Cas may not be involved, since the PRNK construct used in this study contains only a low affinity Cas-binding motif and it therefore may not associate with Cas at a level sufficient to inhibit Cas signaling (Xiong et al., 1998). In any case, PRNK-mediated displacement of Pyk2 away from functional signaling complexes could ultimately lead to an inhibition of Yersinia uptake.

The requirement for Pyk2 in the process of Yersinia uptake appears to be a common feature of $Fak^{-/-}$ cells and macrophages. In both cell types, Pyk2 autophosphorylation becomes elevated upon Yersinia infection with strains that do not express YopH, and YopH efficiently dephosphorylated this site. Interestingly, the level of induction of Pyk2 autophosphorylation appeared to be less pronounced in $Fak^{-/-}$ MEFs than in macrophages. Pyk2 localizes to focal adhesions and becomes activated in response to adhesive signals in $Fak^{-/-}$ cells (Du et al., 2001). This adhesion-dependent activation may have functional consequences for the process of Yersinia uptake by these cells, perhaps by providing a sub-threshold level of Pyk2 activity that can synergize with overexpressed Cas to promote uptake. The downstream effects of Pyk2 phosphorylation in $Fak^{-/-}$ cells probably include recruitment of Src family kinases to the autophosphorylation site, activation of Src and subsequent phosphorylation of other Src- and/or Pyk2-binding proteins such as Cas. We propose that, at least in $Fak^{-/-}$ cells, phosphorylation of Cas through this mechanism creates Crk-binding sites. Subsequent recruitment of Crk and perhaps DOCK180 to the complex can then lead to activation of Rac1 and uptake of *Y. pseudotuberculosis*. It remains to be determined whether this Pyk2-Cas pathway also functions in macrophages or whether it is only active when Fak is not expressed.

Although a Pyk2-Cas-Crk pathway may be responsible for a portion of the Yersinia uptake observed in $Fak^{-/-}$ cells, there

are indications that other Cas-dependent pathways also contribute to this process. The finding that Cas-ΔYXXP was able to promote Fak-independent Yersinia uptake, albeit to a lesser extent than wild-type Cas, indicates that functions independent of the substrate-binding domain of Cas are involved. Evidence suggests that these functions are predominantly associated with the Cas SH3 domain because Cas molecules that do not contain this domain were found to be completely deficient in promoting Yersinia uptake. Numerous proteins in addition to Pyk2 bind to the SH3 domain of Cas, including Fak, PTP-PEST, PTP-1B and C3G (for a review, see O'Neill et al., 2000; Bouton et al., 2001). Thus, although phosphorylation of Cas and subsequent binding to Crk may provide a positive signal for uptake, dephosphorylation of Cas by PTP-PEST or PTP-1B may serve to downregulate this pathway. C3G may also play a central role in this process. This protein, which is a guanine nucleotide exchange factor (GEF) for Rap1, was initially found to associate with Cas in a yeast two-hybrid screen (Kirsch et al., 1998). In yeast, the orthologue of Rap1 has been shown to indirectly activate Cdc42, which can then activate Rac1 (Bos, 1998; Bos et al., 2001). It is tempting to speculate, therefore, that C3G binding to the SH3 domain of Cas could lead to activation of Rap1 in mammalian cells, ultimately resulting in activation of Cdc42 and/or Rac1. This pathway could account for how Cas-ΔYXXP may be competent to promote Yersinia uptake even though it cannot signal to Crk, because Cas-ΔYXXP/C3G complexes could still signal to Cdc42 and/or Rac1. It could also explain why Cas-ΔSH3 is completely deficient in promoting uptake because deletion of the SH3 domain would block both the C3G-Cas and Pyk2-Cas-Crk pathways that lead to Cdc42 and/or Rac1 activation.

Although we have not directly addressed the specific requirements for invasin–β1-integrin association in the Yersinia uptake pathways defined above, it is well established that these interactions serve as the predominant initiator of Yersinia uptake into host cells (Rosqvist et al., 1988b; Isberg, 1989; Marra and Isberg, 1997). Many of the molecular features of Yersinia uptake that have been revealed in this study are common to other integrin-dependent processes such as cell adhesion and migration, as well as Fc-mediated phagocytosis (May and Machesky, 2001). In addition to these common elements, there are also likely to be aspects of Yersinia uptake that are distinct from other integrin-mediated events. One molecule that may function in this manner in Fak$^{-/-}$ cells is Pyk2, which seems to link β1 integrin signals initiated by Yersinia attachment to Src, Cas, Crk and Rac1. The mechanism of activation of Pyk2 by β1 integrins is unclear, particularly because Pyk2 does not contain the N-terminal sequences present in Fak that have been shown to bind to β1 integrin tails (Schaller et al., 1995; Klingbeil et al., 2001). However, in Fak$^{-/-}$ cells, Pyk2 can localize to focal adhesions (Du et al., 2001). This may occur through its association with paxillin (Hiregowdara et al., 1997), which is capable of binding to both β_1 and α_4 integrins (Schaller and Parsons, 1995; Liu et al., 1999; Liu and Ginsberg, 2000). Focal adhesion localization of Pyk2 under these conditions may be important for the Yersinia-dependent activation of Pyk2, as well as propagation of the Pyk2-Cas pathway that promotes Yersinia uptake in the absence of Fak. The mechanism of Pyk2 activation during the course of Yersinia uptake into macrophages, which also express Fak, is even less clear. However, this process may also involve β1 integrin ligation, as it has been previously shown that Pyk2 can become activated upon cell adhesion in cells that express Fak (Lakkakorpi and Vaananen, 1996; Hiregowdara et al., 1997; Ma et al., 1997; Brinson et al., 1998; Hatch et al., 1998; Li et al., 1998; Litvak et al., 2000; Du et al., 2001; Watson et al., 2001).

Several important questions regarding the role of Pyk2 in the process of Yersinia uptake into macrophages must now be addressed. First, the molecular mechanisms by which Pyk2 activation leads to Yersinia uptake remain to be elucidated. Within the framework of this question, it remains to be determined whether Cas and Pyk2 function in concert to promote Yersinia uptake by macrophages. If so, what are the roles of Cas-binding partners such as Crk and C3G? Finally, it is important to determine whether Fak and Pyk2 play independent, redundant or competing roles in this process, since both molecules are expressed in these cells. These questions form the basis for future studies designed to address the molecular mechanisms involved in Yersinia uptake by macrophages.

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