Cellular invasion by *Staphylococcus aureus* reveals a functional link between focal adhesion kinase and cortactin in integrin-mediated internalisation

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Summary

Nosocomial infections by *Staphylococcus aureus*, a Gram-positive pathogen colonising human skin and mucosal surfaces, are an increasing health care problem. Clinical isolates almost invariably express fibronectin-binding proteins that, by indirectly linking the bacteria with host integrin $\alpha_5\beta_1$, can promote uptake of the microorganisms by eukaryotic cells. Integrin engagement by pathogenic fibronectin-binding *S. aureus*, but not by non-pathogenic *S. carnosus*, triggered the recruitment of focal contact-associated proteins vinculin, tensin, zyxin and FAK to the sites of bacterial attachment. Moreover, dominant-negative versions of FAK-blocked integrin-mediated internalisation and FAK-deficient cells were severely impaired in their ability to internalise *S. aureus*. Pathogen binding induced tyrosine phosphorylation of several host proteins associated with bacterial attachment sites, including FAK and the Src substrate cortactin. In FAK-deficient cells, local recruitment of cortactin still occurred, whereas the integrin- and Src-dependent tyrosine phosphorylation of cortactin was abolished. As siRNA-mediated gene silencing of cortactin or mutation of critical amino acid residues within cortactin interfered with uptake of *S. aureus*, our results reveal a novel functional connection between integrin engagement, FAK activation and Src-mediated cortactin phosphorylation. Cooperation between FAK, Src and cortactin in integrin-mediated internalisation of bacteria also suggests a molecular scenario of how engagement of integrins could be coupled to membrane endocytosis.

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Introduction

Integrins are heterodimeric surface receptors of multicellular organisms critically involved in cell-cell and cell-matrix adhesion. In addition to their structural role, integrins transduce vital extracellular information such as cell attachment, matrix composition and matrix rigidity into the cell (Hynes, 2002). More than 20 different integrin heterodimers have been described, with overlapping subunit composition and often redundant binding specificity (van der Flier and Sonnenberg, 2001). Nevertheless, compromising single subunits, such as deleting integrin β1 from the mouse genome, has demonstrated that particular integrins can serve essential functions (Fassler and Meyer, 1995; Hynes, 1996). It is therefore not surprising that these surface-exposed and structurally conserved proteins are exploited by a large number of microbial pathogens to contact host cells and tissues (Hauck, 2002; van der Flier and Sonnenberg, 2001). Prominent examples include the enteropathogenic bacteria *Yersinia enterocolitica* and *Y. pseudotuberculosis* that express the outer membrane protein invasin, a protein with high affinity for the integrin β1 subunit (Isberg and Barnes, 2001). In contrast to the direct integrin-binding of invasin-expressing *Y. enterocolitica*, several other microorganisms are able to indirectly engage integrins. Microbes such as the Gram-positive *Staphylococcus aureus* or *Streptococcus pyogenes* have evolved surface structures that bind to various extracellular matrix (ECM) proteins (Schwarz-Linek et al., 2004) and that have been collectively termed MSCRAMMs (microbial surface components recognising adhesive matrix molecules) (Foster and Hook, 1998; Patti et al., 1994). MSCRAMMs commonly expressed by disease isolates of *S. aureus* are the cell-wall-attached fibronectin-binding proteins A and B (FnBP-A and FnBP-B) that confer a tight association of the bacteria with the ECM protein fibronectin (Fn) (Flock et al., 1987; Jonsson et al., 1991). Fn recruited to the surface of the bacteria can then serve as a molecular bridge linking FnBP-expressing *S. aureus* with a common host Fn receptor, the integrin $\alpha_5\beta_1$ (Joh et al., 1999). As is the case with invasin-expressing *Y. enterocolitica*, FnBP-mediated engagement of integrin β1 readily initiates the clustering of integrins and the internalisation of the bacteria into host cells in vitro (Agerer et al., 2003; Fowler et al., 2000; Sinha et al., 2000; Sinha et al., 1999). Though Fn-binding variants of *S. aureus* predominate in clinical isolates (Peacock et al., 2000), the role of the *S. aureus*-FnBP-Fn-integrin

interaction in the infection process in vivo is not clear-cut (Menzies, 2003). For example, an *S. aureus* mutant with low Fn-binding capacity is attenuated in a rat model of endocarditis (Kuypers and Proctor, 1989), whereas a FnBP-A/FnBP-B double mutant is not impaired in its ability to colonise rat heart valves upon haematogenous spread (Flock et al., 1996). Co-administration of a recombinant, Fn-binding domain of FnBP-A, which blocks *S. aureus* invasion in vitro, inhibits abscess formation in a guinea pig model of wound infection (Menzies et al., 2002). In addition, the expression of FnBPs dramatically enhances the capacity of *S aureus* to colonise the mammary gland of mice and to invade mammary epithelial cells in vivo (Brouillette et al., 2003). Besides a potential role in such acute disease settings, it is likely that the FnBP-mediated invasion by *S. aureus* into eukaryotic cells contributes to the persistence of the microorganism in its host (Menzies, 2003).

How the binding and clustering of integrins by Fn-coated bacteria is translated into an uptake signal is not clear, as β1-containing integrins usually operate in the context of immobilised ECM proteins (van der Flier and Sonnenberg, 2001). Clustering of integrins upon cell adhesion to immobilised ECM proteins is known to trigger the assembly of characteristic protein complexes on the intracellular side of the plasma membrane (Miyamoto et al., 1995a; Miyamoto et al., 1995b; Zaidel-Bar et al., 2003; Zamir and Geiger, 2001). Proteins enriched at integrin-rich focal contact sites include structural components such as talin, vinculin, paxillin, tensin, α-actinin or zyxin, as well as signalling enzymes such as the focal adhesion kinase (FAK), phosphatidylinositol phosphate kinase type1γ, the integrin-linked kinase and Src family kinases that together orchestrate the dynamic linkage between clustered integrins and the actin cytoskeleton (Ling et al., 2002; Zamir and Geiger, 2001). The assembly and disassembly of integrin-associated protein complexes, guided by lipid and protein phosphorylation, are thought to be the main regulatory mechanisms controlling the turnover of focal contacts (Geiger et al., 2001). For example, the protein tyrosine kinase (PTK) FAK is one of the key enzymes highly activated upon integrin-mediated stimulation of cells (for review see Schlaepfer et al., 2004). FAK activation results in autophosphorylation of this protein at tyrosine residue 397 (Y-397) generating a high affinity binding site for the cytoplasmic PTK c-Src. Active FAK and Src PTKs are responsible for the phosphorylation of integrin- or actin-associated proteins such as paxillin, α-actinin and $p130^{CAS}$. Interfering with the activity of the FAK-Src complex impairs cell motility in multiple adherent cell types suggesting that both enzymes are critical for focal contact turnover. Interestingly, integrin β1-mediated internalisation of *S. aureus* depends on Src PTK activity, indicating that turnover of integrin-associated protein complexes is critical for bacterial uptake (Agerer et al., 2003; Fowler et al., 2003). However, it is unclear if the FnBP-triggered uptake of *S. aureus* requires additional structural or signalling components of integrin-rich focal contact sites and which substrates of active Src PTKs might be important for the internalisation process.

Here we show that Fn-binding *S. aureus*, but not *S. carnosus* trigger the recruitment of focal-contact-associated proteins tensin, vinculin, zyxin and FAK. FAK is critical for integrin-mediated internalisation of *S. aureus*, as dominant-negative versions of this PTK block uptake, and FAK-deficient cells are severely impaired in their ability to internalise *S. aureus*. Bacterial binding to integrins also induces enhanced tyrosine phosphorylation of cellular proteins at *S. aureus* contact sites including FAK and the Src substrate cortactin. Importantly, stimulation of cellular integrin $\alpha_5\beta_1$ by either pathogenic *S. aureus* or the physiological ligand fibronectin reveals that the integrin-dependent tyrosine phosphorylation of cortactin is abolished in the absence of FAK. Mutation of critical cortactin amino acids such as tyrosine phosphorylation sites or siRNA-mediated gene silencing of cortactin interfere with uptake of *S. aureus*. Since inhibition of Src family PTKs blocks pathogen-triggered cortactin phosphorylation, our results reveal a novel functional connection between integrin engagement, FAK and Src activation and phosphorylation of cortactin that regulates integrin-mediated internalisation of bacteria.

Materials and Methods

Bacteria

Staphylococcus aureus (Cowan) and non-pathogenic *S. carnosus* TM300 were cultured in tryptic soybean broth (TSB; BD Biosciences, Heidelberg, Germany) and harvested in mid-logarithmic phase. Prior to infection, bacteria were washed once in sterile phosphate-buffered saline (PBS) and adjusted to 1×10^8 cfu/ml in PBS. In some experiments, staphylococci were fluorescently labelled prior to infection as described previously (Agerer et al., 2003; Agerer et al., 2004).

Cell culture and replating assays

The human embryonic kidney cell line 293T (293 cells) was grown in Dulbecco's modified Eagle's medium (DMEM)/10% calf serum (CS) at 37°C, 5% CO_2. Cells were subcultured every 2-3 days. A day prior to infection, 2×10^5 cells/well (gentamicin/lysostaphin protection assay) or 1×10^5 cells/well (immunofluorescence staining) were seeded in poly-L-lysine coated (10 μg/ml) 24-well plates.

Fibroblasts derived from FAK knockout mouse embryos (FAK(–) cells) and FAK re-expressing cells (FAK(+) cells; DA2 cells) (Sieg et al., 1999) were cultured in DMEM/10% fetal calf serum (FCS) supplemented with non-essential amino acids on gelatine-coated (0.1% in PBS) cell culture dishes. Cells were subcultured every 3-4 days. A day prior to infection, 6×10^4 cells (gentamicin/lysostaphin protection assay) or 2×10^4 cells (immunofluorescence staining) were seeded in poly-L-lysine-coated (10 μg/ml) 24-well plates. For biochemical analysis and fibronectin replating, fibroblasts were serum-starved for 20 hours in DMEM containing 0.5% CS. Cells were suspended by limited trypsin/EDTA treatment (0.05% trypsin/2 mM EDTA) and trypsin was inactivated with DMEM containing 0.5 mg/ml soybean trypsin inhibitor. Cells were pelleted, taken up in 0.1% bovine serum albumin in DMEM (suspension medium) and kept in suspension for 45 minutes at 37°C. Then, 1×10^6 cells in suspension medium were seeded in poly-L-lysine-coated (10 μg/ml) or fibronectin-coated (4 μg/ml) 6 cm dishes for 1 hour prior to infection or lysis.

Reagents and antibodies

Cytochalasin D, jasplakinolide, latrunculin B and PP2 were obtained from Calbiochem (Bad Soden, Germany). Bovine fibronectin was from ICN Biomedicals (Eschwege, Germany), NHS-LC-Biotin from Perbio Science (Rockford, IL). Monoclonal antibody (mAb) against phosphotyrosine (clone 4G10) was purchased from Upstate (Lake Placid, NY), mAb against FLAG-tag (clone M2), mAb against β-actin (clone AC-74) and mAb against desmin (clone DE-U-10) from Sigma-Aldrich (Sigma, St Louis, MO), mAb against GFP (clone JL-

8) from BD Biosciences (Heidelberg, Germany), mAb against HA-tag (clone 12CA5) was purified from hybridoma supernatants. Polyclonal antibodies against cortactin (H-191) and Src PTKs (Src-2) were from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies and streptavidin-Cy5 were from Jackson ImmunoResearch (West Grove, PA).

Recombinant DNA constructs and transfection of cells

Expression constructs encoding HA-tagged kinase-inactive mouse FAK (FAK K454M), FAK Y397F, FAK Pro- and GFP-FRNK were as described previously (Sieg et al., 1999). Flag-tagged cortactin WT (wild type), and the respective mutants were kindly provided by Scott Weed (University of Colorado, Denver, CO), cortactin-RFP was from Matthias Selbach (MPI für Infektionsbiologie, Berlin, Germany), FAK-GFP was from Dusko Ilic (UCSF, San Francisco, CA), GFP-zyxin was from J. Wehland (GBF, Braunschweig, Germany), GFP-vinculin and GFP-tensin were provided by Alexander Bershadsky and Benjamin Geiger, respectively (Weizmann Institute, Tel Aviv, Israel), GFP-actin was purchased from BD Biosciences. 293 cells and fibroblast were transfected with plasmids as described previously (Agerer et al., 2003).

For siRNA-mediated gene silencing, a double-stranded (ds) RNA oligonucleotide directed against human cortactin (sense strand: 5′-CATACACAAGCTGAGGGAGdTdT-3′) or a control dsRNA oligonucleotide directed against firefly luciferase (sense strand: 5′-CUUACGCUGAGUACUUCGAdTdT-3′) were obtained from EURIT (Berlin, Germany). 6×10^4 293 cells in 24-well plates were transfected with 2.5 µl of 20 µM dsRNA oligonucleotide using oligofectamine according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Cells were either lysed or used in gentamicin/lysostaphin protection assays 24 hours after oligofectamine transfection.

Infection experiments and gentamicin/lysostaphin protection assay

293 cells and fibroblasts were infected with bacteria at a multiplicity of infection (MOI) of 20. After the indicated times, the culture medium was replaced by DMEM containing 50 µg/ml gentamicin and 20 µg/ml lysostaphin. After 45 minutes incubation at 37°C, cells were washed once with PBS and intracellular bacteria released by incubation in 1% saponin in PBS for 20 minutes at 37°C. Samples were diluted in PBS and plated on TSB agar plates for determination of the recovered colony forming units. In inhibition experiments, pharmacological inhibitors were added to the cells 15 minutes before infection. For biochemical analysis, fibroblasts on poly-L-lysine-coated 6 cm dishes were either left uninfected or were infected with a MOI of 50 with *S. aureus* or *S. carnosus* 10 minutes prior to lysis, cells were treated with 200 µl pervanadate solution (5 mM sodium ortho-vanadate and 0.6% H_2O_2 in water).

Fluorescence staining and microscopy

293 cells and fibroblasts were seeded on acid-washed glass coverslips in 24-well plates and infected with fluorescently labelled staphylococci at a MOI of 10. After infection, cells were washed once with PBS and fixed with 4% paraformaldehyde for 20 minutes at room temperature. For detecting tyrosine-phosphorylated proteins, fixed samples were washed three times with PBS and permeabilised for 5 minutes in PBS, 10% FCS and 0.2% saponin (blocking buffer). Samples were then incubated with monoclonal mouse-α-phosphotyrosine (1:200 of clone 4G10) in blocking buffer. Samples were washed twice with PBS, blocked again for 5 minutes and incubated with Cy3-conjugated goat-α-mouse (1:100 in blocking buffer) for 45 minutes at room temperature. After three washes with PBS, the coverslips were mounted in embedding medium (DaKo, Glastrup, DK) on glass slides and sealed with nail polish. For differentiation between extra- and intracellular bacteria, cells were infected with FITC- and biotin-labelled bacteria essentially as described previously (Agerer et al., 2004). Following infection and fixation, samples were blocked with PBS/10% CS without cell permeabilization and probed with a 1:200 dilution of streptavidin-Cy5 (Molecular Probes, Eugene, OR). Therefore streptavidin-Cy5 reached only extracellular bacteria, resulting in FITC-labelled intracellular and FITC/Cy5-labelled extracellular bacteria.

Time lapse and confocal microscopy

For time-lapse microscopy, 293 cells transfected with GFP-actin were seeded in glass-bottomed chamber slides (Nalge Nunc, Naperville, IL). 24 hours later, cells were infected with rhodamine-labelled *S. aureus* (Cowan) at a MOI of 20. Living and fixed samples were viewed with a LSM510 confocal laser scanning microscope (Zeiss, Oberkochen, Germany). Live samples were incubated in a temperature-controlled chamber at 37°C and GFP and rhodamine signals were recorded at 10-second intervals. For double- and triple-labelled fixed specimens the signals of the fluorescent dyes were serially recorded to avoid bleed-through due to simultaneous excitation. The corresponding images were digitally processed with Photoshop 6 (Adobe Systems, Mountain View, CA) and merged to yield pseudocoloured RGB pictures.

Cell lysis, immunoprecipitation, and western blotting

At the indicated times, cells were washed once with ice-cold PBS and lysed in modified RIPA buffer (25 mM Hepes, pH 7.4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 150 mM NaCl, 20 mM $MgCl_2$, 10% glycerol, 10 mM sodium pyrophosphate, 10 mM p-nitrophenyl phosphate, 100 mM NaF, 1 mM Na_3VO_4, and 10 µg/ml of each aprotinin, leupeptin, pefabloc and pepstatin). Equivalent amounts of the cleared lysates were either added to an equal volume of reducing 2× SDS sample buffer (whole cell lysate; WCL) or used for immunoprecipitation with 3 µg of polyclonal anti-cortactin antibody for 4 hours at 4°C. Immune complexes were precipitated with protein A/G plus agarose (Santa Cruz Biotechnology) and washed three times with Triton buffer (modified RIPA buffer without sodium deoxycholate and SDS). Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Western blotting was performed as described previously (Hauck et al., 2001).

Electron microscopy

FAK(+) or FAK(–) cells were seeded at 2×10^4 cells/well in 24-well plates on acid-washed glass coverslips coated with 5 µg/ml fibronectin. The next day, cells were infected for 1 hour at a MOI of 10 before they were fixed in situ with 2% glutaraldehyde/3% formaldehyde in 0.1 M cacodylate, 0.09 M sucrose, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$, pH 6.9 for at least 1 hour at 4°C. The samples were washed with 20 mM Tris, 1 mM EDTA, pH 7.0 and dehydrated in a graded series of acetone on ice. After critical point drying from liquid CO_2, samples were sputter-coated with 10 nm gold and examined at 5 kV accelerating voltage in a Zeiss DSM982 Gemini field emission scanning electron microscope using the Everhardt Thornley SE detector and the inlense SE detector in a 50:50 ratio. Images were digitally recorded and pseudocoloured in Adobe Photoshop 6.

Results

Actin dynamics during integrin-mediated internalisation of *S. aureus*

The integrin-mediated internalisation of fibronectin-binding *Staphylococcus aureus* by human epithelial cells depends on

the actin cytoskeleton, as cytochalasin D, which blocks actin filament elongation by direct binding to F-actin barbed ends, severely interferes with uptake (Fig. 1A) (Agerer et al., 2003; Sinha et al., 1999). Importantly, both inhibiting actin monomer incorporation into F-actin by latrunculin B and inhibiting the depolymerization of F-actin by jasplakinolide had a severe negative effect on integrin-mediated internalisation of *S. aureus,* suggesting that the dynamic turnover of actin cytoskeleton structures is crucial (Fig. 1A). To analyse actin dynamics during staphylococcal entry in more detail, we transfected 293 cells with cDNA encoding a GFP-actin fusion protein. Actin localisation in the transiently transfected cell population upon infection with rhodamine-labelled *S. aureus* was then monitored by time-lapse confocal microscopy. Interestingly, *S. aureus* induced localised accumulation of GFP-actin within minutes after contact with the host cell surface (Fig. 1B), whereas infection of GFP-actin-expressing cells with *S. carnosus* did not lead to localised actin polymerisation (data not shown) (Agerer et al., 2003). In the case of *S. aureus*-infected cells, the accumulation of actin persisted at the cell periphery, while the bacteria were taken up deeper into the cell and lost actin association (Fig. 1B; small arrow). The process of internalisation of *S. aureus* was often completed within 3-5 minutes. In addition, in some cases (<10% of the observed interactions) bacterial attachment triggered massive actin-based protrusions from the cell surface leading to bacterial engulfment (Fig. 1B; arrowhead) suggesting that there might be additional modes of staphylococcal entry into the cell.

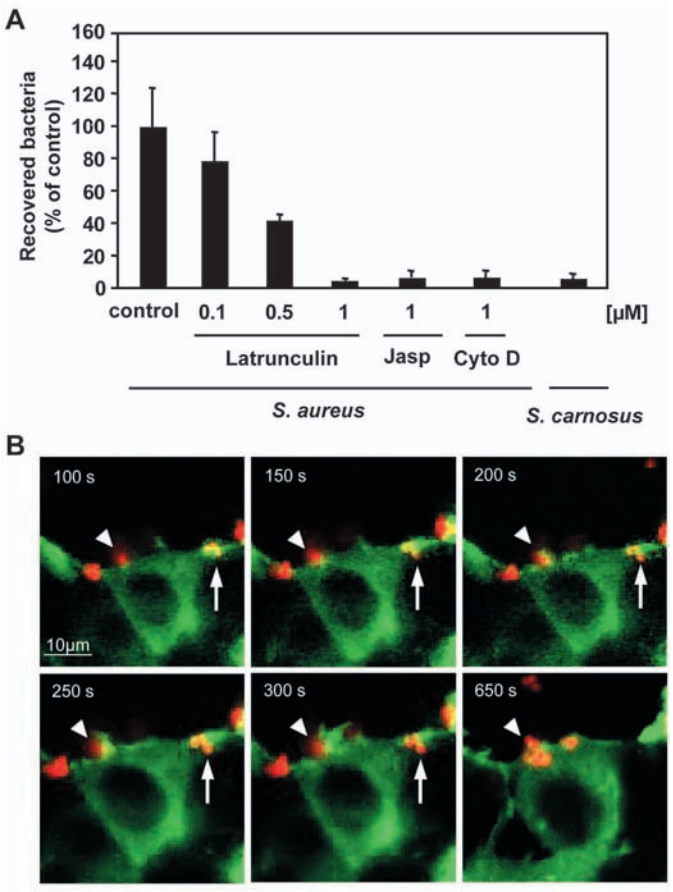

Fig. 1. *S. aureus* internalisation requires host cell actin dynamics. (A) 293 cells were infected with *S. aureus* or *S. carnosus* at a MOI 20 for 2 hours in the presence of the indicated concentrations of latrunculin, cytochalasin D, or jasplakinolide. Living intracellular bacteria were counted by gentamicin/lysostaphin protection assays. Values are means±s.d. of three independent experiments done in triplicate. (B) 293 cells were transfected with GFP-actin and infected with rhodamine-labelled *S. aureus*. Using time-lapse microscopy, the dynamics of GFP-actin in the transfected 293 cells 30 minutes after infection were recorded. Invasive bacteria induced transient local accumulation of polymerised actin (arrows). In some cases, massive actin-filled membrane protrusions wrapped around invasive bacteria (arrowheads). Elapsed time, in seconds, from the beginning of the recording is indicated.

Staphylococcus aureus recruits focal adhesion proteins to the site of entry

To investigate the possibility that invasive *S. aureus* initiate the assembly of focal contact-like protein complexes upon integrin engagement, 293 cells were transiently transfected with GFP-tensin or GFP-zyxin, and infected with rhodamine-labelled *S. aureus*. Interestingly, both GFP-tensin (Fig. 2A) and GFP-zyxin (Fig. 2C) were strongly recruited to the site of bacterial attachment, when the cells were infected with *S. aureus*. Since the infection process was not synchronized, recruitment was evident within 30-120 minutes after the addition of the bacteria and at any time point was seen in about 10% of the cell-associated bacteria. In contrast, infection of these cells with non-pathogenic *S. carnosus* did not result in recruitment of tensin or zyxin (Fig. 2A,C). Quantification of the fluorescence signals obtained separately in the GFP or rhodamine channels clearly showed a sharp increase in the local concentration of GFP-tensin over a distance of 1-2 μm in the vicinity of attached *S. aureus* (Fig. 2B). Cell-associated *S. carnosus* did not affect the local distribution of tensin (Fig. 2B). Similar results were obtained for GFP-vinculin (Fig. S1 in supplementary material). These results demonstrated that integrin engagement by pathogenic *S. aureus* induces localised recruitment of focal contact proteins and suggested that these proteins might be involved in actin rearrangements guiding staphylococcal internalisation.

FAK activity is critical for integrin-mediated uptake of *S. aureus*

The focal adhesion kinase (FAK) is an important modulator of integrin-dependent focal contacts thereby orchestrating cell spreading, cell migration and integrin-initiated signalling events (for a review, see Hauck et al., 2002b). In particular, FAK seems to regulate the turnover of focal adhesion protein complexes critically contributing to the dynamics of cell attachment sites. To investigate if FAK is required for the integrin-initiated internalisation of *S. aureus*, 293 cells were transiently transfected with an empty control vector (pcDNA) or different FAK mutants that were either impaired in their kinase activity (FAK K454M), were not capable of autophosphorylation (FAK Y397F), or lacked several proline residues necessary for association with SH3-containing proteins such as $p130^{CAS}$ or Graf (FAK Pro^{-}) (Hauck et al., 2002b). Gentamicin/lysostaphin protection assays revealed that

overexpression of either FAK mutant severely reduced the internalisation of *S. aureus* (Fig. 3A). In all cases, uptake of *S. aureus* by cells transfected with FAK mutants was less than 40% compared to mock transfected cells (pcDNA). Though the transfected genes were equally well expressed, the transfection efficiency usually fell between 60 and 80% of the total cell population (Fig. 3A and data not shown). Therefore, the observed 60-70% reduction in uptake corresponded to a block of internalisation by nearly all transfected cells. To further corroborate these findings we transiently expressed the FAK-related non-kinase (FRNK), a well-characterised endogenous inhibitor of FAK, in 293 cells. As observed for the FAK mutants, FRNK-expressing cells showed a remarkable reduction in the uptake of *S. aureus* (Fig. 3B), together indicating an important role for FAK in integrin-mediated uptake of *S. aureus*.

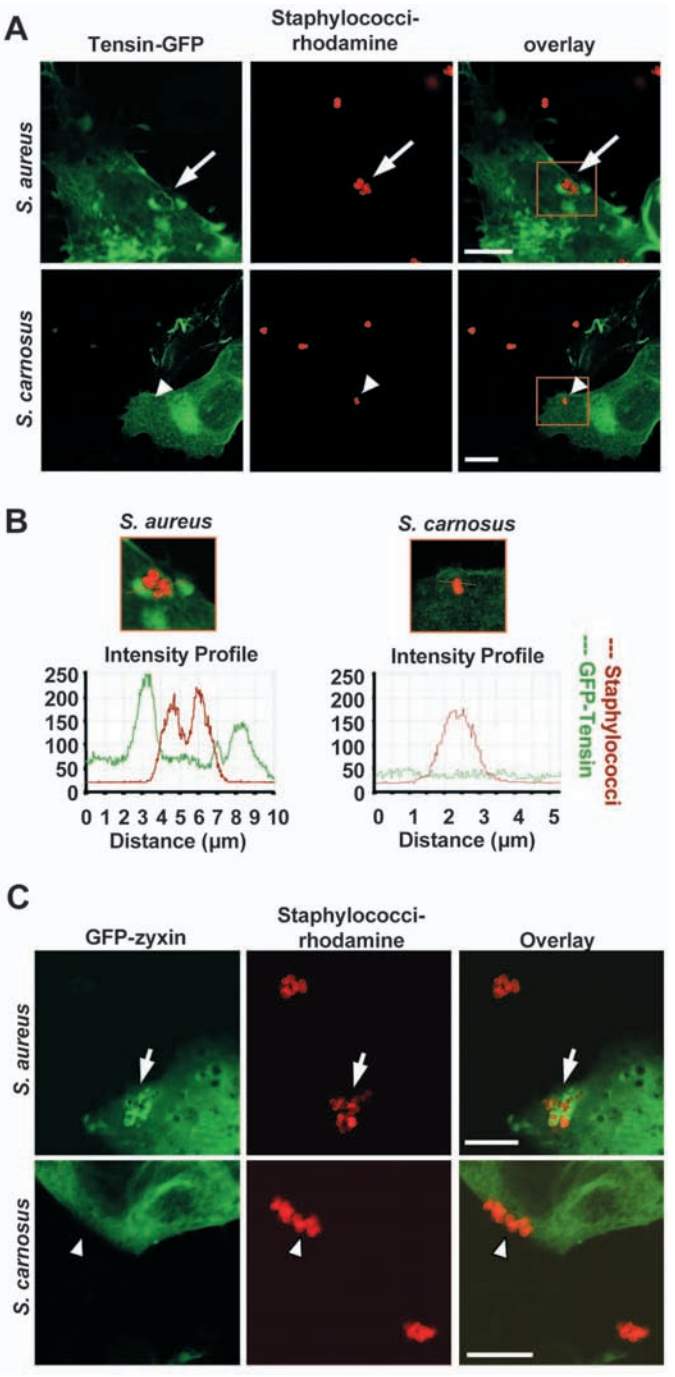

Fig. 2. Accumulation of focal contact marker proteins in the vicinity of attached *S. aureus*. (A) 293 cells were transfected with GFP-tensin and infected for 1 hour with rhodamine-labelled *S. aureus* or *S. carnosus* at a MOI 20. After fixation, samples were examined by confocal microscopy. Cell-associated *S. aureus* (arrows) or *S. carnosus* (arrowheads) are indicated. Bars, 10 μm. (B) Local recruitment of GFP-tensin (green line) to cell-associated bacteria (red line) was quantified by plotting the fluorescence intensity as detected in the GFP or rhodamine channels, respectively, against the distance. (C) 293 cells were transfected with GFP-zyxin, infected and processed as in A. Bars, 10μm.

FAK-deficient cells are resistant to invasion by *S. aureus*

If FAK is critical for *S. aureus* entry into eukaryotic cells then we hypothesised that FAK-deficient cells should be resistant to integrin-mediated uptake of this pathogen. To test this, mouse fibroblasts derived from FAK-deficient embryos [FAK(–) cells] and HA-FAK re-expressing FAK(–) cells [FAK(+) cells] were employed in a gentamicin/lysostaphin protection assays. Importantly, in FAK re-expressing cells the kinetics of invasion of *S. aureus* was comparable to that of human epithelial cells or mouse fibroblasts (Fig. 4A) (Agerer et al., 2003). Importantly, non-pathogenic *S. carnosus* was not internalised by FAK(+) cells, demonstrating that internalisation into mouse fibroblasts is a pathogen-specific process (Fig. 4A). In contrast to FAK(+) cells, there was hardly any invasion of *S. aureus* in FAK(–) fibroblasts in gentamicin/lysostaphin protection assays, demonstrating the critical role of FAK in staphylococcal uptake (Fig. 4B). To further verify the results

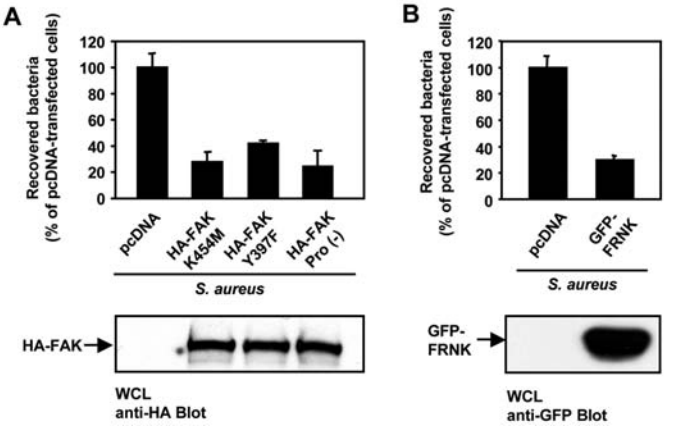

Fig. 3. Interference with FAK function blocks uptake of pathogenic *S. aureus*. (A) 293 cells were transfected with HA-FAK K454M, HA-FAK Y397F, HA-FAK Pro(–), or the empty expression vector (pcDNA). After 2 days, cells were infected with *S. aureus* for 2 hours at MOI of 20 and the number of internalised bacteria was determined by gentamicin/lysostaphin protection assays. Values are means±s.d. of three independent experiments done in triplicate. (Lower panel) Western blotting of whole cell lysates (WCL) with anti-HA-tag antibody shows the expression of HA-tagged FAK constructs. (B) 293 cells were transfected with GFP-FRNK or the empty expression vector (pcDNA) and used as in A. Values are means±s.d. of two independent experiments done in triplicate. (Lower panel) WCLs were probed with anti-GFP antibody.

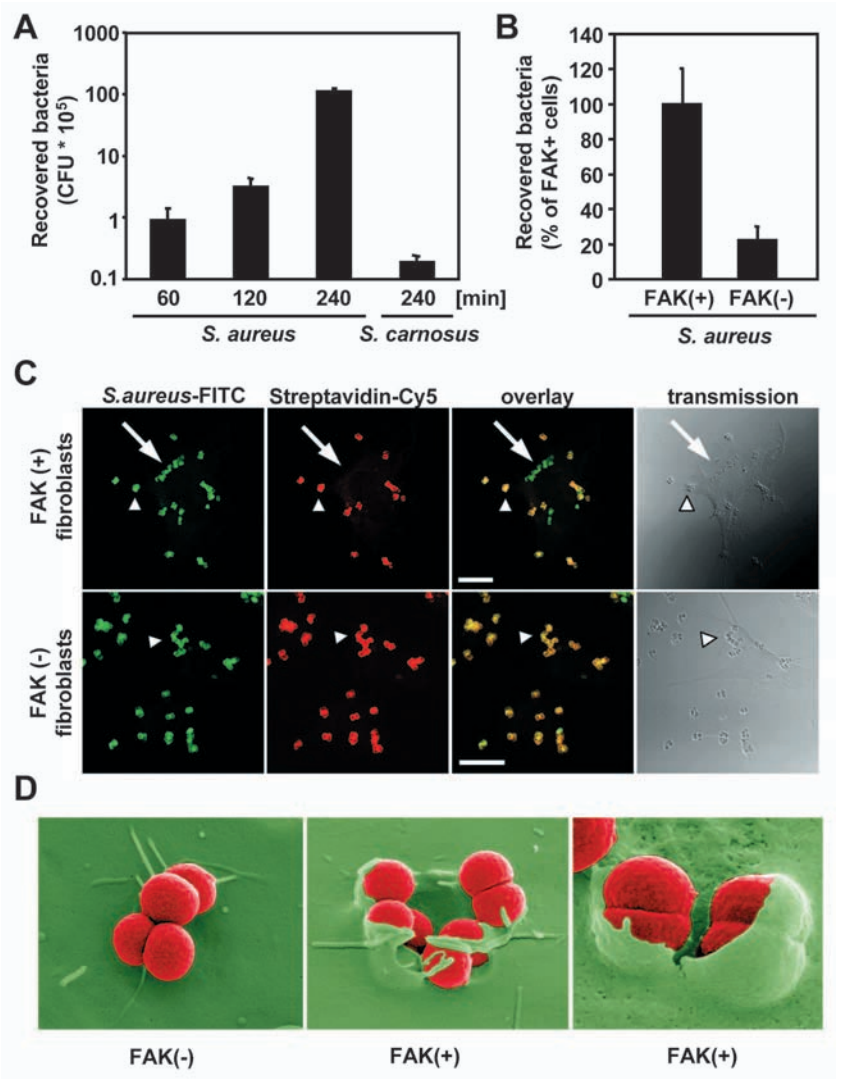

Fig. 4. FAK-deficient cells do not support *S. aureus* invasion. (A) Internalisation of *S. aureus* or *S. carnosus* by FAK-re-expressing fibroblasts was measured at the indicated times following infection by gentamicin/lysostaphin protection assays. Values are means±s.d. of two independent experiments done in triplicate. (B) FAK-re-expressing [FAK(+)] or FAK-deficient [FAK(–)] mouse fibroblasts were infected with *S. aureus* for 2 hours and employed in gentamicin/lysostaphin protection assays. Values are means±s.d. of three independent experiments done in triplicate (C) FAK(+) or FAK(–) fibroblasts were infected with FITC- and biotin-labelled *S. aureus* for 2 hours. After fixation, extracellular bacteria were detected by addition of streptavidin-Cy5. Extracellular bacteria (arrowheads) stain positive with both FITC and Cy5, whereas intracellular bacteria (arrows) are labelled with FITC only. Bars, 10 μm. (D) FAK(–) and FAK(+) cells were infected for 1 hour with *S. aureus* and analysed by scanning electron microscopy. Pseudocoloured images depict bacteria in red and the fibroblast surface in green.

of the antibiotic protection assay, we differentially stained infected samples of FAK-deficient and FAK-expressing cells for extra- and intracellular bacteria (Agerer et al., 2004). Confocal microscopy revealed that within 2 hours after infection a large proportion of the cell-associated bacteria was localised inside the FAK(+) cells (Fig. 4C), whereas only in rare cases were intracellular bacteria found in FAK(–) cells. Clearly, the pathogens bound equally well to both FAK(+) and FAK(–) cells (Fig. 4C) suggesting that FAK does not modulate receptor affinity for the bacteria, but rather is required for the internalisation of the integrin-bound *S. aureus* into the cell. This observation was also confirmed by scanning electron microscopy of FAK(+) and FAK(–) cells infected with *S. aureus* for 1 hour. Whereas similar amounts of the microorganisms were attached to both cell types, only in FAK(+) fibroblasts were membrane protrusions found around the bacteria. which finally lead to internalisation of the pathogens (Fig. 4D). In addition, large membrane invaginations were formed around attached bacteria in the case of FAK(+) cells, that were completely absent in FAK(–) cells (Fig. 4D). Taken together, these results confirmed an essential role for FAK in integrin-mediated uptake of pathogenic *S. aureus* and suggest that cell protrusions as well as membrane invaginations are both required for bacterial internalisation, and these might be controlled by FAK activity.

FAK is recruited to cell-associated *S. aureus* and displays enhanced tyrosine phosphorylation

During focal adhesion turnover FAK acts locally at focal adhesion sites, where it is recruited to by determinants in its C-terminal domain (Hildebrand et al., 1993; Klingbeil et al., 2001). To investigate if FAK also localises to sites of staphylococcal attachment to eukaryotic cells, FAK-deficient fibroblasts were transfected with GFP-FAK. Upon infection of these cells with *S. aureus*, a prominent recruitment of FAK to the vicinity of the attached bacteria could be observed (Fig. 5A). FAK recruitment to clustered integrins at the cell membrane is a critical step required for its activation that is reflected by enhanced phosphorylation of several tyrosine residues within FAK, in turn leading to enhanced tyrosine phosphorylation of downstream effectors. To gain insight into the tyrosine phosphorylation status of cellular proteins upon *S. aureus* infection, FAK-expressing fibroblasts were serum-starved and re-plated for 1 hour on poly-L-lysine to minimise engagement of integrins by matrix proteins and to allow maximal integrin-mediated interaction with pathogenic *S. aureus*. Poly-L-lysine-attached cells were left uninfected or were infected for 1 hour with *S. aureus* or *S. carnosus*, before samples were lysed and analysed by western blotting with an anti-phosphotyrosine antibody. Several proteins clearly showed a marked increase in tyrosine phosphorylation upon infection with *S. aureus* within 60 minutes (Fig. 5B). Most strikingly, proteins with an apparent molecular mass of ~210 kDa, ~116 kDa, ~80 kDa and ~60 kDa displayed enhanced tyrosine phosphorylation in response to *S. aureus* compared to the same proteins from uninfected or *S. carnosus*-infected cells. Reprobing of the membrane identified one of the tyrosine

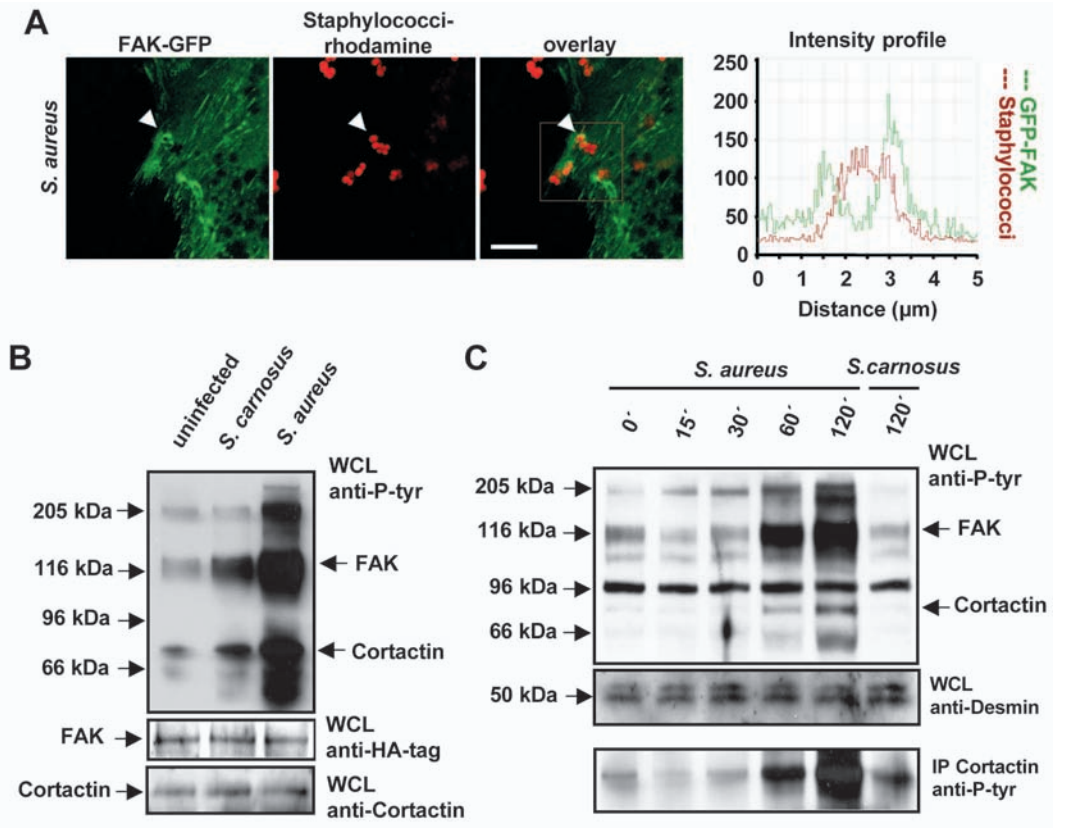

Fig. 5. Recruitment and phosphorylation of FAK accompanies *S. aureus* invasion. (A) FAK-deficient fibroblasts were transfected with GFP-FAK. Transfected cells were infected with rhodamine-labelled *S. aureus* at MOI 20 for 1 hour. The arrowheads point to accumulation of GFP-FAK in the vicinity of cell-attached *S. aureus*. Local recruitment of GFP-FAK (green line) to cell-associated bacteria (red line) was quantified by plotting the fluorescence intensity as detected in the GFP or rhodamine channels, respectively, against the distance. Bar, 10 μm. (B) FAK re-expressing fibroblasts were plated on poly-L-lysine, and either kept uninfected or infected at MOI 50 for 1 hour with *S. aureus* or *S. carnosus*, respectively. Samples were treated with pervanadate 10 minutes prior to lysis, and whole cell lysates (WCL) were analysed by western blotting with a monoclonal anti-phosphotyrosine (P-tyr) antibody (upper panel). Membranes were stripped and reprobed with anti-HA antibody (middle panel) or anti-cortactin antibody (lower panel). (C) FAK re-expressing cells were treated as in B and infected for the indicated times. WCLs were analysed by western blotting with anti-P-tyr antibody (upper panel) or anti-desmin antibody (middle panel). The same samples were immunoprecipitated (IP) with anti-cortactin antibodies and precipitates were analysed with anti-P-tyr antibody (lower panel).

phosphorylated proteins as FAK (~120 kDa) and another as the actin-binding protein cortactin (~80 kDa), and also demonstrated equal protein content of the analysed samples (Fig. 5B). To investigate the kinetics of enhanced tyrosine phosphorylation during *S. aureus* infection, FAK(+) cells were either left uninfected or infected for different times with *S. aureus* or non-pathogenic *S. carnosus*. Interestingly, tyrosine phosphorylation of FAK and other proteins increased in a time-dependent manner upon infection with *S. aureus*, while there was barely any increase in tyrosine phosphorylation detectable in cells infected with non-pathogenic *S. carnosus* (Fig. 5C). The kinetics of FAK tyrosine phosphorylation closely resembled the kinetics of staphylococcal internalisation by these cells (compare Fig. 4A) further supporting the idea that FAK activity is involved in the integrin-mediated uptake process. Importantly, similar kinetics of enhanced tyrosine phosphorylation were observed for several other proteins, including cortactin, suggesting that these events might be linked to *S. aureus*-induced FAK activation (Fig. 5C). The membranes used to investigate phosphorylation were reprobed with anti-desmin antibodies, which demonstrated equal protein content in the samples (Fig. 5C). Together, these results indicate that FAK activity is modulated during *S. aureus* entry into eukaryotic cells and suggest a potential connection between FAK and cortactin in the internalisation of integrin-associated particles.

The entry site of *S. aureus* is enriched in phosphotyrosine-containing proteins and cortactin

The presence of activated FAK in the vicinity of cell-associated *S. aureus* should result in enhanced tyrosine phosphorylation of downstream effectors of the FAK-Src complex. Indeed, when FAK(+) cells were infected with *S. aureus* for 1 hour, immunofluorescent staining revealed a massive increase in tyrosine phosphorylation in the vicinity of cell-attached bacteria (Fig. 6A). In contrast, although the basal level of tyrosine phosphorylation was higher in FAK cells, infection with *S. aureus* did not result in enhanced local concentrations of phosphorylated tyrosines (Fig. 6A). Moreover, infection of FAK(+) cells with non-pathogenic *S. carnosus* did not induce local increases in tyrosine phosphorylation of cellular proteins (see Fig. S2 in supplementary material). To investigate if cortactin might be one of the tyrosine kinase substrates recruited to staphylococcus entry sites, 293 cells were transiently transfected with RFP-cortactin prior to infection with staphylococci. Importantly, 60 minutes after infection with *S. aureus* a prominent recruitment of RFP-cortactin to the site of bacterial internalisation was observed, whereas *S. carnosus* did not affect the cellular distribution of cortactin (Fig. 6B). Again, quantitative analysis of the spatial distribution of cortactin pointed to a locally confined accumulation of cortactin within a 1-2 μm surrounding the invading bacteria (Fig. 6C). As cortactin showed an increase in

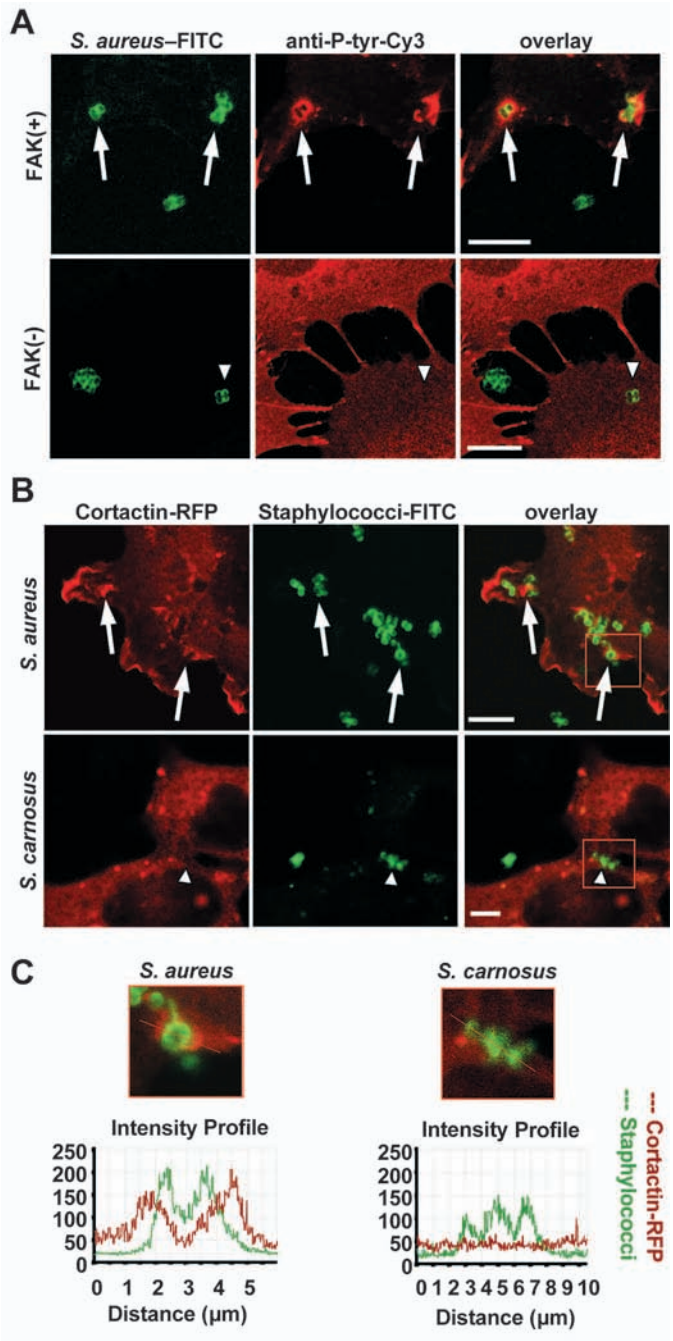

Fig. 6. Accumulation of phosphotyrosine-containing proteins and cortactin at the sites of *S. aureus* invasion. (A) FAK(+) or FAK(–) fibroblasts were infected with FITC-labelled *S. aureus* for 1 hour. After fixation and permeabilization, samples were stained with monoclonal anti-phosphotyrosine (P-tyr) antibodies. Whereas *S. aureus* induced massive accumulation of tyrosine-phosphorylated proteins in FAK(+) cells (arrows), no bacteria-associated tyrosine phosphorylation was evident in FAK(–) cells (arrowheads). (B) 293 cells were transfected with RFP-cortactin and infected for 1 hour with FITC-labelled staphylococci. In contrast to *S. carnosus* (arrowheads), *S. aureus* induced accumulation of cortactin (arrows). Bars, 10 µm. (C) Local recruitment of cortactin-RFP (green line) to cell-associated bacteria (red line) was quantified by plotting the fluorescence intensity along the indicated red lines as detected in the FITC- or RFP-channels, respectively, against the distance.

tyrosine phosphorylation upon *S. aureus* infection and was specifically enriched at the sites of staphylococcal entry, this protein might play a decisive role in the integrin-mediated internalisation process.

Interference with cortactin function blocks cellular invasion by *S. aureus*

Cortactin is a Src kinase substrate and actin binding protein that organises the cortical actin cytoskeleton and influences membrane association of actin filaments by directing the activities of the Arp2/3 complex and dynamin (Weed and Parsons, 2001). To investigate if cortactin has a functional role in integrin-mediated uptake of *S. aureus*, siRNA-mediated silencing of cortactin expression was performed. 24 hours after treatment of 293 cells with a cortactin-directed double-stranded RNA oligonucleotide, cortactin protein levels had decreased by more than 80% compared to cells treated with a control oligonucleotide (Fig. 7A). The reduced cortactin expression also resulted in a strong impairment of *S. aureus* uptake by the cells, demonstrating a functional role for this actin binding protein (Fig. 7A). To further explore which molecular determinants of cortactin are required for the integrin-mediated internalisation of *S. aureus*, we transiently transfected 293 cells with two mutant forms of cortactin, one impaired in its ability to bind the Arp2/3 complex (cortactin W22A) and the other no longer able to bind dynamin (cortactin W525K) (Schafer et al., 2002). Both forms were equally well expressed by the transfected cells and both reduced the internalisation of *S. aureus* by about 60% compared to cells transfected with the empty control vector (pcDNA) (Fig. 7B). As increased tyrosine phosphorylation of cortactin correlates with enhanced internalisation of *S. aureus*, we wondered whether cortactin tyrosine phosphorylation has a functional role during staphylococcal entry. Accordingly, 293 cells were transiently transfected with mutants of cortactin, in which either three C-terminal tyrosine residues (cortactin Y421/466/482) or the major c-Src phosphorylation site (cortactin Y421F) were replaced by phenylalanine. Gentamicin/lysostaphin protection assays conducted with these cells demonstrated that overexpression of cortactin Y421F severely reduced internalisation of *S. aureus,* by ~70% (Fig. 7C). Furthermore, overexpression of cortactin Y421/466/482F reduced *S. aureus* uptake by ~80% in comparison with 293 cells transfected with the control vector (pcDNA; Fig. 7C). These results demonstrate that the presence and proper function of cortactin, in particular tyrosine phosphorylation, dynamin binding and Arp2/3 recruitment, are required for efficient internalisation of *S. aureus*. As the uptake process was dependent on FAK and cortactin, the possibility existed that both proteins are functionally connected.

Cortactin tyrosine phosphorylation in response to integrin engagement requires both FAK and Src PTKs

Although FAK and cortactin are both responsive to integrin engagement, serve as Src substrates and are involved in cell motility, a functional connection between these two proteins has not been reported (Parsons, 2003; Vuori and Ruoslahti, 1995; Weed and Parsons, 2001). FAK could influence the recruitment of cortactin to *S. aureus* attachment sites, or FAK

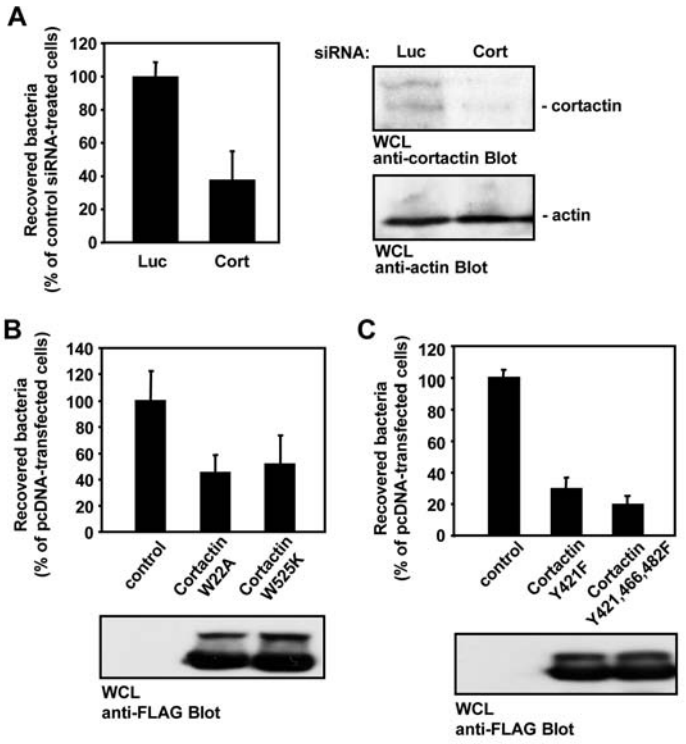

Fig. 7. Interference with cortactin expression or function inhibits invasion of *S. aureus*. (A) 293 cells were transfected with siRNA directed against either cortactin (Cort) or firefly luciferase (Luc) as a control. 24 hours later, cells were employed in gentamicin/lysostaphin protection assays with *S. aureus*. Values are means±s.d. of two independent experiments done in triplicate. Western blotting of whole cell lysates (WCL) with anti-cortactin antibody demonstrates gene silencing of cortactin (upper panel). The same membrane was probed with anti-actin antibodies as a loading control (lower panel). (B) 293 cells were transfected with the empty control vector (pcDNA) or cortactin mutants interfering with Arp2/3 association (W22A) or dynamin binding (W525K). Transfected cells were employed in gentamicin/lysostaphin protection assays with *S. aureus*. Values are means±s.d. of three independent experiments done in triplicate. (Lower panel) Western blotting of whole cell lysates (WCL) of the transfected cells with anti-FLAG antibody demonstrates expression of cortactin mutants. (C) 293 cells were transfected with the empty control vector (pcDNA), cortactin mutated at tyrosine residue 421 (Y421F), or mutated at three C-terminal tyrosine residues (Y421,466,482F). Cells were infected with *S. aureus* for 2 hours and the number of internalised bacteria was determined in gentamicin/lysostaphin protection assays. Values are means±s.d. of three independent experiments done in triplicate. Whole cell lysates (WCL) probed with anti-FLAG antibody demonstrate the expression of transfected cortactin constructs (lower panel).

could either directly or indirectly, via the association with Src PTKs, modulate the phosphorylation status of cortactin. Since accumulation of RFP-cortactin in the vicinity of attached staphylococci occurred in both FAK(+) and FAK(–) cells, FAK does not seem to be involved in directing the subcellular localisation of cortactin (see Fig. S3 in supplementary material). Next, we examined whether integrin-initiated tyrosine phosphorylation of cortactin is modulated by the presence of FAK. We stimulated FAK(+) or FAK(–) fibroblasts by plating onto fibronectin-coated culture dishes and monitored cortactin tyrosine phosphorylation. Importantly, cortactin tyrosine phosphorylation was absent in suspended cells, but increased upon replating of the FAK(+) cells (Fig. 8A). Clearly, attachment of fibroblasts to poly-L-lysine, a cell attachment substrate that does not engage integrins, was sufficient to cause basal tyrosine phosphorylation of cortactin. However, plating of these cells onto fibronectin confirmed that integrin engagement results in enhanced tyrosine phosphorylation of cortactin (Fig. 8A). In contrast, integrin stimulation in FAK(–) cells did not lead to enhanced cortactin tyrosine phosphorylation demonstrating that FAK is critical for the integrin-dependent aspect of cortactin phosphorylation. In addition, FAK(–) and FAK(+) fibroblasts were left uninfected or were infected for 60 minutes with *S. aureus*. Western blotting revealed that cortactin phosphorylation increased upon *S. aureus* infection of FAK-expressing cells, whereas the basal level of cortactin tyrosine phosphorylation was unchanged following infection of FAK-deficient fibroblasts (Fig. 8B). Furthermore, the increase in cortactin tyrosine phosphorylation upon fibronectin replating (see Fig. S4 in supplementary material) or in response to *S. aureus* (Fig. 8C) was completely abolished in the presence of the specific Src PTK inhibitor PP2. Together, these results suggest that an active FAK-Src complex influences cortactin tyrosine phosphorylation, but not its localisation, upon integrin stimulation by either immobilised fibronectin or fibronectin-coated bacteria, and reveal a novel FAK/Src-cortactin signalling axis regulating integrin internalisation.

Discussion

Integrins are exploited by numerous viruses and bacterial pathogens for entry into eukaryotic host cells. Here, we show that FnBP-expressing *S. aureus* take advantage of integrin-regulated actin dynamics to trigger their uptake. In particular, bacterial engagement of the integrin $\alpha_5\beta_1$ directs the assembly of a cytoplasmic protein complex characterised by the presence of tensin, vinculi, and zyxin. In addition, the integrin-associated protein tyrosine kinase FAK, as well as the actin-binding protein cortactin, are recruited to invasive bacteria and co-operate to mediate the integrin-dependent internalisation of *S. aureus*. By engagement of integrin $\alpha_5\beta_1$ the bacterial pathogens seem to exploit the ability of this Fn receptor to dynamically associate with the actin cytoskeleton and to generate the pulling forces to promote staphylococcal uptake by the eukaryotic host cell.

Several recent investigations have demonstrated that cellular focal contacts possess functional and morphological diversity (Geiger et al., 2001). Based on morphological aspects, cell attachment sites have been subdivided into focal complexes, mature focal adhesions and fibrillar adhesions (Geiger et al., 2001). In particular tensin, a protein strongly recruited to the sites of *S. aureus* attachment to integrin $\alpha_5\beta_1$, serves as a distinctive constituent of fibrillar adhesions (Zamir et al., 2000). Functionally, integrin $\alpha_5\beta_1$-containing fibrillar adhesions are involved in the organisation of the extracellular matrix, as they co-align with fibronectin fibrils, and genetic

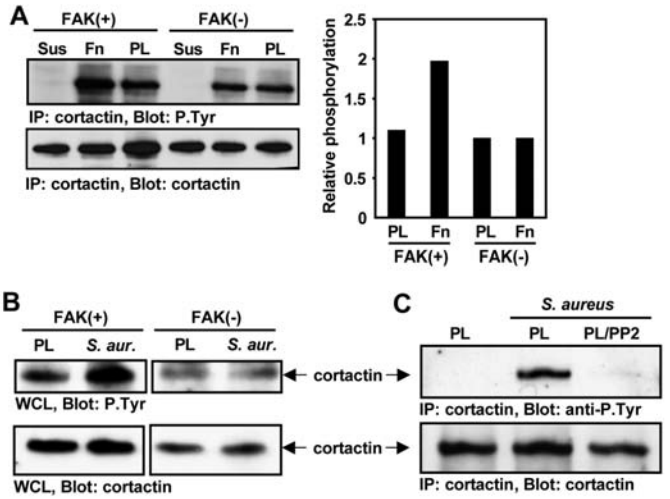

Fig. 8. FAK and Src cooperate in integrin-initiated tyrosine phosphorylation of cortactin. (A) Serum-starved FAK(+) and FAK(–) cells were either kept in suspension (Sus) or seeded on fibronectin (Fn) or poly-L-lysine (PL)-coated dishes for 30 minutes. Samples were treated with pervanadate for 5 minutes before lysis. After cortactin immunoprecipitation (IP), samples were analysed by western blotting with monoclonal anti-phosphotyrosine antibody (P.Tyr; upper panel). Following stripping, the blot was re-probed with polyclonal anti-cortactin antibodies (lower panel). The graph shows the ratio between phosphorylated cortactin and the total amount of cortactin present in the immunoprecipitates. For comparison, the ratio of FAK(–) cells plated onto poly-L-lysine was set to 1. (B) Serum-starved FAK(+) or FAK(–) cells were seeded onto poly-L-lysine-coated dishes and infected with *S. aureus* for 1 hours or left uninfected. Whole cell lysates (WCL) were analysed by western blotting and probed with monoclonal anti-phosphotyrosine antibody (P.Ttyr; upper panel). After stripping, membranes were re-probed with polyclonal anti-cortactin antibodies (lower panel). (C) Serum-starved FAK(+) cells were seeded onto poly-L-lysine-coated dishes and either left uninfected or infected with *S. aureus* for 1 hour in the presence or absence of 5 μM PP2. After cortactin immunoprecipitation (IP), samples were analysed by western blotting with monoclonal anti-phosphotyrosine antibody (P.Tyr; upper panel). Following stripping, the blot was re-probed with polyclonal anti-cortactin antibodies (lower panel).

ablation of integrin β_1 results in defects in the assembly of a fibrillar network of extracellular Fn (Danen et al., 2002; Wennerberg et al., 1996). Cellular pulling forces generated via an integrin $\alpha_5\beta_1$-mediated linkage to the actin-myosin network seem to be critical for Fn fibril formation, as force-induced conformational changes are essential to expose cryptic multimerization sites within the second type III repeat of Fn (Sechler et al., 2001). It is interesting to note that the focal adhesion kinase has been demonstrated to play a key role in the formation of a fibrillar Fn matrix. FAK-deficient cells in vitro, as well as FAK-deficient mouse embryos in vivo, fail to properly assemble Fn fibrils (Ilic et al., 2004). In line with the defect of FAK-deficient cells to properly organise a Fn matrix, we observed that these cells are also deficient in the ability to internalise *S. aureus*. As one would expect, *S. aureus* still binds equivalently to FAK-expressing and FAK-deficient cells. However, scanning electron microscopy reveals that in FAK-deficient cells, no membrane invaginations are formed below the attached bacteria, suggesting that integrin-linkages to the actin-myosin network are disrupted and pulling forces are not provided.

FAK has also been implicated in integrin-mediated internalisation processes in other systems. For example, uptake of spent photoreceptor outer segment fragments by retinal pigment epithelium is mediated by integrin $\alpha_v\beta_5$ and requires the presence of FAK (Finnemann, 2003). As is true for integrin $\alpha_5\beta_1$-mediated uptake of *S. aureus*, this internalisation process is accompanied by local recruitment and increased tyrosine phosphorylation of FAK (Finnemann, 2003). Also, FAK is involved in the internalisation of enteropathogenic *Y. enterocolitica* upon invasin-mediated engagement of β_1 integrins and the protein F1-triggered uptake of *Streptococcus pyogenes* via fibronectin-binding integrins (Alrutz and Isberg, 1998; Ozeri et al., 2001). These observations are in line with the view that signals co-ordinated by FAK are critical for the internalisation of diverse particles attached to integrins.

Besides the forces needed to pull in integrin-attached bacteria, the cells have to enclose the particles with a membrane protrusion generated by actin polymerization. In this context, we observed that cortactin, an actin-bundling protein, is highly enriched at the sites of bacterial attachment. As cortactin can directly associate with and activate the Arp2/3 complex, a key cellular mediator of actin polymerisation (Pollard, 2003; Weaver et al., 2002), this protein could play a critical role in the uptake process. Indeed, overexpression of a mutant cortactin (cortactin W22A) unable to associate with the Arp2/3 complex (Weed et al., 2000) severely compromises integrin-mediated internalisation. Interestingly, cortactin and the Arp2/3 complex also seem to be involved in guiding *Shigella flexneri* uptake by human cells (Bougneres et al., 2004). Invasion by *S. flexneri* is promoted by association with cellular receptors such as integrin β_1 or CD44 and the type III secretion system-mediated injection of bacterial effectors (Watarai et al., 1996; Skoudy et al., 2002). In addition, the bacteria seem to exploit a similar actin-modulating cellular machinery involving activation of Src family PTKs as well as local recruitment of vinculin and cortactin (Bougneres et al., 2004; Dehio et al., 1995; Tran Van Nhieu et al., 1997). In this context, it is interesting to note that the adaptor molecule Crk influences the uptake of *Shigella*, presumably by localising tyrosine phosphorylated cortactin to the vicinity of invading bacteria (Bougneres et al., 2004). Furthermore, $p130^{CAS}$, an adapter molecule binding to proline-rich residues in the FAK C-terminal domain and a prominent substrate of the active FAK-Src complex, can associate with Crk. As mutations of the C-terminal proline-rich sites in FAK (FAK Pro-) lead to a strong impairment of *S. aureus* uptake, a similar role for Crk in this context can be envisioned. However, localisation of cortactin to the sites of *S. aureus* attachment was independent of FAK suggesting that other routes to cortactin recruitment exist. Clearly, whereas *S. flexneri* maximally activates this system inducing massive actin accumulation and membrane rearrangements at the site of bacterial entry, such dramatic membrane protrusions are only seen in a minor fraction of *S. aureus*-infected cells. This observation suggests that the fine-tuning of actin polymerization at the site of bacterial contact might differ between these pathogens. It is tempting to speculate that the more physiological way used by *S. aureus* to engage a cell surface receptor, rather than injecting proteins via

a type III secretion system as is the case for *S. flexneri*, results in more confined actin polymerisation.

In addition to directing actin polymerisation by recruitment of the Arp2/3 complex, cortactin has also been shown to link the cortical actin cytoskeleton with molecules involved in membrane endocytosis (McNiven et al., 2000). Cortactin directly binds to dynamin-2, a GTPase that regulates the pinching-off of endocytotic vesicles at the plasma membrane (van der Bliek, 1999). In podosomes, specialised attachment sites predominantly found in osteoclasts and macrophages, but also present in cells transformed by constitutively active versions of the Src PTK dynamin-2, has been found to co-localise with cortactin and to be involved in the organisation of these actin-rich structures (Ochoa et al., 2000). The association between cortactin and dynamin-2 is mediated by the SH3 domain of cortactin that binds to proline-rich motifs in the C-terminal part of dynamin (McNiven et al., 2000). Our observation that overexpression of a mutant cortactin, impaired in binding to dynamin-2, severely affects integrin-mediated uptake of *S. aureus* points to the exciting possibility that cortactin might coordinate actin polymerisation and membrane protrusion via the Arp2/3 complex with the severing of the endocytic vesicle containing invasive staphylococci via dynamin-2.

Unexpectedly, the investigation of the FnBP-triggered uptake of *S. aureus* reveals a novel functional connection between FAK and cortactin. In particular, FAK plays a critical role in relaying integrin $\alpha_5\beta_1$-initiated signals to cortactin. Though both FAK and cortactin are substrates of activated Src and both are involved in regulating cell migration, a functional connection between these two molecules has never been described (Parsons, 2003; Vuori and Ruoslahti, 1995; Weed and Parsons, 2001). Whereas the basal level of cortactin tyrosine phosphorylation is unaffected by the presence of FAK, FAK-deficient cells are unable to increase cortactin tyrosine phosphorylation in response to integrin engagement. Accordingly, *S. aureus*-triggered tyrosine phosphorylation of cortactin is abolished in the absence of FAK. These data demonstrate that FAK is essential in connecting an integrin $\alpha_5\beta_1$-initiated stimulus with tyrosine phosphorylation of cortactin. Phosphorylation of cortactin at tyrosine residues, in particular Tyr-421, is mediated by Src family kinases and is known to affect the actin cross-linking activity of this protein (Huang et al., 1997). As integrin-mediated FAK activation is tightly coupled with the generation of an active FAK-Src complex (Schlaepfer et al., 1994) and *S. aureus*-triggered phosphorylation of cortactin is blocked by the Src PTK inhibitor PP2, we envision that Fn-initiated integrin clustering leads to FAK-dependent local recruitment of Src and subsequent Src-mediated tyrosine phosphorylation of cortactin. These data provide novel insight into the regulation of actin dynamics at focal contact sites and in particular suggest a molecular scenario of how integrin engagement might be coupled to endocytosis. It is of particular interest that both FAK and cortactin have been implicated in the regulation of cell motility and tumour cell invasion. More specifically, FAK is overexpressed in a number of invasive human tumours and interference with FAK function abolishes the invasive motility of v-Src-transformed cells in vitro and in vivo (Gabarra-Niecko et al., 2003; Hauck et al., 2002a; Hsia et al., 2003). In addition, the human cortactin gene (*EMS1*) has been found to be amplified in human cancer (Schuuring et al., 1992) and overexpression of cortactin in fibroblasts promotes in vitro invasion (Patel et al., 1998). The functional cooperation between these two molecules observed during integrin-mediated internalisation of pathogenic *S. aureus* might therefore also be of relevance in other settings such as cell migration events during embryonic development or the invasive motility of transformed cells.

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