First publ. in: Journal of Biological Chemistry 278 (2003), 43, pp. 42524–42531

Integrin-mediated Invasion of *Staphylococcus aureus* into Human Cells Requires Src Family Protein-tyrosine Kinases*

Received for publication, February 27, 2003, and in revised form, June 23, 2003
Published, JBC Papers in Press, July 31, 2003, DOI 10.1074/jbc.M302096200

Franziska Agerer‡§, Antje Michel§¶, Knut Ohlsen¶, and Christof R. Hauck‡‖

From the ‡Zentrum für Infektionsforschung and the ¶Institut für Molekulare Infektionsbiologie, Universität Würzburg, Röntgenring 11, 97070 Würzburg, Germany

***Staphylococcus aureus*, a common cause of nosocomial infections, is able to invade eukaryotic cells by indirectly engaging β_1 integrin-containing host receptors, whereas non-pathogenic *Staphylococcus carnosus* is not invasive. Here, we identify intracellular signals involved in integrin-initiated internalization of *S. aureus*. In particular, the host cell actin cytoskeleton and Src family protein-tyrosine kinases (PTKs) are essential to mediate *S. aureus* invasion. Src PTKs are activated in response to pathogenic *S. aureus*, but not *S. carnosus*. In addition, pharmacological and genetic interference with Src PTK function reduces bacterial internalization. Importantly, Src PTK-deficient cells are resistant to *S. aureus* invasion, demonstrating the essentiality of host Src PTKs in integrin-mediated uptake of this pathogen.**

The Gram-positive microorganism *Staphylococcus aureus* is a common cause of nosocomial infections (1, 2). Introduction of staphylococci is facilitated by indwelling medical devices that provide a surface for colonization and a platform for further dissemination. Due to widespread and often multiple antibiotic resistance of *S. aureus*, infections with this microbe are increasingly difficult to treat.

A prominent characteristic of different *S. aureus* isolates associated with disease is the expression of extracellular matrix (ECM)[1]-binding proteins that have been collectively termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (3, 4). In particular, the cell wall-attached fibronectin-binding proteins A and B (FnBPA and FnBPB) confer a tight association of the bacteria with the ECM protein fibronectin (Fn) (5, 6). The interaction of FnBPs with fibronectin is mediated by direct protein-protein interactions between multiple Fn-binding domains of the bacterial proteins connecting as extended antiparallel β-strands to the fibronectin type 1 modules in the amino-terminal domain of Fn (7). As Fn is a common constituent of the extracellular matrix in different tissues and is also abundant in serum, it is thought that Fn deposition on introduced materials such as bone-implanted metals promotes the attachment of *S. aureus* on medical devices (8).

Eukaryotic cells also possess specific surface receptors that bind to Fn. Most prominently, the integrin $\alpha_5\beta_1$ serves as a Fn receptor on multiple cell types. Integrin $\alpha_5\beta_1$ recognizes a short peptide motif, the Arg-Gly-Asp (RGD) sequence, found within one of the type III repeats of Fn. As staphylococcal FnBP associates with the N terminus of Fn independently of the RGD sequence (7, 9), simultaneous association of both *S. aureus* and human cells with Fn should be possible. Indeed, it has been shown recently that Fn acts as a molecular bridge, linking FnBP-expressing *S. aureus* with integrin $\alpha_5\beta_1$ on the surface of human cells (for review see Ref. 10). This interaction not only tightly anchors *S. aureus* to its eukaryotic host cell, but also promotes the internalization of the microorganisms by human epithelial and endothelial cells (11–13) as well as mouse fibroblasts (14). It is interesting to note, that integrin β_1-containing receptors are well known for their role in cell adhesion and for their signal transduction capacity in response to cell attachment to the ECM (15). However, ligands for these integrins are usually immobilized and, hence, integrin β_1 is not considered an endocytic receptor. Therefore, not much is known about the intracellular signals responsible for this integrin-initiated internalization process.

In this study, we have analyzed the intracellular signals governing integrin β_1-mediated internalization of *S. aureus*. We not only find an essential role for host cell PTKs and the actin cytoskeleton, but identify Src family PTKs to play a critical role in mediating *S. aureus* invasion-promoting signals. Thus, Src kinases are activated in response to pathogenic *S. aureus*, but not to non-pathogenic *S. carnosus*, and pharmacological and genetic interference with Src PTK function reduces bacterial internalization. Importantly, Src PTK-deficient cells are resistant to *S. aureus* invasion, underlining the essentiality of host Src family PTKs in integrin β_1-mediated uptake of *S. aureus*.

EXPERIMENTAL PROCEDURES

Bacteria—S. aureus (Cowan) and non-pathogenic *S. carnosus* 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 PBS and adjusted to 1×10^8 cfu/ml in PBS. In some experiments, staphylococci were fluorescently labeled prior to infection. Therefore, bacteria (1×10^9/ml) were washed three times with PBS and suspended in PBS containing 0.2 μg/ml 5-(6)-carboxyfluorescein-succinylester or 5-(6)-carboxytetramethylrhodamine-succinylester, respectively, (Molecular Probes, Eugene, OR) for 20 min at room temperature in the dark. Labeled bacteria were extensively washed with PBS prior to use.

Cell Culture—The human embryonic kidney cell line 293T was grown in DMEM/10% calf serum (CS) at 37 °C, 5% CO_2. Cells were

* This study was supported by funds from the BMBF (01 KI 8906/0) and Fonds der Chemischen Industrie (to C. R. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Both authors contributed equally to this work.

‖ To whom correspondence should be addressed: Zentrum für Infektionsforschung, Universität Würzburg, Röntgenring 11, 97070 Würzburg, Germany. Tel.: 49-(0)-931-312137; Fax: 49-(0)-931-312578; E-mail: christof.hauck@mail.uni-wuerzburg.de.

[1] The abbreviations used are: ECM, extracellular matrix; CS, calf serum; FCS, fetal calf serum; Fn, fibronectin; FnBP, fibronectin-binding protein; PFA, paraformaldehyde; PTK, protein-tyrosine kinase; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; mAb, monoclonal antibody; GST, glutathione *S*-transferase; MOI, multiplicity of infection; FITC, fluorescein isothiocyanate; SYF, Src-, Yes-, Fyn-deficient fibroblasts.

subcultured every 2–3 days. A day prior to infection, 2×10^5 cells/well were seeded in poly-L-lysine coated (20 μg/ml) 24-well plates in DMEM/10% CS (gentamicin/lysostaphin protection assay and immunofluorescence staining) or 1×10^6 cells/dish were seeded in poly-L-lysine coated 6-cm dishes and serum-starved for 20 h in DMEM containing 0.5% CS (cell lysates).

Fibroblasts derived from Src, Yes, Fyn triple knock-out mouse embryos (SYF cells; Ref. 16) and SYF + c-Src cells were cultured in DMEM/10% fetal calf serum (FCS) supplemented with non-essential amino acids on gelatin-coated (0.1% in PBS) cell culture dishes.

Reagents and Antibodies—Cytochalasin D, genistein, AG957 and the Src-specific inhibitor PP2 were obtained from Calbiochem (Bad Soden, Germany). GRGDS and SDGRG peptides were from Sigma (Taufkirchen, Germany). Fibronectin from bovine serum was from ICN Biomedicals (Eschwege, Germany). Rabbit antiserum against *S. aureus* was produced by immunoGlobe (Himmelstadt, Germany). Monoclonal antibody (mAb) against Csk (clone 52) was obtained from BD Biosciences (Heidelberg, Germany), mAb against phosphotyrosine (clone 4G10) from Upstate (Lake Placid, NY). MAbs against integrin β_1 (clone P5D2), against ICAM1 (clone P2A4), and against h-lamp-2 (clone H4B4) were from the Developmental Studies Hybridoma Bank (University of Iowa, IA). Polyclonal phospho-Src antibody P-Tyr-418 (recognizing phosphorylated Tyr-419 in human c-Src) was from BIOSOURCE International (Nivelle, Belgium), polyclonal antibodies against Src PTKs (Src-2) were from Santa Cruz Biotechnology (Santa Cruz, CA). mAb against Src (clone 2-17) was purified from hybridoma supernatants. Prior to invasion inhibition experiments, antibodies were cleared from sodium azide by 10 washes with PBS using Vivaspin concentrators 50K (Vivascience, Hannover, Germany).

Production of Recombinant FnBPA B Domain—The sequences comprising the FnBPA B domain (amino acids 303–568 of FnBPA) were amplified from *S. aureus* Cowan chromosomal DNA using the primer pair FnBPA-sense 5′-GAAGTTATCAGTCGACAAAGATGGTATTGGGAATTATTATGCC-3′ and FnBPA-anti 5′-ATGGTCTAGAAAGCTTTAAGAGGACTCAGTGTATCCTCCAAC-3′ according to Massey *et al.* (9). The resulting fragments were cloned in pDNR-Dual using the In-Fusion PCR Cloning Kit (BD Biosciences, Palo Alto, CA) and transferred by Cre-mediated recombination into pGEX4Ti-LoxP containing a LoxP site in-frame with the GST coding sequence (assembled with the help of the Creator-acceptor-vector-construction Kit; BD Biosciences) to yield GST-FnBPA-B. GST and GST-FnBPA-B were purified after isopropyl-1-thio-β-D-galactopyranoside induction of *Escherichia coli* BL-21 by standard procedures using glutathione-agarose FF (Amersham Biosciences, Uppsala, Sweden).

Recombinant DNA Constructs and Transfection of Cells—Expression constructs encoding kinase-inactive c-Src (Src K297M), Csk WT, and kinase-inactive Csk (Csk K222M) were kindly provided by David Schlaepfer (Scripps Research Institute, La Jolla, CA). 293T cells were transfected with standard calcium-phosphate co-precipitation method using 5 μg of the respective constructs or empty control vector pcDNA3.1 (Invitrogen, Carlsbad, CA). Cells were employed in infection experiments 48 h after transfection.

Infection Experiments and Gentamicin/Lysostaphin Protection Assay—For gentamicin/lysostaphin protection assays, 293T cells (2×10^5 cells/well) or fibroblasts (8×10^4 cells/well) were infected with bacteria at an MOI of 20. After the indicated times, the culture medium was replaced by DMEM/10% CS containing 50 μg/ml gentamicin and 20 μg/ml lysostaphin. After 45 min of incubation at 37 °C, cells were washed once with PBS and intracellular bacteria released by incubation in 1% saponin in PBS for 20 min at 37 °C. Samples were diluted in PBS and plated on TSB agar plates for determination of the recovered colony forming units (cfu). In inhibition experiments, pharmacological inhibitors or antibodies were added to the cells 15 min before infection. For infection of serum-starved cells, bacteria were preincubated with PBS containing 10 μg/ml bovine fibronectin for 10 min at room temperature, washed three times with PBS, and added to the cells at an MOI of 50.

Fluorescence Staining—293T cells and fibroblasts were seeded on acid-washed glass-coverslips in 24-well plates and infected with an MOI of 10. After infection, cells were washed once with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. Paraformaldehyde-fixed cells were washed three times with PBS and afterward incubated for 5 min in PBS, 10% FCS, and 0.2% saponin (blocking buffer) in order to permeabilize cellular membranes and to block unspecific binding sites. Suitable dilutions of polyclonal rabbit-α-*S. aureus* (1:200) and monoclonal mouse-α-h-lamp-2 (1:40 of clone H4B4) were added in blocking buffer for 1 h at room temperature. Samples were washed twice with PBS, blocked again for 5 min and incubated with Cy3-conjugated goat-α-mouse and FITC-conjugated goat-α-rabbit (diluted 1:100 in blocking buffer; Dianova, Hamburg, Germany) for 45 min 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.

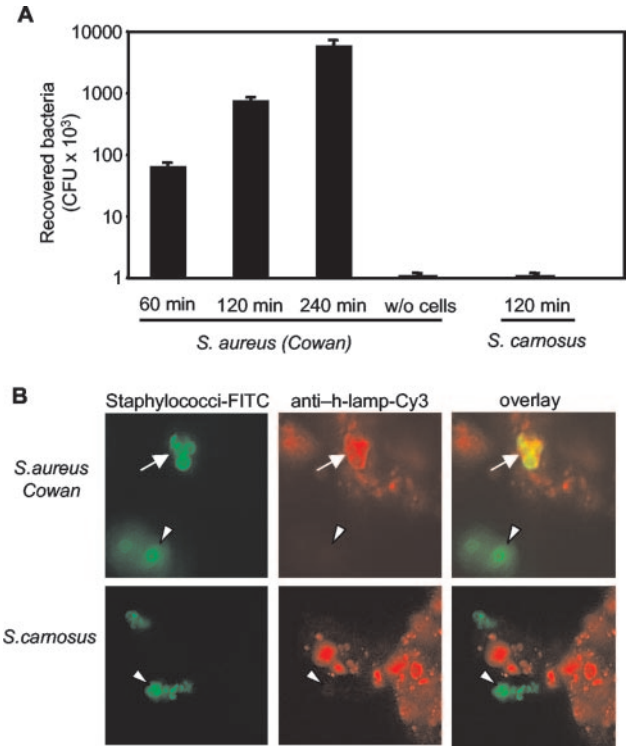

FIG. 1. ***S. aureus* Cowan invades human 293T cells.** *A*, the internalization of *S. aureus* Cowan and *S. carnosus* was determined after the indicated times of infection by gentamicin/lysostaphin protection assays. The graph shows mean values ± S.D. of two independent experiments done in triplicate. As a further control, *S. aureus* Cowan was treated with gentamicin/lysostaphin in the absence of cells (w/o cells). *B*, immunofluorescence staining of cells infected for 2 h with FITC-labeled *S. aureus* Cowan (*upper row*) or *S. carnosus* (*lower row*), respectively. After fixation, cells were stained with monoclonal antibody against h-lamp1. Confocal microscopy detected extracellular bacteria (*arrowhead*) and intracellular *S. aureus* Cowan co-localizing with the late endosomal/phagosomal marker h-lamp1 (*small arrow*).

For differentiating between extra- and intracellular bacteria, cells were infected with FITC-labeled bacteria. Following infection and fixation, samples were stained with polyclonal rabbit-anti-*S. aureus* and goat-anti-rabbit-Cy5 in PBS, 10% FCS without permeabilization of the cells. Therefore, antibodies reached only extracellular bacteria resulting in FITC-labeled intracellular and FITC/Cy5-labeled extracellular bacteria. The samples were viewed with a Zeiss confocal laser scanning microscope (Zeiss, Heidelberg, Germany). For double-labeled 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 Photoshop6 (Adobe Systems, Mountain View, CA) and merged to yield pseudo-colored RGB pictures.

Cell Lysis and Western Blotting—At the indicated times, infected cells were washed once with ice-cold PBS and lysed in modified radioimmune precipitation assay 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, 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 added to an equal volume of reducing 2× SDS sample buffer, the proteins were separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Western blotting was performed as described (17).

Immunoprecipitation and Src in Vitro Kinase Assay—3 μg monoclonal anti-Src antibody/sample were added to cleared lysates containing equivalent amounts of protein and incubated for 4 h at 4 °C. After addition of protein A/G plus agarose (Santa Cruz Biotechnology) and 1 h incubation at 4 °C, samples were washed twice with radioimmune precipitation assay buffer and twice with Triton buffer (25 mM Hepes (pH 7.4), 1% Triton X-100, 150 mM NaCl, 20 mM $MgCl_2$, 10% glycerol, 10 mM

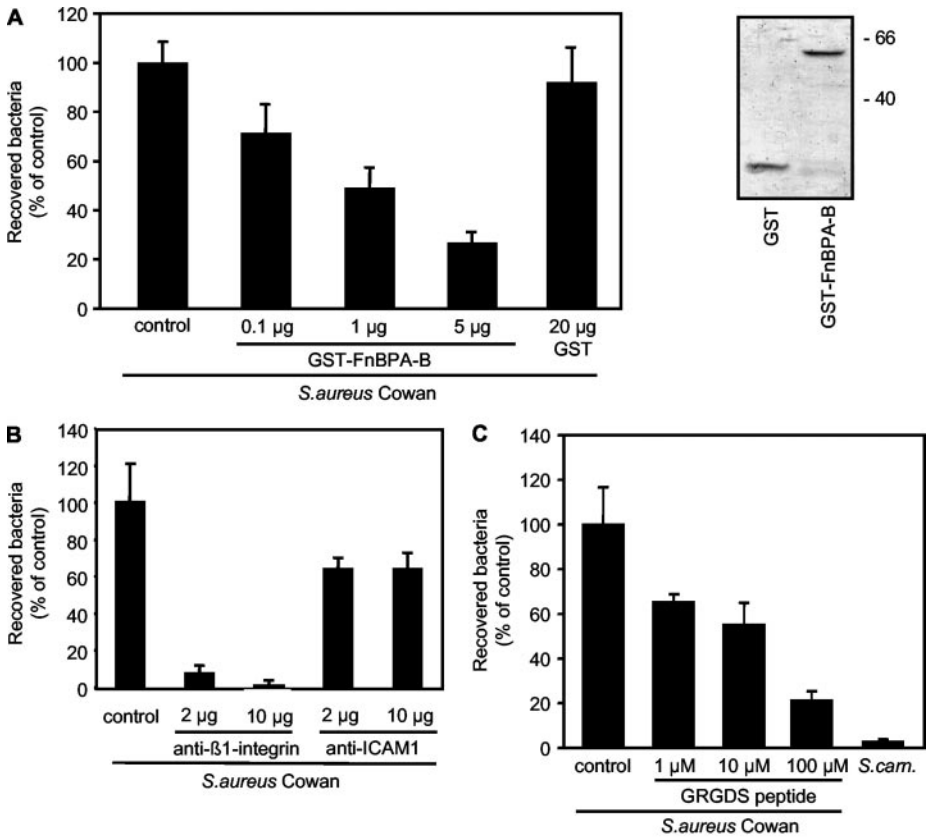

FIG. 2. ***S. aureus* Cowan invasion is initiated by FnBPs targeting host cell integrins.** *A*, 293T cells were infected for 2 h with *S. aureus* Cowan in the presence of the indicated concentrations of a GST fusion protein of the FnBPA B domain (GST-FnBP-B) or 20 μg of GST. Bacterial invasion was determined by gentamicin/lysostaphin protection assays. The graph shows mean values ± S.D. of two independent experiments done in triplicate. The *panel* on the *right* shows a Coomassie Blue-stained SDS-PAGE gel loaded with 5 μg of GST or GST-FnBPA-B, respectively. *B*, 293T cells were infected for 2 h with *S. aureus* Cowan in the absence or presence of 2 μg or 10 μg of anti-β_1 integrin or anti-ICAM 1 monoclonal antibodies. Bacterial invasion was determined by gentamicin/lysostaphin protection assays. The graph shows mean values ± S.D. of two independent experiments done in triplicate. *C*, gentamicin/lysostaphin protection assays were performed in the presence of the indicated concentrations of an GRGDS peptide. *S. aureus* Cowan invasion was determined after 2 h. The graph shows mean values ± S.D. of three independent experiments done in triplicate.

sodium pyrophosphate, 100 mM NaF, 1 mM Na_3VO_4, and 10 μg/ml of each aprotinin, leupeptin, pefabloc, and pepstatin). For Western blot analysis, the precipitates were taken up in reducing 2× SDS sample buffer and analyzed as described above. For *in vitro* kinase assays, precipitates were further washed twice with kinase buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 1% Nonidet P-40, 1 mM Na_3VO_4, 5 mM dithiothreitol). Samples were then incubated at 30 °C for 20 min in kinase buffer containing 10 μCi of [γ-^{32}P]ATP/sample. The kinase reaction was stopped by addition of reducing 4× SDS sample buffer, proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and analyzed by autoradiography.

RESULTS

S. aureus Cowan Invades Human Cells—*S. aureus* has been reported to invade human non-professional phagocytes such as epithelial cells and fibroblasts (11, 14). Indeed, human 293T cells took up *S. aureus* Cowan, a clinical isolate from a septic arthritis patient, already within 60 min following infection and the number of internalized bacteria increased further over a 4 h period as measured in gentamicin/lysostaphin protection assays (Fig. 1*A*). When the infection proceeded for more than 4 h, cytotoxicity of 293T cells was observed suggesting that growth of bacteria and potential release of staphylococcal toxins was detrimental to the cultured cells after longer incubations (data not shown). Therefore, internalization assays were limited to a 2-h period. Interestingly, *S. carnosus*, a commensal species not associated with disease, was not internalized by the cells (Fig. 1*A*) indicating that invasion is a specific pathogen-induced process. Bacteria treated with gentamicin/lysostaphin in the absence of 293T cells could not be recovered demonstrating that both *S. aureus* and *S. carnosus* were sensitive to the antibiotic treatment and that only cell-associated, intracellular bacteria were able to survive the presence of gentamicin/lysostaphin (Fig. 1*A*). Importantly, fluorescence staining of infected cells showed the intracellular location of *S. aureus* as indicated by co-localization of the bacteria with the late endosomal/lysosomal marker h-lamp-2 (Fig. 1*B*). In contrast, *S. carnosus* was not detected intracellularly in infected cultures and did not co-localize with h-lamp-2 (Fig. 1*B*). These results demonstrated that *S. aureus* Cowan triggered internalization into human non-professional phagocytes and confirmed the results obtained with gentamicin/lysostaphin protection assays.

S. aureus Cowan Invasion Is Mediated by FnBPs Targeting Cellular Integrins—It has been reported recently, that *S. aureus* invasion into human cells is predominantly initiated by cell wall-attached FnBPs that recruit Fn to the surface of the bacteria (11, 12, 14). Consistent with this view, a FnBPA and FnBPB double mutant of *S. aureus* strain 8325–4 is severely attenuated in its ability to invade cells *in vitro* (11, 12, 14). In addition, Fn-binding domains derived from FnBPA can block Fn recruitment to the bacterial surface and thereby interfere with the attachment to and invasion into human cells (9, 10). To address if the internalization of the *S. aureus* Cowan strain used in this study relies on FnBP-directed interactions, we performed gentamicin/lysostaphin protection assays in the presence of the B-domain of FnBPA (as a GST fusion protein encompassing amino acids 303–568 of FnBPA; GST-FnBPA-B) that has been shown to competitively block FnBP-mediated events (9). Indeed, addition of GST-FnBPA-B inhibited the internalization of *S. aureus* Cowan into human epithelial cells in a dose-dependent manner, whereas addition of GST alone had no influence on bacterial invasion (Fig. 2*A*).

Fn can act as a bridge and indirectly link the bacteria to integrins on human cells. Supporting this view, the efficient internalization of *S. aureus* was inhibited by monoclonal anti-β_1 integrin antibodies, but not isotype-matched control antibodies directed against ICAM-1 (Fig. 2*B*). In addition, a GRGDS-peptide that functions as competitive inhibitor of β_1 integrin ligands was blocking *S. aureus* uptake in a dose-dependent manner (Fig. 2*C*), whereas a reverse order control peptide (SDGRG) had no effect (data not shown). Taken together, these data supported the view that the observed inva-

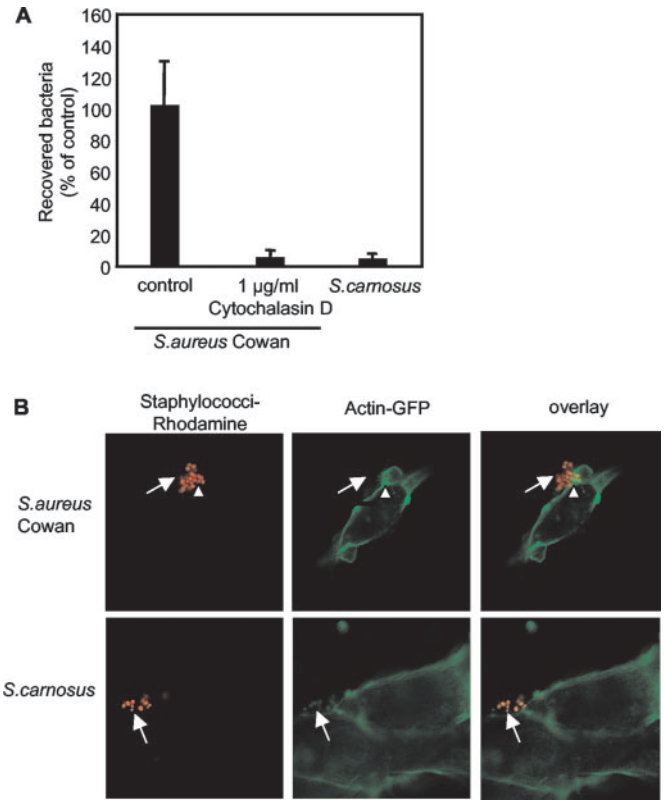

FIG. 3. ***S. aureus* invasion requires the host cell actin cytoskeleton.** *A*, 293T cells were infected for 2 h with *S. aureus* Cowan or *S. carnosus* in the presence of 1 μg/ml cytochalasin D and intracellular bacteria were enumerated with the help of gentamycin/lysostaphin protection assays. The graph shows mean values ± S.D. of two independent experiments done in triplicate. *B*, confocal laser scanning microscopy of GFP-actin-expressing 293T cells infected with rhodamine-labeled *S. aureus* Cowan or *S. carnosus* (*small arrow*). The *arrowhead* points to F-actin accumulations in the vicinity of attached *S. aureus*.

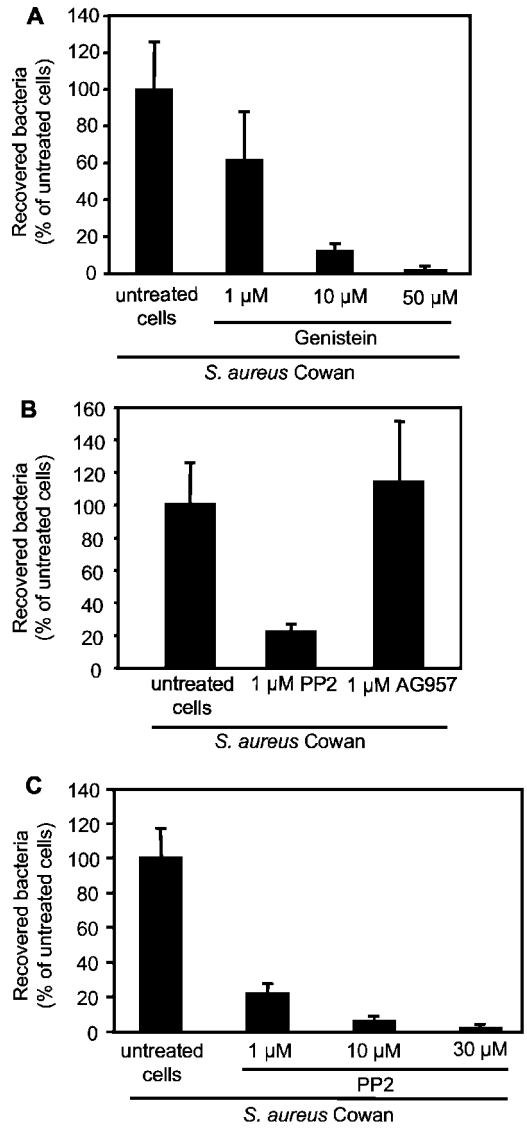

FIG. 4. **Host protein tyrosine kinase activity is essential for *S. aureus* internalization.** *A*, 293T cells were pretreated with the indicated concentrations of genistein and infected for 2 h with *S. aureus*. Intracellular bacteria were determined by gentamicin/lysostaphin protection assays. The graph shows mean values ± S.D. of two independent experiments done in triplicate. *B*, prior to infection, 293T cells were treated with 1 μM PP2 (inhibitor of Src family PTKs) or 1 μM AG957 (inhibitor of c-Abl). After 2 h of infection, intracellular *S. aureus* were determined by gentamicin/lysostaphin protection assays. The graph shows mean values ± S.D. of three independent experiments done in triplicate. *C*, prior to infection with *S. aureus* for 2 h, 293T cells were pretreated with the indicated concentrations of PP2. Intracellular bacteria were determined by gentamicin/lysostaphin protection assays. The graph shows mean values ± S.D. of three independent experiments done in triplicate.

sion of *S. aureus* in this system was due to FnBP-FN-β_1 integrin interactions.

S. aureus Invasion Relies on the Host Cell Actin Cytoskeleton—Integrins connect to multiple intracellular signaling pathways. Therefore, we wondered which integrin-initiated signals contribute to the internalization of *S. aureus*. Pharmacological inhibition of actin cytoskeleton dynamics by cytochalasin D potently diminished *S. aureus* invasion suggesting that it is the link between integrins and the actin cytoskeleton that is required for internalization of the microorganisms (Fig. 3*A*). To investigate actin dynamics during infection, 293T cells were transfected with a construct encoding actin fused to green fluorescent protein (Actin-GFP) and infected for 30 min with rhodamine-labeled *S. aureus* Cowan or *S. carnosus*, respectively. Confocal microscopy revealed a localized accumulation of polymerized actin in the vicinity of attached *S. aureus* (Fig. 3*B*). This actin polymerization seemed to be transient as it could only be observed during a short time window (20–60 min) during the initial phase of the interaction. In contrast to *S. aureus* Cowan, actin polymerization was not detectable at occasional contact zones between *S. carnosus* and 293T cells (Fig. 3*B*). These results suggested that attachment of *S. aureus* to host cell integrins induces dynamic rearrangements of the actin cytoskeleton that are involved in bacterial invasion.

S. aureus Cowan Invasion Requires Host Protein-tyrosine Kinase Activity—As integrins lack intrinsic enzymatic activity, integrin clusters at cellular adhesion sites are enriched in distinct signaling enzymes, such as PTKs that transduce integrin-initiated signals into the cell (18). Indeed, enhanced protein tyrosine phosphorylation is one of the early cellular responses following integrin stimulation. To address the role of cellular PTKs in *S. aureus* invasion, we employed genistein as a general inhibitor of PTK activity. Pretreatment of 293T cells with increasing concentrations of genistein blocked *S. aureus* internalization in a dose-dependent manner supporting the view that integrin-induced host PTKs play a critical role in regulating *S. aureus* host cell invasion (Fig. 4*A*). Several host PTKs have been shown to be activated in response to integrin engagement by ECM proteins (19, 20). To investigate whether particular integrin-stimulated kinases are involved in *S. aureus* invasion, we blocked Src family PTKs or the PTK c-Abl with the specific inhibitors PP2 and AG957, respectively (Fig.

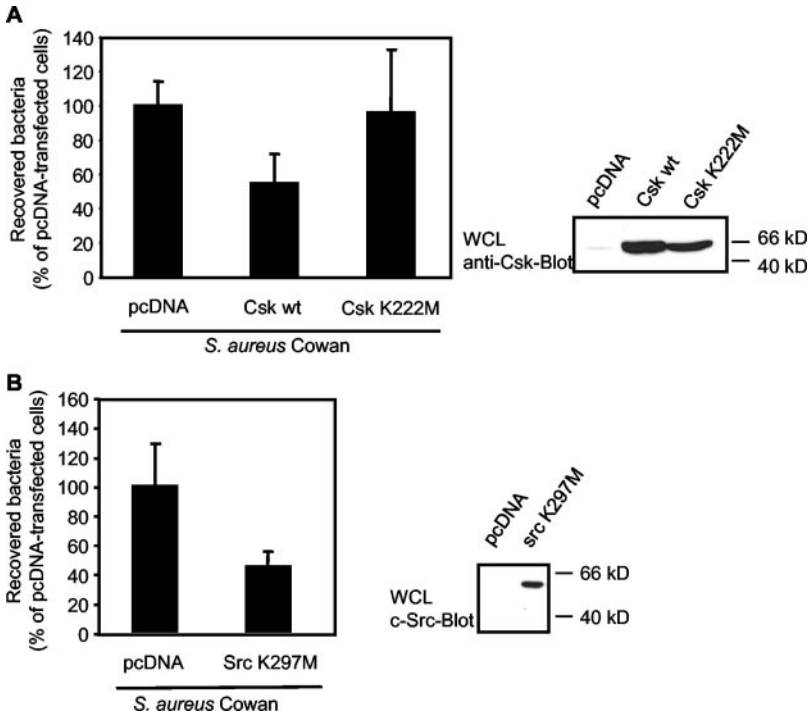

FIG. 5. **Interference with Src family kinase function blocks invasion of *S. aureus* Cowan.** *A*, 293T cells were transfected with constructs encoding wild-type C-terminal Src kinase (*Csk WT*), a kinase-inactive form of Csk (*Csk K222M*), or the control vector (pcDNA). Transfected cells were employed in gentamicin/lysostaphin protection assays with *S. aureus* Cowan. The graph shows mean values ± S.D. of two independent experiments done in triplicate. Western blotting of whole cell lysates (*WCL*) of the transfected cells with monoclonal anti-Csk antibody demonstrates expression of transfected Csk, respectively. *B*, 293T cells transfected with the empty control vector (pcDNA) or kinase-inactive c-Src (Src K297M) were infected with *S. aureus* and the number of internalized bacteria was determined in gentamicin/lysostaphin protection assays. The graph shows mean values ± S.D. of three independent experiments done in triplicate. Western blotting of whole cell lysates (WCL) with monoclonal anti-c-Src antibody demonstrates expression of transfected Src.

4*B*). Clearly, inhibition of Src PTKs by PP2 strongly impaired uptake of *S. aureus*, whereas AG957 had no effect (Fig. 4*B*). Importantly, PP2 blocked uptake of the pathogens in a dose-dependent manner resulting in a more than 90% inhibition at 10 μM of the inhibitor (Fig. 4*C*). These results suggested that Src family kinases were involved in the integrin-mediated internalization of *S. aureus*.

Src Activity Is Critical for Integrin-mediated Uptake of S. aureus—To further confirm a role of Src PTKs in *S. aureus* invasion, we choose a genetic approach to interfere with Src function. Overexpression of the C-terminal Src kinase (Csk), a negative regulator of Src PTK activity that tyrosine phosphorylates Src at a regulatory site at Tyr-529 (Tyr-527 in chicken c-Src), has been shown to inhibit Src PTK activity and to block Src PTK-dependent cellular events (21). Therefore, 293T cells were transiently transfected with expression plasmids encoding wild-type Csk (Csk WT) or a kinase-inactive form of Csk (Csk K222M) as control. Gentamicin/lysostaphin-protection assays with the transfected cells revealed that overexpression of Csk WT impaired the internalization of *S. aureus* by ~45% in comparison with 293T cells transfected with a control vector (pcDNA) (Fig. 5*A*). In addition, the kinase-inactive form of Csk, though expressed at equivalent levels as Csk WT, did not influence staphylococcal invasion (Fig. 5*A*) supporting the view that Src PTK activity is required for integrin-mediated uptake of *S. aureus*. It is important to stress that the used transfection protocol led to transgene expression in about 60–75% of the cell population as measured by FACS analysis of GFP-transfected 293T cells (data not shown). Therefore, the 45% reduction in overall invasion of *S. aureus* in transfected 293T cells corresponds to about 60–75% reduction of invasion in the transfected cell population.

To corroborate these findings, we employed a dominant-negative, kinase-inactive version of c-Src (Src K297M) and overexpressed this mutant in 293T cells. Notably, cells expressing Src K297M showed a prominent decrease in uptake of *S. aureus* (Fig. 5*B*) further indicating a critical role for Src kinase activity in integrin-mediated internalization of *S. aureus* and supporting our previous results obtained with the pharmacological inhibitor PP2 and overexpression of Csk.

Src-deficient Cells Are Resistant to Invasion by S. aureus—If Src PTKs play an essential role in integrin-mediated uptake of *S. aureus*, we hypothesized that Src-deficient cells should be resistant to staphylococcal invasion. To test this hypothesis, we employed fibroblasts derived from Src, Yes, and Fyn-deficient mouse embryos (SYF cells; (16)). SYF cells lack all three Src kinase family members that are normally expressed in this cell type. As a control, SYF cells re-expressing c-Src (SYF + c-Src cells) were used. Importantly, *S. aureus* was able to invade SYF + c-Src fibroblasts with similar kinetics as observed for 293T cells (Fig. 6*A*). Also, the total number of *S. aureus* recovered after gentamicin/lysostaphin treatment from SYF + c-Src cells was comparable to the number of bacteria recovered from 293T cells. Again, *S. carnosus* was not internalized by SYF + c-Src cells indicating that invasion into fibroblasts is a pathogen-induced process (Fig. 6*A*). Importantly, when the Src PTK-deficient SYF cells were employed in infection assays with *S. aureus*, the staphylococci barely invaded these cells verifying the critical role Src family PTKs play in *S. aureus* invasion via integrins (Fig. 6*B*). Differential fluorescence staining of intracellular and extracellular bacteria in infected cultures revealed that SYF + c-Src cells contained intracellular *S. aureus* after 2 h (Fig. 6*C*). Infected cultures of SYF cells contained equivalent amounts of bacteria that attached to the surface of the cells. However, the fluorescence staining revealed that almost all of these staphylococci were extracellularly localized (Fig. 6*C*). Taken together these results demonstrate a critical contribution of Src family PTKs to the integrin-mediated invasion of pathogenic *S. aureus*.

Enhanced Src Activity in Response to S. aureus Infection—The lack of *S. aureus* invasion in SYF cells as well as the inhibitory effects of PP2 treatment and Src K297M overexpression, respectively, implied that Src kinase activity might drive the integrin-mediated uptake of pathogenic staphylococci. To test if Src kinase activity is altered upon infection of cells, we investigated the activation status of Src in SYF + c-Src cells by two means: (i) using phosphospecific antibodies to detect phosphorylation of Src at regulatory tyrosine residues, and (ii) biochemically by *in vitro* kinase assays. Accordingly, serum-starved SYF + c-Src cells were plated on poly-L-lysine coated dishes to minimize integrin

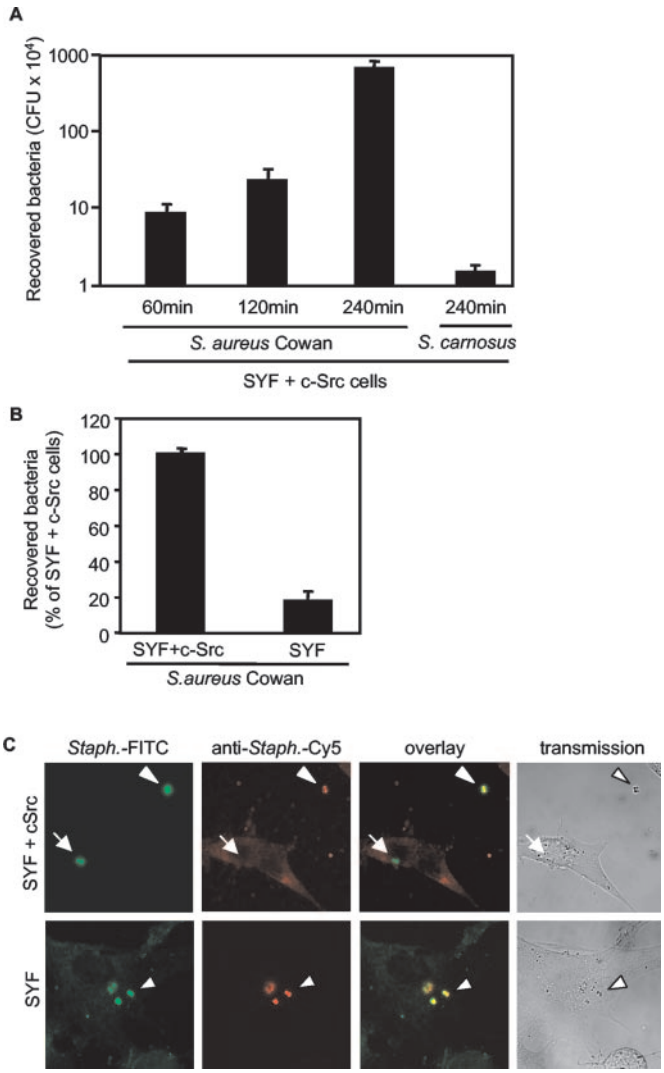

FIG. 6. **Src-deficient cells are resistant to *S. aureus* invasion.** *A*, invasion of *S. aureus* and *S. carnosus* in SYF + c-Src fibroblasts was measured at the indicated times following the infection by gentamicin/lysostaphin protection assays. The graph shows mean values ± S.D. of two independent experiments done in triplicate. *B*, SYF and SYF + c-Src cells were infected with *S. aureus* Cowan for 2 h and employed in gentamicin/lysostaphin protection assays. The graph shows mean values ± S.D. of three independent experiments done in triplicate. *C*, SYF and SYF + c-Src cells were infected with FITC-labeled staphylococci for 2 h. After fixation, extracellular bacteria were stained with polyclonal anti-*S. aureus* antibodies and Cy5-coupled secondary reagents. Extracellular bacteria (*arrowheads*) can be distinguished by their double-staining with FITC and Cy5 from FITC-only labeled intracellular bacteria (*small arrow*).

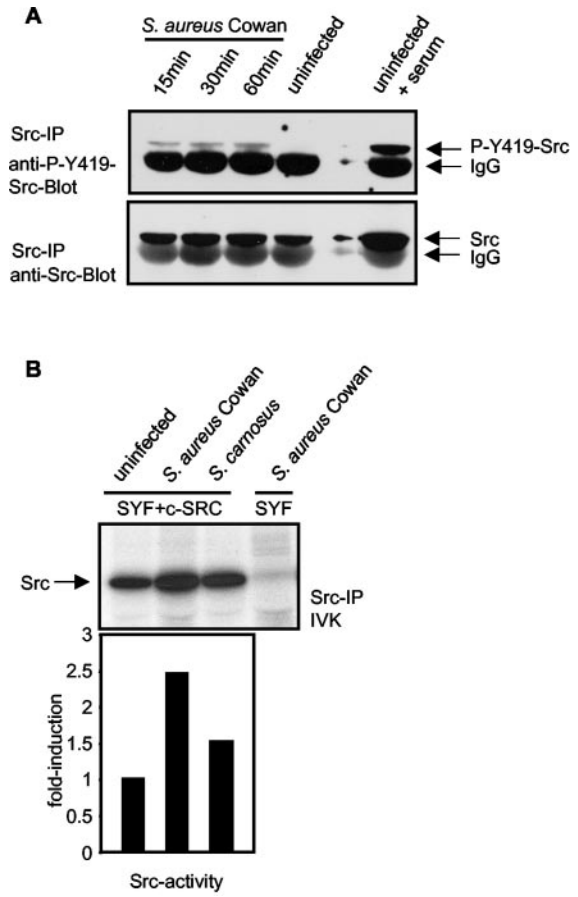

FIG. 7. **Cellular Src kinase activity is increased upon *S. aureus* infection.** *A*, serum-starved SYF + c-Src cells were seeded onto poly-L-lysine coated dishes and infected with *S. aureus* Cowan for the indicated times or left uninfected before lysis. After Src immunoprecipitation (*Src-IP*), samples were sequentially analyzed in Western blots with phosphospecific anti-Src P-Y-419 antibodies (*upper panel*) and monoclonal anti-Src antibodies (*lower panel*). Cells plated in the presence of serum served as a positive control for maximum Src activation. *B*, SYF + c-Src cells were infected with *S. aureus* Cowan or *S. carnosus* for 1 h or left uninfected. In addition, SYF cells were infected with *S. aureus* for 1 h. After lysis, Src was immunoprecipitated (Src-IP) and employed in an *in vitro* kinase autophosphorylation assay (*IVK*). Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes and Src activity detected by autoradiography. The membranes were probed with monoclonal anti-Src antibodies to detect the amounts of immunoprecipitated Src. The graph shows the fold increase of kinase activity corrected for the amount of kinase present in the immunoprecipitates of a representative experiment of three experiments.

engagement by the cell culture substrate. Two hours after plating, cells were infected for the indicated times with Fn-pretreated *S. aureus* or left uninfected and then lysed. After immunoprecipitation with a Src-specific monoclonal antibody, the samples were analyzed by Western blotting with a phosphospecific antibody recognizing the phosphorylated tyrosine residue 419 of human c-Src (Src Tyr-419; Tyr-416 in chicken Src). This tyrosine is situated in the activation loop of the Src kinase domain and its phosphorylation is indicative of active Src (22). As demonstrated in Fig. 6*A*, phosphorylation of Tyr-419 was elevated in *S. aureus*-infected cells compared with uninfected cells plated on poly-L-lysine. However, the proportion of pathogen-induced Src Tyr-419 phosphorylation in the infected cultures comprised only a minor fraction of the total amount of Src, as maximal Src Tyr-419 phosphorylation in cells plated in the presence of serum was about 10-fold higher (Fig. 7*A*). This small, but significant increase in Src activity is in line with the view, that integrin-bound staphylococci provide a locally confined and transient stimulus. The same membrane was reprobed with anti-Src antibodies to demonstrate equivalent amounts of total Src (phosphorylated and un-phosphorylated Src) in the samples (Fig. 7*A*). To analyze the enzymatic activity of Src directly, Src immunoprecipitates from uninfected, *S. aureus*- or *S. carnosus*-infected SYF + c-Src cells or *S. aureus*-infected SYF cells, respectively, were analyzed by *in vitro* kinase assays. Though uninfected and cells infected with Fn-pretreated *S. carnosus* exhibited a basal level of Src kinase activity, *in vitro* kinase activity of Src was about 2.5-fold elevated upon infection with Fn-pretreated *S. aureus* compared with uninfected cells (Fig. 7*B*) demonstrating that enzyme activity is changed upon staphylococcal infection. As expected, no Src kinase activity was detected in *S. aureus*-infected SYF cells (Fig. 7*B*). Taken together, these results provide evidence that Src enzymatic activity is enhanced upon infection with pathogenic staphylococci supporting our hypothesis that cellular Src kinase

function critically contributes to the integrin-mediated invasion of *S. aureus*.

DISCUSSION

Integrin $\alpha_5\beta_1$ is an essential cell surface receptor that mediates the attachment of eukaryotic cells to the extracellular matrix protein fibronectin (23). Similar to other integrins, this receptor is clustered at focal adhesion (FA) structures, where it helps to integrate the attachment of the cell to the extracellular matrix with the organization of the intracellular cytoskeleton. Though FA structures are critical for the mechanical support of the cell, they are highly dynamic and integrin binding to ECM proteins can trigger cytoskeletal rearrangements (15). Indeed, indirect engagement of $\alpha_5\beta_1$ integrin by FnBPs of *S. aureus* induces the actin-dependent uptake of this microorganism by its human host cell. Our results demonstrate that the integrin-initiated invasion of *S. aureus* critically depends on additional host cell factors and we have identified cellular Src PTKs to be essential for this process. In particular, Src kinase activity is enhanced upon infection with *S. aureus* and staphylococcal invasion into epithelial cells and fibroblasts is severely diminished after pharmacological or genetic inhibition of this non-receptor tyrosine kinase. Importantly, Src-deficient fibroblasts are resistant to staphylococcal invasion, demonstrating the essential role of this enzyme in integrin-triggered invasion.

Integrin-mediated internalization has been observed to be triggered by other pathogens as well (for review see (24)). In particular, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* employ an outer membrane protein termed invasin that directly associates with β_1 integrins and induces uptake into eukaryotic cells (25). The entry process seems to be important *in vivo*, as invasin-deficient bacteria are re-isolated in lower numbers from infected animals at the initial stage of the infection (26). It is thought that invasin allows the orally ingested *Yersinia* to overcome the intestinal barrier by exploiting β_1 integrins on M cells to gain access to Peyer's plaque-associated lymph nodes (27).

Interestingly, the human-specific pathogens *Neisseria gonorrhoeae* and *Neisseria meningitidis* express outer membrane proteins that, similar to staphylococcal MSCRAMMs, mediate binding to ECM proteins and indirectly connect the microbes with host cell integrins (28). In addition, invasive *Streptococcus pyogenes* indirectly engages host cell integrins via fibronectin (29, 30). The localized recruitment of integrin β_1 underneath attached bacteria induces the internalization of the streptococci into epithelial cells and fibroblasts (31), again demonstrating that these integrins can trigger uptake of bound particles.

Integrin β_1 is connected to the actin cytoskeleton by way of additional FA-associated cytoskeletal molecules such as tensin, vinculin, talin, paxillin, α-actinin, cortactin, and filamin that are recruited to clustered, ligand-bound integrins in a hierarchical manner (32). In addition, signaling molecules partition to FA structures, most prominently the focal adhesion kinase (FAK) and Src family PTKs (32). It is interesting to point out that several of the FA-associated cytoskeletal proteins are substrates for the tyrosine kinase activities of FAK and Src (33–35). Though a comprehensive view of the regulatory phosphorylation events in the context of FA remodeling is emerging (36), the nature of signaling molecules involved in integrin internalization is unclear. Previously, it has been shown that Src family PTKs can function to uncouple the linkage between an integrin cytoplasmic domain and the actin cytoskeleton (37). In addition, FAK seems to function in the dynamic turnover of FA during directed cell migration (38). Therefore, it has been speculated that Src kinases together with FAK enhance the surface availability of integrins for bacterial binding by releasing the receptors from FA structures (25). As in our experimental setup the cells are plated onto a poly-L-lysine coated substrate that does not engage integrins, these receptors should be readily available to the bacteria. Therefore, Src kinase activity seems to play a more direct role in the integrin-mediated internalization process, presumably by regulating actin-associated proteins involved in the cytoskeletal rearrangements that accompany bacterial uptake.

Though *S. aureus* possesses multiple extracellular matrix binding proteins (4), *in vitro* invasion of eucaryotic cells seems to be predominantly mediated by the Fn-binding proteins FnBPA and FnBPB (11, 12). However, the role of the *S. aureus* FnBP-Fn-integrin interaction for the infection process *in vivo* is not yet understood. It is plausible that the indirect attachment of the microorganisms to host cell integrins could also promote the internalization of the microbe *in vivo*, thereby sheltering the bacteria from certain immune defenses. Indeed, in 163 clinical isolates of *S. aureus* either one or both FnBP genes were detected and presence of both FnBPs seems to be correlated with invasive types of disease when compared with strains derived from nasal carriers (39). However, as engagement of integrins is connected to a multitude of cellular responses including alterations in gene expression, cell morphology, and cell proliferation, it is tempting to speculate that staphylococcal binding to host cell integrins could also stimulate processes in addition to cellular uptake. How such responses will affect the dynamic interaction between the pathogen and its host in the course of disease will be a challenging task for future investigations.

Acknowledgments—We thank P. Soriano (FHCRC, Seattle, WA) for the SYF cells, D. Schlaepfer (TSRI, San Diego, CA) for cDNA constructs, and J. Hacker (Institut für Molekulare Infektionsbiologie, Würzburg, Germany) for continuing support and comments on the manuscript.

REFERENCES

1. Steinberg, J. P., Clark, C. C., and Hackman, B. O. (1996) *Clin. Infect. Dis.* **23,** 255–259
2. Lowy, F. D. (1998) *N. Engl. J. Med.* **339,** 520–532
3. Patti, J. M., Allen, B. L., McGavin, M. J., and Hook, M. (1994) *Annu. Rev. Microbiol.* **48,** 585–617
4. Foster, T. J., and Hook, M. (1998) *Trends Microbiol.* **6,** 484–488
5. Flock, J. I., Froman, G., Jonsson, K., Guss, B., Signas, C., Nilsson, B., Raucci, G., Hook, M., Wadstrom, T., and Lindberg, M. (1987) *EMBO J.* **6,** 2351–2357
6. Jonsson, K., Signas, C., Muller, H. P., and Lindberg, M. (1991) *Eur. J. Biochem.* **202,** 1041–1048
7. Schwarz-Linek, U., Werner, J. M., Pickford, A. R., Gurusiddappa, S., Kim, J. H., Pilka, E. S., Briggs, J. A., Gough, T. S., Hook, M., Campbell, I. D., and Potts, J. R. (2003) *Nature* **423,** 177–181
8. Fischer, B., Vaudaux, P., Magnin, M., el Mestikawy, Y., Proctor, R. A., Lew, D. P., and Vasey, H. (1996) *J. Orthop. Res.* **14,** 914–920
9. Massey, R. C., Kantzanou, M. N., Fowler, T., Day, N. P., Schofield, K., Wann, E. R., Berendt, A. R., Hook, M., and Peacock, S. J. (2001) *Cell Microbiol.* **3,** 839–851
10. Joh, D., Wann, E. R., Kreikemeyer, B., Speziale, P., and Hook, M. (1999) *Matrix Biol.* **18,** 211–223
11. Sinha, B., Francois, P. P., Nusse, O., Foti, M., Hartford, O. M., Vaudaux, P., Foster, T. J., Lew, D. P., Herrmann, M., and Krause, K. H. (1999) *Cell Microbiol.* **1,** 101–117
12. Dziewanowska, K., Patti, J. M., Deobald, C. F., Bayles, K. W., Trumble, W. R., and Bohach, G. A. (1999) *Infect. Immun.* **67,** 4673–4678
13. Jett, B. D., and Gilmore, M. S. (2002) *Infect. Immun.* **70,** 4697–4700
14. Fowler, T., Wann, E. R., Joh, D., Johansson, S., Foster, T. J., and Hook, M. (2000) *Eur. J. Cell Biol.* **79,** 672–679
15. Schwartz, M. A., and Ginsberg, M. H. (2002) *Nat. Cell Biol.* **4,** E65–E68
16. Klinghoffer, R. A., Sachsenmaier, C., Cooper, J. A., and Soriano, P. (1999) *EMBO J.* **18,** 2459–2471
17. Hauck, C. R., Hunter, T., and Schlaepfer, D. D. (2001) *J. Biol. Chem.* **276,** 17653–17662
18. Aplin, A. E., Howe, A., Alahari, S. K., and Juliano, R. L. (1998) *Pharmacol. Rev.* **50,** 197–263
19. Parsons, J. T., and Parsons, S. J. (1997) *Curr. Opin. Cell Biol.* **9,** 187–192
20. Lewis, J. M., Baskaran, R., Taagepera, S., Schwartz, M. A., and Wang, J. Y. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 15174–15179
21. Imamoto, A., and Soriano, P. (1993) *Cell* **73,** 1117–1124
22. Owens, D. W., McLean, G. W., Wyke, A. W., Paraskeva, C., Parkinson, E. K., Frame, M. C., and Brunton, V. G. (2000) *Mol. Biol. Cell* **11,** 51–64
23. Hynes, R. O. (1996) *Dev. Biol.* **180,** 402–412
24. Hauck, C. R. (2002) *Med. Microbiol Immunol.* **191,** 55–62
25. Isberg, R. R., and Barnes, P. (2001) *J. Cell Sci.* **114,** 21–28

26. Pepe, J. C., and Miller, V. L. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6473–6477
27. Clark, M. A., Hirst, B. H., and Jepson, M. A. (1998) *Infect. Immun.* **66,** 1237–1243
28. Hauck, C. R., and Meyer, T. F. (2003) *Curr. Opin. Microbiol.* **6,** 43–49
29. Ozeri, V., Rosenshine, I., Mosher, D. F., Fassler, R., and Hanski, E. (1998) *Mol. Microbiol.* **30,** 625–637
30. Talay, S. R., Zock, A., Rohde, M., Molinari, G., Oggioni, M., Pozzi, G., Guzman, C. A., and Chhatwal, G. S. (2000) *Cell Microbiol.* **2,** 521–535
31. Ozeri, V., Rosenshine, I., Ben-Ze'Ev, A., Bokoch, G. M., Jou, T. S., and Hanski, E. (2001) *Mol. Microbiol.* **41,** 561–573
32. Miyamoto, S., Teramoto, H., Coso, O. A., Gutkind, J. S., Burbelo, P. D., Akiyama, S. K., and Yamada, K. M. (1995) *J. Cell Biol.* **131,** 791–805
33. Izaguirre, G., Aguirre, L., Hu, Y. P., Lee, H. Y., Schlaepfer, D. D., Aneskievich, B. J., and Haimovich, B. (2001) *J. Biol. Chem.* **276,** 28676–28685
34. Brown, M. T., and Cooper, J. A. (1996) *Biochim. Biophys. Acta.* **1287,** 121–149
35. Thomas, S. M., and Brugge, J. (1997) *Annu. Rev. Cell Dev. Biol.* **13,** 513–609
36. Zamir, E., and Geiger, B. (2001) *J. Cell Sci.* **114,** 3583–3590
37. Felsenfeld, D. P., Schwartzberg, P. L., Venegas, A., Tse, R., and Sheetz, M. P. (1999) *Nat. Cell Biol.* **1,** 200–206
38. Hauck, C. R., Hsia, D. A., and Schlaepfer, D. D. (2002) *IUBMB Life* **53,** 115–119
39. Peacock, S. J., Day, N. P., Thomas, M. G., Berendt, A. R., and Foster, T. J. (2000) *J. Infect.* **41,** 23–31