

# Staphylococcal Fibronectin Binding Protein Interacts with Heat Shock Protein 60 and Integrins: Role in Internalization by Epithelial Cells

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We reported previously that internalization of *Staphylococcus aureus* by nonprofessional phagocytes involves an interaction between fibronectin (Fn) binding protein (FnBP) and the host cell, resulting in signal transduction, tyrosine kinase activity, and cytoskeletal rearrangement (K. Dziewanowska, J. M. Patti, C. F. Deobald, K. W. Bayles, W. R. Trumble, and G. A. Bohach, *Infect. Immun.* 67:4673–4678, 1999). The goal of the present study was to identify the host molecules responsible for uptake of the organism through an interaction with FnBP. First, Fn was required for internalization. Addition of small amounts of exogenous Fn stimulated the uptake of *S. aureus* by HEP-2 cells, which are deficient in Fn synthesis. Fn antibodies blocked internalization of the organism by MAC-T cell monolayers, a bovine epithelial cell line which expresses Fn. Second, a monoclonal antibody (MAb) specific for  $\beta_1$  integrins dramatically reduced *S. aureus* invasion, suggesting that the formation of a Fn bridge linking the host cell  $\beta_1$  integrin and FnBP precedes internalization. However, ligand blotting of cell membrane proteins with a functional fragment of FnBP consistently identified an additional ~55-kDa receptor on both human and bovine epithelial cells. This protein was purified and identified by N-terminal microsequencing as heat shock protein 60 (Hsp60). The interaction between FnBP and Hsp60 also occurred when the whole cells were used. Cell membrane localization of Hsp60 was confirmed by biotinylation with an agent nonpermeable to the cell membrane. Pretreatment of epithelial cells with a MAb specific for eukaryotic Hsp60 significantly reduced internalization of *S. aureus*. Combined, these results suggest that the FnBP binds directly to both Hsp60 and Fn and is linked to  $\beta_1$  integrins through a Fn bridge. The simultaneous involvement of Fn and two host cell ligands,  $\beta_1$  integrins and Hsp60, suggests that FnBP is a multifunctional adhesin that mediates internalization in a manner similar to that proposed for OpaA, the *Neisseria gonorrhoeae* FnBP homolog (J. P. M. van Putten, T. D. Duensing, and R. L. Cole, *Mol. Microbiol.* 29:369–379, 1998).

Bacteria have developed various mechanisms for inducing internalization into nonprofessional phagocytes (8, 9). A shared requirement is that a molecular interaction must occur between a bacterial surface adhesin and a host ligand in the cytoplasmic membrane. This interaction must be of sufficiently high affinity to induce signal transduction through the membrane, resulting in cytoskeletal rearrangements and uptake of the organism (14, 37). For some organisms, a simple model involving the binding of a bacterial adhesin to its host receptor is sufficient to induce uptake. *Yersinia* species and *Listeria monocytogenes* are examples of organisms with this pattern of uptake. The adhesins on these facultative intracellular pathogens bind directly to integrins or E-cadherin, respectively, with an affinity that is sufficient to induce internalization (15, 24).

A number of reports have described bacterial surface adhesins, which adhere to host extracellular matrix (ECM) proteins. The ECM binding proteins are termed MSCRAMMS, and *Staphylococcus aureus* expresses several of these proteins with different ligand specificities (17, 18, 27). We demonstrated previously that the *S. aureus* surface adhesin responsible for stimulating signal transduction upon uptake by nonprofes-

sional phagocytes is one of its MSCRAMMs, fibronectin (Fn) binding protein (FnBP) (6). Our data were confirmed in two additional publications (22, 28). This finding raised several questions regarding the nature of the molecular interactions at the host cell surface.

Fn is the ECM protein commonly associated with integrins. It is known that Fn is bivalent and can serve as a bridging molecule between FnBP and the host cell integrins (17, 26, 39). Although other bacteria use Fn as a link to adhere to host tissues, the mechanisms by which this linkage could induce internalization are less clear. For example, Tran Van Nhieu and Isberg showed that coating of *S. aureus* with Fn did not lead to efficient internalization (37). It was proposed that the binding affinity between Fn and integrins was not sufficient to induce uptake. Interestingly, at least one organism, *Neisseria gonorrhoeae* has overcome this limitation by employing a heparin-containing accessory coreceptor, which is necessary to induce maximal internalization by HEP-2 cells (39).

The present study was initiated to identify potential cellular ligands and other molecular requirements for uptake of *S. aureus* by nonprofessional phagocytes. Using a variety of methods, we found that FnBP binds directly to heat shock protein 60 (Hsp60) on the membranes of human and bovine epithelial cells. Fn and  $\beta_1$  integrins are also required for maximal uptake. Based on these combined results, a potential model to explain the molecular interactions leading to uptake of *S. aureus* is proposed.

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TABLE 1. *S. aureus* strains used in this study

Strain	Relevant genotype <sup>a</sup>	Relevant phenotype or properties <sup>a</sup>	Reference
8325-4	<i>fnbA</i> <sup>+</sup> <i>fnbB</i> <sup>+</sup>	FnBPA <sup>+</sup> FnBPB <sup>+</sup> ; NCTC 8325, cured of prophages, plasmid free	25
DU5883	<i>fnbA::Tc<sup>r</sup>fnbB::Em<sup>r</sup></i>	FnBPA <sup>-</sup> FnBPB <sup>-</sup> ; isogenic mutant of 8325-4	10
DU5883(pFnBPA4)	<i>Ap<sup>r</sup> Cm<sup>r</sup> fnbpA<sup>+</sup></i>	FnBPA <sup>+</sup> FnBPB <sup>-</sup> ; DU5883 with multicopy shuttle plasmid carrying wild-type <i>fnbpA</i>	10
DU5875	<i>spa::Tc<sup>r</sup></i>	Spa <sup>-</sup> ; isogenic mutant of 8325-4	23

<sup>a</sup> *fnbpA* and *fnbpB* refer to the structural genes encoding the two staphylococcal FnBPs, A and B, and *Spa* refers to staphylococcal protein A.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The *S. aureus* strains used in this study are listed in Table 1. All strains were grown in Todd-Hewitt broth (Difco Laboratories) for 18 h at 37°C with aeration, in the presence of antibiotics when necessary. The bacterial cells were grown to stationary phase, collected by centrifugation, and washed with sterile 150 mM phosphate-buffered saline (PBS) (pH 7.2). The washed pellets were resuspended to their original volumes in invasion medium (described below), diluted 1:100 with the same medium, and returned to 37°C for 1 to 2 h to induce logarithmic growth.

**Cell culture.** An established bovine mammary epithelial cell line (MAC-T) (12) was cultured as described previously (6). Briefly, the cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL), supplemented with 10% heat-inactivated fetal bovine serum (HyClone), insulin (5 µg/ml), hydrocortisone (5 µg/ml), penicillin (100 U/ml), and streptomycin sulfate (100 µg/ml). HEP-2 (ATCC CCL 23) and Caco-2 (ATCC HTB 37) cells were cultured in the same medium except that insulin and hydrocortisone were omitted. The cells were seeded to 24-well culture plates (6 × 10<sup>4</sup>/well) or to 100-mm-diameter culture dishes (2 × 10<sup>6</sup>/dish) (Costar) and incubated at 37°C under 6% CO<sub>2</sub>. When nearly confluent, the monolayers were washed once with sterile PBS and incubated overnight (at 37°C under 6% CO<sub>2</sub>) in DMEM (1.0 ml/well).

**Internalization assays.** For most bacterial internalization assays, the following standard assay was employed. Confluent cell monolayers (approximately 2.3 × 10<sup>5</sup> MAC-T cells/well or 4.0 × 10<sup>5</sup> HEP-2 cells/well) were washed once with DMEM containing 1% bovine serum albumin (BSA) and inoculated with bacteria suspended in the same medium (multiplicity of infection [MOI], 10 to 50). After incubation for 1 h (at 37°C under 6% CO<sub>2</sub>), the wells were washed once with PBS and 1.0 ml of invasion medium, supplemented with 100 µg of gentamicin per ml, was added to each well. The plates were incubated for an additional 1 h to kill extracellular bacteria. The monolayers were washed four times with sterile PBS, detached from the plates by treatment for 5 min at 37°C with 100 µl of trypsin solution (0.25%) in Hanks' balanced salt solution (HBSS) (Gibco-BRL), and then lysed by adding 900 µl of sterile deionized water. The cell lysates were serially diluted 10-fold and plated in triplicate on Todd-Hewitt agar plates to quantify intracellular staphylococci.

To assess internalization in the presence of various potential effectors, the standard assay described above was modified as follows. Prior to inoculation with bacteria, the nearly confluent monolayers were preincubated for 30 min (at 37°C under 6% CO<sub>2</sub>) in DMEM containing 1% BSA, plus one of the following: bovine Fn (1 to 100 nM) (Calbiochem), protein G-purified immunoglobulin G1 LK-1 murine monoclonal antibody (MAb) specific for eukaryotic Hsp60 (recombinant human Hsp60 was used as the immunogen; the epitope is located between residues 383 and 447 of human Hsp60, so that the antibody identifies Hsp60 in eukaryotes but not in prokaryotes) (StressGen Corp.), bovine Fn antiserum prepared in rabbits (Biogenesis Inc.), nonimmune rabbit serum (Sigma), human β<sub>1</sub> integrin ascites prepared in mice (clone P4C10; Gibco-BRL), or nonimmune murine ascites. The cultures were then infected with bacteria and processed by the standard internalization assay method.

An additional modification was made in some experiments to assess the effect of ligand-induced receptor translocation on internalization. The protocol for these experiments was based on the receptor downmodulation method described by Rozdzinski and Tuomanen (30). Wells in 96-well microtiter plates (A1/2; Costar) were coated by adding 50 µl of one of the following reagents dissolved in PBS containing 2 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub> (DPBS): LK-1 Hsp60 MAb (10 µg/ml), Du-D4 functional FnBP Fn binding fragment (16) (100 µg/ml), or 0.1% BSA. The plates were incubated for 1 h at room temperature and then overnight at 4°C. After the plates were washed three times with DPBS, MAC-T cells (5 × 10<sup>4</sup>) in 100 µl of invasion medium-0.1% BSA were added to each well. The plates were incubated (at 37°C under 6% CO<sub>2</sub>) for 1 h to allow attachment and spread of the cells. Subsequently 100 µl of bacterial cell suspension (approximately 3.5 × 10<sup>5</sup> cells) in DMEM-0.1% BSA was added to achieve a MOI between 20 and 30. The plates were incubated for 30 min at 37°C under 6% CO<sub>2</sub> to allow internalization of the bacterial inoculum and then processed by the standard internalization assay method.

**SDS-PAGE and blotting.** Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5 or 10% acrylamide gel slabs. Proteins were visualized within the gels by staining with Coomassie blue R-250 or by silver staining.

For some experiments, the resolved proteins were transferred to nitrocellulose (pore size, 0.45 µm; Schleicher & Schuell) or Immobilon P (Millipore Corp.)

membranes in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad), using standard methods, and were detected by immunoblotting or ligand blotting. For ligand blotting, potential membrane receptors were probed with Du-D4 peptide (10 µg per ml of blocking solution [3% BSA, 150 mM Tris HCl (pH 7.5), 150 mM NaCl]) followed by a rabbit polyclonal Du-D4 antiserum. Hsp60 immunoblot analyses were conducted using the LK-1 MAb (see above).

**Purification of staphylococcal FnBP.** Wild-type *S. aureus* expresses two highly conserved forms of FnBP, FnBPA and FnBPB (10). Each is encoded by a separate gene, designated *fnbpA* and *fnbpB*, respectively. *S. aureus* DU5885 (pFnBPA4) is a complemented deletion mutant that harbors the *fnbpA* gene on a recombinant plasmid and overexpresses FnBPA, and it was used to purify the protein for this present study.

*S. aureus* DU5883(pFnBPA4) was grown in Todd-Hewitt broth plus chloramphenicol (5 µg/ml) to a density of ca. 5 × 10<sup>7</sup> cells per ml. The cells were collected by centrifugation, washed twice with PBS, and resuspended (12 g of wet weight) in 50 ml of 0.7 M sucrose solution (pH 6.5) containing 0.02 M maleate, 0.02 M MgCl<sub>2</sub>, 2 × Bacto Antibiotic Medium 3 (Difco Laboratories), and 200 µg of lysostaphin (Sigma). The suspension was incubated for 15 to 30 min, until cell wall lysis was complete. The suspension was clarified by centrifugation for 30 min at 14,000 × g and then dialyzed exhaustively against water (4°C). FnBP was partially purified from the retentate by preparative isoelectric focusing in crushed Sephadex G-50, using Ampholine (pH 3.5 to 10.0) (Amersham Pharmacia Biotech AB) (11). Gel fractions were analyzed by SDS-PAGE (10% acrylamide) and immunoblotting with rabbit polyclonal serum specific for FnBP (see above). Fractions containing FnBP with only minor impurities were subjected to further purification (to homogeneity) by precipitation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (60% saturation). The purified protein was dialyzed against 50 mM ammonium acetate and lyophilized.

**Isolation of epithelial cell membrane proteins.** MAC-T and HEP-2 cells were grown to near confluency (2.8 × 10<sup>7</sup> MAC-T cells/dish and 5.5 × 10<sup>7</sup> HEP-2 cells/dish) in 100-mm-diameter culture dishes, washed twice with HBSS, and then detached by addition of 1 mM EDTA in HBSS for 20 min at 37°C. The detached cells were washed twice with HBSS-5 mM MgCl<sub>2</sub>-0.5 mM CaCl<sub>2</sub> and were either processed immediately or frozen at -80°C until use.

To isolate crude membrane protein preparations, the cell pellet was suspended in 10 volumes of 10 mM Tris (pH 7.5) containing 1 mM EDTA and protease inhibitor cocktail (Boehringer GmbH, Mannheim, Germany). After being incubated for 20 min on ice, the suspension was transferred to, and lysed using, a Dounce homogenizer. Cell lysis was monitored by light microscopy; 10 strokes were usually sufficient. Cell debris was removed by centrifugation initially for 10 min at 1,000 × g, and the membrane fraction was recovered by centrifugation of the supernatant at 100,000 × g for 1 h at 4°C. Proteins in the membrane fraction pellet (derived from 2 × 10<sup>7</sup> cells) were dissolved by incubation (3 h at 4°C) in 50 mM HEPES buffer (pH 7.5) containing 100 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, and protease inhibitor cocktail. The preparation was clarified by centrifugation for 20 min at 100,000 × g and analyzed by SDS-PAGE and electroblotting or used in ligand affinity experiments.

**Identification of potential epithelial cell membrane receptors by using an affinity gel matrix.** Affi-Gel 102 was purchased from Bio-Rad, and purified FnBP (see above) was ligated to the gel as specified by the manufacturer. Typically, 0.25 ml of Affi-Gel 102 and 200 µg of FnBP were used. An aliquot of solubilized membrane proteins (100 µl) derived from 2 × 10<sup>7</sup> cells was diluted 10-fold with 50 mM HEPES buffer (pH 7.5) containing 100 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, and protease inhibitor cocktail. The protein preparation was then mixed with 100 µl of FnBP-Affi-Gel 102 in a 1.7-ml centrifuge tube and incubated for 3 h at 4°C on a rocker. The gel was washed extensively with 50 mM HEPES buffer (pH 7.5) containing 100 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, and protease inhibitor cocktail. The bound proteins were dissociated from the gel by adding SDS-PAGE sample buffer and analyzed by SDS-PAGE.

**Biotinylation of surface membrane proteins.** Biotinylation reactions were performed on nearly confluent cultures of MAC-T cells or HEP-2 cells. The cells were treated with sulfo-*N*-hydroxysuccinimide-long-chain spacer arm (sulfo-NHS-LC-biotin; Pierce), which is not permeable to the cell membrane, or with the negative control reagent sulfo-NHS-acetate as recommended by the manufacturer. Membrane proteins from cells exposed to the biotinylation reagents were extracted by the method described above for isolation of membrane proteins. The solubilized and biotinylated membrane proteins were analyzed by

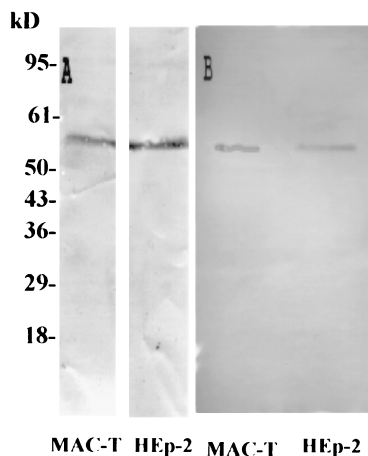


FIG. 1. Ligand blotting for FnBP receptors on epithelial cells. Proteins in MAC-T or HEp-2 cell membrane preparations were resolved by SDS-PAGE and transferred to membranes. The proteins were probed with Du-D4 peptide followed by Du-D4 rabbit antiserum (A) or with Hsp60 MAb (B).

SDS-PAGE and electroblotting. For the localization of proteins, the blots were incubated with a streptavidin-alkaline phosphatase conjugate (Sigma) or with specific antibodies.

**Microsequencing.** For N-terminal sequencing reactions, proteins were resolved by a double SDS-PAGE separation (20). Crude membrane fractions from MAC-T and HEp-2 cells were resolved first in SDS-PAGE preparative gels (10% acrylamide), and the proteins were localized by staining with Coomassie brilliant blue. The desired protein bands were excised. After the gel had been shrunk by soaking in 50% ethanol–50% stacking gel buffer, the excised band was subjected to an additional electrophoresis step, using an SDS-PAGE gel (7.5% acrylamide). Proteins were electroblotted to Immobilon P membranes (Millipore Corp.) backed by nitrocellulose membranes. Proteins on the Immobilon P membrane were localized by staining with Coomassie brilliant blue, and those on the nitrocellulose membrane were probed with Du-D4 peptide and Du-D4 rabbit antiserum. The protein band colocalizing on both blots was excised from the Immobilon P membrane, and N-terminal sequencing was performed by the Edman procedure using a 477A Sequencer.

**Interaction between FnBP and Hsp60 on whole cells.** Nearly confluent MAC-T cell monolayers (in 100-mm-diameter culture dishes) were inoculated with *S. aureus* DU5883 or *S. aureus* DU5883(pFnBPA4) at a MOI of 50. After 1 h of incubation at 37°C under 6% CO<sub>2</sub>, the monolayers were washed three times with HBSS and the cells were lysed by being suspended in 1.5 ml of 50 mM HEPES buffer containing 1% Triton X-100, 1% deoxycholate, 1 mM EDTA, and protease inhibitor cocktail. The lysate was transferred to a 2.0-ml centrifuge tube, and insoluble material was recovered by centrifugation for 10 min at 10,000 × *g*. The pellet was washed three times with lysing solution, solubilized with SDS-PAGE sample buffer, and analyzed by SDS-PAGE, immunoblotting, or ligand blotting, using Hsp60 MAb or Du-D4 peptide probes, as described above.

## RESULTS

**A functional fragment of FnBP binds to Hsp60 from epithelial cell membrane fractions.** Previous studies in our laboratory have demonstrated that FnBP is essential for efficient internalization within mammary epithelial cells (6). To identify the cell receptor for *S. aureus*, a ligand-blotting method was employed. Cell membrane proteins, obtained from MAC-T and HEp-2 cells lysates, were resolved by SDS-PAGE (10% acrylamide) and transferred to nitrocellulose. Proteins on the nitrocellulose were probed with Du-D4, a peptide fragment corresponding to the functional ECM binding molecular regions of FnBP. The location of the bound Du-D4 was determined by immunoblotting with Du-D4 rabbit antiserum. This assay revealed that Du-D4 bound significantly to only one major protein band, with an apparent size of ~55 kDa (Fig. 1A).

Membrane preparations were obtained from MAC-T, HEp-2, or Caco-2 cells. The partially purified membrane proteins were solubilized and incubated with an affinity gel matrix

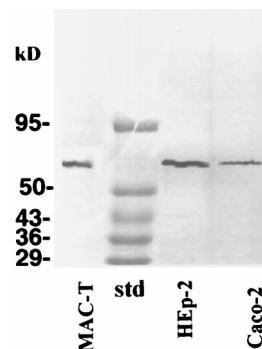


FIG. 2. Affinity purification of potential host cell receptors on FnBP-Affi-Gel 102. Solubilized membrane fractions from MAC-T cells, HEp-2 cells, or Caco-2 cells were incubated with FnBP-Affi-Gel 102. The gel was then washed extensively. Proteins on the gel were eluted, analyzed by SDS-PAGE, stained, and in some experiments subjected to N-terminal sequence determinations. Control BSA-Affi-Gel 102 conjugates did not retain proteins from cell lysates (results not shown). std, protein standards.

prepared by ligation of FnBP to Affi-Gel 102. From all three cell types, a protein with an apparent size of ~55 kDa was retained by the gel (Fig. 2). Control Affi-Gel 102 preparations, derivatized with BSA, did not retain proteins from cell lysates (results not shown).

The identity of this protein was determined by N-terminal microsequencing after being transferred to an Immobilon P membrane. The unambiguous and identical sequence AKDV KFGADA was obtained for the proteins isolated from either MAC-T cells or HEp-2 cells. A search of PIR International Pattern Match, protein databases, and Bioinformatics Tool ([www-nbrf.georgetown.edu/pirwww/search/patmatch.html](http://www-nbrf.georgetown.edu/pirwww/search/patmatch.html)) revealed an exact match with the 10 N-terminal amino acids of human and bovine Hsp60. The identity of the proteins from MAC-T and HEp-2 cells was further confirmed by immunoblotting with the LK-1 Hsp60 MAb (Fig. 1B).

**Hsp60 is exposed on the cytoplasmic membrane of epithelial cells.** A potential role for Hsp60 in mediating bacterial adherence and uptake would require its presence on the host cell surface. Although initial experiments (described above) demonstrated the binding of FnBP to Hsp60 in a membrane fraction, it was possible that Hsp60 originated from contamination by a mitochondrial fraction. Thus, to confirm the cell membrane localization and surface exposure of Hsp60, human and bovine epithelial cells were treated with sulfo-NHS-LC-biotin, a biotin derivative which is not permeable to cell membrane. Therefore, any biotinylation observed is attributed to proteins on the cell surface. Furthermore, addition of a biotin tag specifically to the cell surface proteins would be expected to generate two pools of Hsp60 (biotinylated and nonbiotinylated), one derived from the cytoplasmic membrane and the other derived from the intracellular location. As expected, two bands were detected in immunoblots of lysates from surface-biotinylated MAC-T and HEp-2 cells probed with an Hsp60 MAb (Fig. 3). The two bands corresponded to nonbiotinylated Hsp60 (lower band) and its biotinylated derivative (upper band). The latter band is absent in the blot of a control MAC-T cell preparation treated with sulfo-NHS-acetate. These results confirm the cell surface membrane localization of Hsp60 and are consistent with a potential for Hsp60 to interact with FnBP on *S. aureus* cells.

**Interaction of FnBP and Hsp60 on whole cells.** In an experiment to determine the specificity of the Hsp60 interaction with FnBP, MAC-T cell monolayers were incubated with *S.*

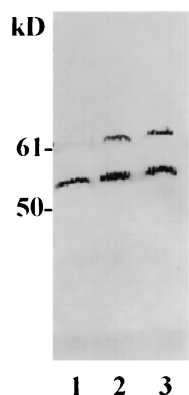


FIG. 3. Immunoblot of biotin-tagged and nontagged Hsp60. Intact MAC-T or HEP-2 cells were biotinylated with sulfo-NHS-LC-biotin (lanes 2 and 3) or treated with sulfo-NHS-acetate (for MAC-T cells only) as a negative control (lane 1). Cell lysates were resolved by SDS-PAGE and probed with an Hsp60 MAb.

*aureus* DU5883, a mutant deficient in FnBP synthesis, or its isogenic strain complemented with a plasmid harboring *fnbpA*, the structural gene for FnBPA. The monolayers and bacteria were coincubated for 30 min. The cells were washed to remove nonadherent bacteria, and the MAC-T cells were lysed. The cell lysate was centrifuged to pellet bacterial cells plus any attached MAC-T cell proteins. The resulting pellet was washed exhaustively and analyzed by SDS-PAGE and immunoblotting. The results in Fig. 4 indicate that Hsp60 binds to the surface of intact *S. aureus* cells expressing FnBP. A protein, which migrated in gels with an apparent size of ~55 kDa and was identified as Hsp60 by immunoblotting, was consistently detected in cell lysates generated using this technique. This interaction was specific for FnBP since *S. aureus* DU5883, the FnBP-deficient mutant, did not bind to Hsp60. Similarly, preincubation of *S. aureus* (pFnBPA4) with Du-D4 antiserum blocked the association of Hsp60 with the bacterial cell surface.

**Interaction with membrane Hsp60 is required for maximal internalization of *S. aureus* by epithelial cells.** To determine whether interaction of FnBP with Hsp60 could affect internalization of the organism, the effect of a MAb specific for human Hsp60 was assessed. First, MAC-T cell cultures were preincubated with the Hsp60 MAb or a murine acites control and then infected with *S. aureus* DU5875, a strain expressing FnBP (but not protein A). The results of these experiments are summarized in Fig. 5A and indicate that the addition of the Hsp60 MAb to culture supernatants significantly reduced the efficiency of internalization by the epithelial cells in a dose-dependent manner.

Second, we determined the effect of ligand-induced receptor translocation of Hsp60 from the epithelial-cell apical surface. The ability of some membrane proteins to translocate toward their ligands can be used to differentially affect the receptor density on the apical and basal surfaces of cells. As such, it is often possible to reduce the receptor density on the apical membranes of cells by incubating them on surfaces coated with specific ligands or antibodies (30). We predicted that if interaction of Hsp60 with *S. aureus* FnBP was involved in internalization, translocation of Hsp60 from the apical surfaces of the epithelial cell monolayers should reduce internalization. To address this possibility, MAC-T cells were seeded into wells coated with Hsp60 MAb, Du-D4 peptide (positive control), or BSA (negative control). The results summarized in Table 2

consistently demonstrated, as expected, that Du-D4, the functional fragment of FnBP, reduced internalization by causing translocation of its receptor. A significant reduction was also caused by the Hsp60 antibody, suggesting that an interaction of FnBP with Hsp60 on monolayers is essential for the most efficient internalization of *S. aureus*.

**Fn and integrins are required for efficient internalization of *S. aureus* by MAC-T and HEP-2 epithelial cells.** FnBP was initially recognized for its ability to promote adherence to host tissues through its high affinity for Fn. Since integrins also bind Fn and since other bacteria are internalized through  $\beta_1$  integrin signaling (26, 39), it was important to assess the role of Fn in staphylococcal internalization. The effects of adding exogenous Fn varied depending on the cell types used and whether they expressed Fn. For example, initial experiments in which MAC-T cell cultures were first depleted of exogenous Fn and then supplemented with known quantities of Fn showed that internalization was blocked in the presence of very small quantities of exogenous Fn (Fig. 6A). It was suspected that this result was due to saturation of host cell integrins with bovine Fn produced by the MAC-T cells. Supplementing cultures with additional soluble Fn presumably results in its binding to FnBP, making it impossible for the staphylococcal adhesin to interact with Fn on the MAC-T cell surface. We subsequently confirmed this prediction by using two different methods. First, we showed that MAC-T cells express their own endogenous Fn by immunoblot analyses of membrane proteins using Fn antiserum (results not shown). Second, additional experiments showed that uptake of *S. aureus* by MAC-T cells was reduced in a dose-dependent manner when cultures were preincubated with a commercially available Fn antiserum (Fig. 5B).

Interestingly, a different effect was observed with HEP-2 cells, a cell line noted for lack of Fn expression (26). Experiments using HEP-2 cells showed a clear dose-response effect in which small quantities of Fn (up to 5 nM) stimulated uptake (Fig. 6). These results were consistent with a model in which Fn helps link *S. aureus* FnBP to the epithelial-cell surface by binding to integrins. Higher Fn concentrations blocked uptake.

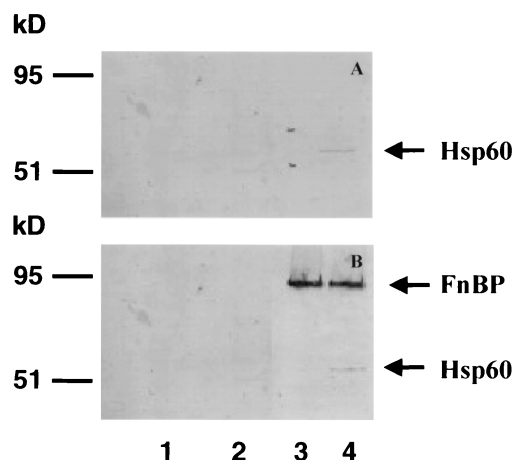


FIG. 4. FnBP-Hsp 60 interaction on cell surfaces. MAC-T cells were incubated for 30 min with no bacteria (lane 1), *S. aureus* DU5883 (lane 2), *S. aureus* DU5883(pFnBPA4) preincubated with Du-D4 rabbit antiserum (lane 3), or *S. aureus* DU5883(pFnBPA4) (lane 4). After lysis of the MAC-T cells, the residual bacterial cell suspension and attached proteins were recovered by centrifugation and the pellets were solubilized in SDS-PAGE sample buffer, resolved by SDS-PAGE, transferred to a membrane, and probed with Hsp60 MAb (A) or Du-D4 followed by Du-D4 antiserum (B). The protein bands shown in the figure correspond to Hsp 60 (apparent size, ~55 kDa) (A and B) and *S. aureus* FnBPA (104 kDa) (B).

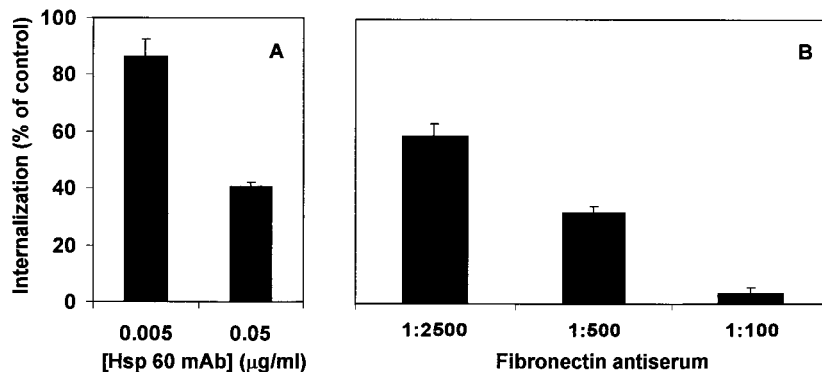


FIG. 5. Effect of Hsp60 MAb (A) or bovine Fn rabbit antiserum (B) on internalization of *S. aureus* DU5875 by MAC-T cells. MAC-T cell monolayers were preincubated for 30 min with serum or ascites proteins at the dilution or quantities indicated (MOI = 20). The number of internalized bacteria was determined in a standard internalization assay, and the results are presented as a percentage of the number in identical control cultures treated with nonimmune ascites (A) or normal rabbit serum (B).

This effect is consistent with the function of Fn in this system as a bifunctional molecule in which a blocking effect at high concentrations would be due to saturation of the integrin-FnBP system.

$\beta_1$  integrins were also required for efficient internalization of *S. aureus* DU5875 by HEp-2 cells. Preincubation of monolayers with the  $\beta_1$  integrin MAb caused a dramatic reduction in internalization to levels similar to those in the absence of exogenous Fn (Fig. 7). We were unable to demonstrate a similar requirement for integrins with the bovine MAC-T cells since MAb raised against either bovine or human  $\beta_1$  integrins did not block internalization (results not shown). The reason for this is unclear and is currently a topic of investigation by our laboratories.

DISCUSSION

We previously demonstrated that *S. aureus* could be internalized by nonprofessional phagocytes through a process requiring FnBP binding to the host cell surface. Our results showed that binding of FnBP to the host cell induced a genistein-sensitive signal transduction and cytoskeletal rearrangement (6), effects similar to those seen with other well-characterized intracellular organisms (29). This present study was performed to identify potential ligands for *S. aureus* on epithelial cells. Using a variety of techniques, we obtained evidence for a complex interaction at the bacterial and epithelial cell surfaces involving at least four molecules, FnBP, Fn,  $\beta_1$  integrins, and Hsp60. The interactions of FnBP with Fn and integrins have been demonstrated previously and are considered to be crucial for binding to host tissues (17, 26). However, the extension of these observations to defining a role for Fn and integrins in internalization of *S. aureus* plus the potential contribution of Hsp60 in this process were less obvious prior to the beginning of this study.

TABLE 2. Effect of downmodulating Hsp60 on internalization of *S. aureus* 8325-4 by MAC-T cells

Ligand	Internalization (% of control) in:	
	Exp I	Exp II
BSA	100 <sup>a</sup>	100 <sup>a</sup>
Du-D4	7.8 ± 2	19 ± 2.6
Hsp60 MAb	19.3 ± 3.1	36 ± 3.9

<sup>a</sup> 100% corresponds to 1.3 × 10<sup>4</sup> CFU/well (MOI = 15).

To identify host cell ligand proteins that could bind directly to FnBP, we used standard ligand affinity binding techniques that had been used to identify receptors for a number of other intracellular bacterial adhesins (15, 30, 37, 38). The only protein that consistently bound to purified FnBP, cell-associated FnBP, or Du-D4, a functional fragment of FnBP, was bovine or human Hsp60, depending on the cell source. Although the primary sequences of FnBPA and FnBPB are not identical, the predicted functional residues within the region encompassed by the Du-D4 peptide are conserved (17). This suggests that

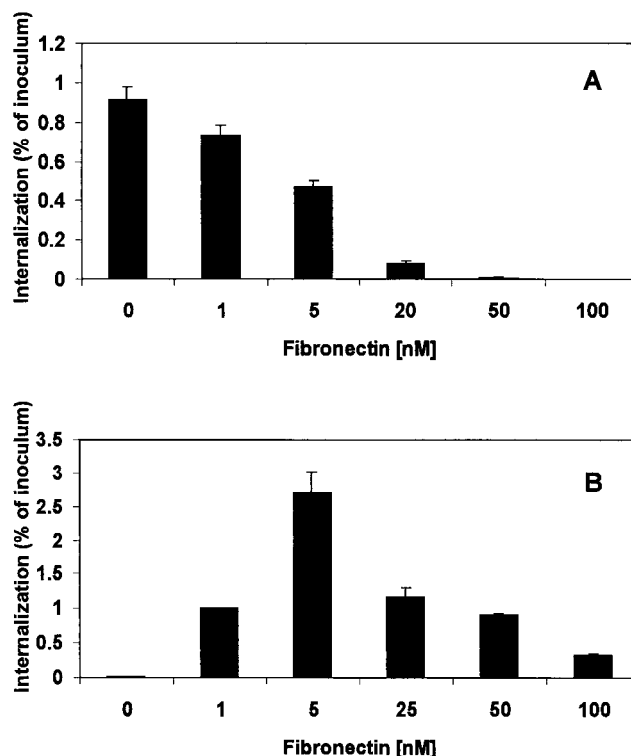


FIG. 6. Effect of Fn on internalization of staphylococci by HEp-2 or MAC-T cells. Monolayers were incubated for 30 min with Fn, at the concentrations shown, prior to infection with the bacterial suspension (MOI = 25). The number of internalized bacteria was determined by the standard invasion assay, and the results are presented as the percentage of the inoculum used.

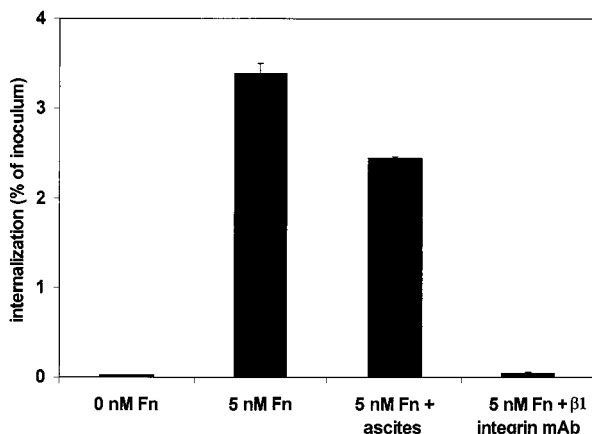


FIG. 7. Effect of  $\beta_1$  integrin MAb on internalization of *S. aureus* DU5875 by HEp-2 cells. HEp-2 monolayers were preincubated with or without 5 nM Fn plus nonimmune ascites (1:200) or  $\beta_1$  integrin MAb (1:200) for 30 min prior to infection with the bacterial suspension (MOI = 17). The number of internalized bacteria was determined by the standard invasion assay, and the results are presented as the percentage of inoculum used.

both FnBPs have the ability to interact with Hsp60, although only purified FnBPA was used in the present study. Interestingly, these results are consistent with those of Tompkins et al. (36), who reported the isolation of a 50-kDa protein from human endothelial cells that was capable of binding to *S. aureus* cells in vitro. Although the protein in that former study was not identified, its properties, including the ability to interact directly with the surface of *S. aureus*, resembled those of Hsp60 in the present study.

Its direct binding to FnBP, the staphylococcal adhesin responsible for internalization, raises the possibility that Hsp60 is involved in the adherence and internalization pro-

cesses. In support of this possibility was our demonstration that a MAb raised against Hsp60 significantly reduced internalization of *S. aureus* by MAC-T cells. Hsp60 is a 57-kDa protein that was originally well characterized because of its function as a molecular chaperonin and its association with the mitochondrial matrix. However, a number of recent reports have documented the presence of Hsp60 not only in the mitochondria but also in several extramitochondrial sites including the cytoplasmic membrane (3, 5, 13, 19, 21, 31, 32, 42). Although many studies have associated the surface expression of Hsp60 with stressed, apoptotic, or transformed cells, membrane Hsp60 is now considered to be typical of eukaryotic cells in general (33, 34).

To be involved in *S. aureus* internalization by nonprofessional phagocytes, Hsp60 would have to possess several important properties. The first, localization on the cell surface, was confirmed by using several techniques. The topology of Hsp60 in the cytoplasmic membrane is not well understood, but a transmembrane prediction program (Prediction of Transmembrane Regions and Orientation, ISREC Bioinformatics Server, Lausanne, Switzerland [www.ch.embnet.org/cgi-bin.TMPRED\_form.html]) showed that Hsp60 has at least two potential transmembrane helices (results not shown). Although mitochondrial Hsp60 is believed to form heptameric rings (2, 7, 35, 40), it is unclear if membrane Hsp60 aggregates in a similar fashion.

A second requirement for internalization is that Hsp60 should be able to either (i) facilitate signaling by another membrane protein or (ii) to transduce a signal directly. The direct binding of FnBP to Hsp60 does not preclude a potential role for other host ligands or ECM such as Fn and fibrinogen, both of which bind to FnBP (41). In fact, the results of the present study, in addition to the documented ability of Fn and integrins to mediate the adherence of *S. aureus* (4), suggest that Hsp60 could function as a coreceptor with integrins linked through Fn (Fig. 8A). A similar model has been proposed by

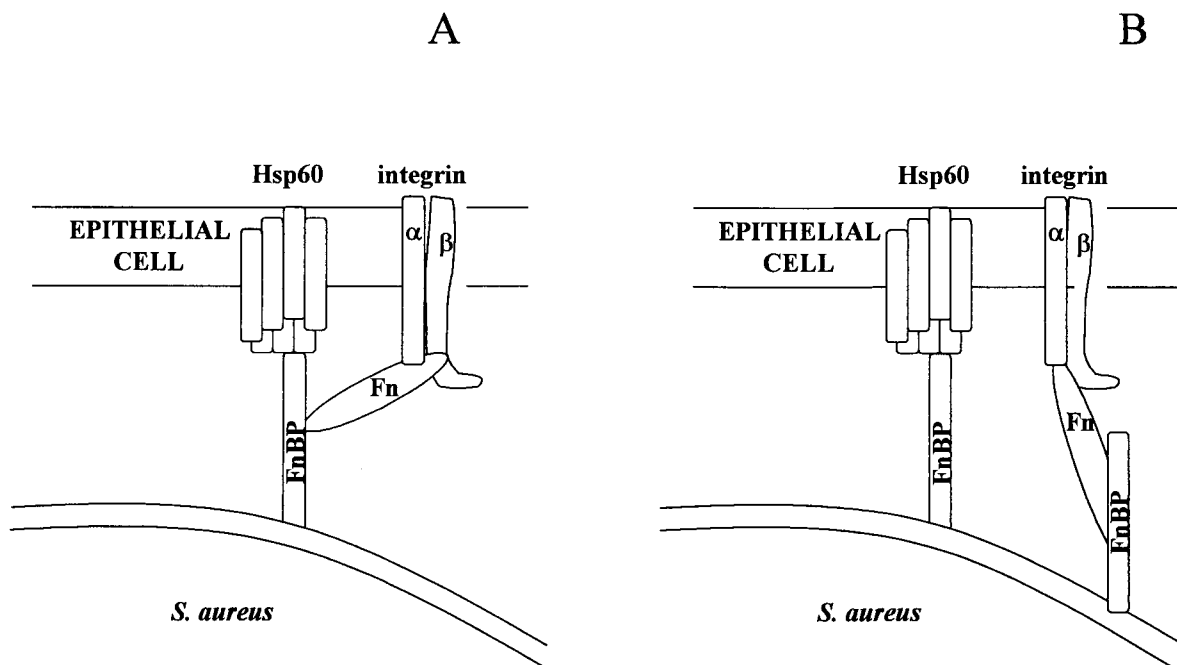


FIG. 8. Potential models for FnBP receptor binding leading to internalization. (A) FnBP interacts with integrin and Hsp60 coreceptors through a Fn linkage. (B) FnBP interacts independently with Hsp60 and the Fn-integrin complex.

van Putten et al. (39) for *Neisseria gonorrhoea* internalization. OpaA, a gonococcal FnBP, mediates uptake through  $\beta_1$  integrin signaling. The gonococcal model involves a Fn bridge linking OpaA and the integrin, with the Fn linkage stabilized by a host cell glycosaminoglycan coreceptor. The coligand is suspected to bind simultaneously to OpaA and Fn. Binding of bacterial FnBPs to a coligand (i.e., Hsp60 or glycosaminoglycan) would be one potential mechanism for organisms to overcome the low-affinity interaction between Fn and  $\beta_1$  integrins. This notion is consistent with the results of Tran Van Nhieu and Isberg (37) indicating that Fn alone is insufficient to induce the uptake of microorganisms including *S. aureus*. It was postulated that this inability was due to a low-affinity binding of Fn to the integrin, in contrast to high-affinity bacterial ligands such as *Yersinia* invasin, which binds directly to integrins.

Another possibility is that the binding of FnBP to Hsp60 occurs independently of  $\beta_1$  integrins (Fig. 8B). This scenario would most probably require a direct or indirect role for Hsp60 in signal transduction. Although Hsp60 has several potential phosphorylation sites and can be phosphorylated by protein kinase A (21), it has not been reported to directly transduce a signal following binding to extracellular ligands. However, Hsp60 is known to control the activity of Src tyrosine kinases. Phosphorylation of E, A, and Y random polymers by c-Src kinase was stimulated 20-fold in the presence of Hsp60 (1). Also, Hsp60 interacts with p21<sup>ras</sup>, a signaling molecule in the cytoplasmic membrane (5, 13). Whether any of these activities is involved in internalization of *S. aureus* into epithelial cells is currently under investigation in our laboratories.

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#### ADDENDUM IN PROOF

We recently became aware of a supporting publication (B. Sinha, P. P. François, O. Nüße, M. Foti, O. M. Hartford, P. Vaudaux, T. J. Foster, D. L. Lew, M. Hermann, and K.-H. Krause, *Cell. Microbiol.* 1:101–117, 1999) in which the authors provided evidence of a role for host cell integrins and fibronectin in internalization of *S. aureus* by a variety of nonprofessional phagocytes.

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