Involvement of FAK/Src complex in the processes of *Escherichia coli* phagocytosis by insect hemocytes

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Abstract Recently we demonstrated that lipopolysaccharide promotes activation of the Ras/mitogen-activated protein cascade in hemocytes and that phagocytosis of *Escherichia coli* by insect hemocytes is mediated by an integrin-dependent process [Foukas et al. (1998) J. Biol. Chem. 273, 14813–14818]. Here we report data concerning the focal adhesion kinase (FAK) tyrosine phosphorylation status in hemocytes in response to *E. coli*. We demonstrate that *E. coli*-triggering stimulates a significant increase in tyrosine phosphorylation of FAK in hemocytes. Furthermore, immunoblotting analysis using anti-Y397 demonstrated intense FAK activity at the Y397/SH2-binding site in hemocytes treated with *E. coli*. In addition, antibody-mediated inhibition of FAK and Src-kinase has been shown to abolish FAK phosphorylation and *E. coli* phagocytosis, indicating a specific role for the FAK/Src complex in the processes of promoting cell phagocytosis. These findings expand the known signaling functions of FAK and provide insight into signal transduction events associated with hemocyte phagocytosis in response to *E. coli*. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Focal adhesion kinase; Focal adhesion kinase/Src complex; Phagocytosis; Hemocyte; *Ceratitis capitata*

1. Introduction

Phagocytosis is characterized by the uptake of large particles, parts of cells and even whole microorganisms or other cells. In most higher animals, phagocytosis is a protective mechanism, rather than a mode of feeding. Phagocytosis in mammals is mainly achieved by macrophages, monocytes and neutrophils, whereas in insects it is achieved by granular cells and plasmatocytes [1,2]. Their function is to ingest and digest pathogens that gain entry to the body. In addition, macrophages play a scavenging role, ingesting damaged cells, aging red blood cells and debris from the circulation.

Phagocytosis of a microbe by a macrophage is an extremely complex and diverse process and requires multiple successive interactions between the phagocyte and the microbe [1]. The microbe internalization by a phagocytotic cell depends on specific signals. These can be released either directly from the microbe or generated by the cell surface receptors which mediate the microbe recognition [3].

To our knowledge, investigations conducted over the last decade have begun to unravel the molecular basis of phagocytosis in mammals [3]. The phagocytosis processes in mammalian macrophages are mediated mainly by the Fc family of receptors [4,5]. The extracellular activation of Fc receptors triggers their intracellular tyrosine phosphorylation on the immunoreceptor tyrosine-based activation motif (ITAM domain) by members of the Src-kinase family. This phosphorylation on tyrosine residues creates binding sites for the SH2-containing p72Syk protein kinase, [6–8]. This signal couples p72Syk protein kinase on the Fc receptors, which in turn stimulates a plethora of intracellular signaling pathways. The mechanism by which Fc γ receptors trigger the polymerization of actin and induce phagosome formation is not known, although PI-3 kinase, the Rho family of GTPases, protein kinase C and motor proteins appear to participate.

In insects, substantial progress in understanding the signaling required for *Escherichia coli* recognition and internalization by *Ceratitis capitata* hemocytes has been achieved [9]. Exploring possible signaling events associated with bacteria phagocytosis by insect hemocytes, we have shown that lipopolysaccharide (LPS) can cause a strong and prompt activation of extracellular signal-regulated protein kinase (ERK1) in hemocytes. In addition, we showed that the SH2/SH3 adapter protein Drk, a homolog protein to the mammalian GRB2, is implicated in the transmission of LPS signaling, indicating that the Ras/mitogen-activated protein kinase (MAPK) cascade is activated. However, the cell-free LPS internalization into the hemocytes differs from the cell-associated LPS internalization. For *E. coli* internalization β3 integrin receptors and cytoskeletal rearrangements are required, as judged by inhibitors of *E. coli* internalization in the presence of the RGD peptide, β3 integrin antibodies and cytochalasin D [9].

Integrins are a family of transmembrane receptors which mediate cell adhesion, but also function as bi-directional signal transducers across the plasma membrane. In integrin-mediated signaling events the activation of cellular proteins including most prominently focal adhesion kinase (FAK) and Src family kinases is well-documented [10]. In this paper we will focus on the FAK and Src kinases and their possible involvement in coupling integrin receptors to intracellular signaling pathways leading to *E. coli* phagocytosis by insect hemocytes.

To assess the potential role of FAK and Src kinases in the phagocytosis process, we have initially investigated FAK

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Abbreviations: LPS, lipopolysaccharide; FAK, focal adhesion kinase; ERK1, extracellular signal-regulated protein kinase; MAP, mitogen-activated protein; TBS, Tris-buffered saline; Fn, fibronectin

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phosphorylation following hemocyte activation by *E. coli*. We found that FAK increases its tyrosine phosphorylation level, indicating its participation in the signaling events, which are generated during this procedure. In addition, *E. coli* promotes autophosphorylation/SH2-binding site, Y397, and antibody-mediated inhibition of FAK and Src has been shown to result in blockage of *E. coli* phagocytosis by insect hemocytes and phosphorylation of FAK indicating a role for the FAK/Src complex in the processes of promoting cell phagocytosis.

2. Materials and methods

2.1. Materials and antibodies

Affinity-purified rabbit polyclonal antibodies to ERK1 (K23), FAK (C903) which recognizes the C-terminal end, c-Src (N-16) which recognizes the N-terminal end, and MCP1 (R-17) as well as the rabbit ABC staining system were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-phosphotyrosine monoclonal antibody (mAb) recombinant RC20:HRPO and the anti-β3 mAb raised against a peptide corresponding to amino acids 16–223 of the extracellular domain of the mouse β3 integrin subunit as well as PC12 cell lysate and mouse macrophage lysate were purchased from Transduction Laboratories (Lexington, KY, USA). Rabbit (polyclonal) anti-FAK (pY397) phosphospecific antibody was from Biosource International (Camapillo, CA, USA). Donkey anti-rabbit IgGs (Amerlex-M magnetic separation reagent) were from Amersham Pharmacia Biotech (UK). Eight-well glass slides were from Nunc (Naperville, IL, USA). Other materials were obtained as indicated and were of the highest grade available.

2.2. Isolation, culture and homogenization of C. capitata hemocytes

C. capitata were reared as described previously [11]. Isolation and homogenization of larval hemocytes were performed as described elsewhere [12]. Protein was assayed by the method of Bradford [13]. To prepare suspended hemocytes, isolated hemocytes were suspended in Grace's insect medium (Sigma) and then cultured for 1 h non-adherently in a rotator at 25°C. Incubations were also performed non-adherently in a rotator at 25°C. Hemocyte monolayers were prepared by allowing 10^5 cells to adhere on cell culture wells for 15 min at 25°C, followed by washing with 100 μl of insect Ringer's solution (128 mM NaCl, 18 mM $CaCl_2$, 1.3 mM KCl, 2.3 mM $NaHCO_3$, pH 7) three times to remove non-adherent hemocytes. Hemocytes were cultured in Grace's insect medium.

2.3. Stimulation of hemocytes with fibronectin (Fn) and mAbs against β3 integrin

Suspended hemocytes, which had been cultured for 1 h at 25°C, were incubated with Fn (10 μg/ml) or mAbs against β3 integrin (10 μg/ml) for 10 min. After incubations, hemocytes were washed twice with cold Ringer's solution and then prepared for immunoprecipitation as described below.

2.4. E. coli-induced tyrosine phosphorylation of hemocyte proteins

Suspended hemocytes which had been cultured for 1 h at 25°C, were challenged with *E. coli* for the indicated times. *E. coli* cells were suspended in Grace's insect medium ($\sim 5\times10^8$ cells/ml) and then were added to the suspended hemocytes (ratio *E. coli*:hemocyte, 50:1). After incubations, hemocytes were washed twice with cold insect Ringer's solution, then lysed in boiling electrophoresis sample buffer (60 mM Tris–HCl, pH 6.8, 10% (v/v) glycerol, 2% (v/v) sodium dodecyl sulfate (SDS), 10 mM dithiothreitol) and processed for immunoblot analysis. In a duplicate experiment the cells were lysed in lysis buffer prior to immunoprecipitation and immunoblot analysis (see below).

2.5. Osmotic loading of FAK and c-Src antibodies

Intracellular loading of FAK antibodies or c-Src antibodies was achieved by promoting uptake of extracellular proteins by incubation in a hypertonic medium, followed by lysis of cytoplasmic pinosomes in a hypotonic solution by the method of Okada and Rechsteiner [14]. Briefly, hemocytes in suspension, prepared as described above, were washed with Ringer's solution and then incubated for 10 min in a hypertonic medium containing 0.5 M sucrose, 10% (w/v) polyethylene glycol 1000 and FAK IgGs, c-Src IgGs or MCP1 IgGs (30 μg/ml) in Grace's insect medium. Hemocytes were then rinsed with a hypotonic solution of diluted Grace's medium:water (6:4) and incubated in the hypotonic medium for 2 min. After rinsing three times with normal Grace's medium, hemocytes were allowed to recover for 3 h before further procedures were performed. After cell recovery, suspended hemocytes were challenged with *E. coli* for 10 or 30 min. *E. coli* cells were suspended in Grace's insect medium ($\sim 5\times10^8$ cells/ml) and then were added into the suspended hemocytes (ratio *E. coli*:hemocyte, 50:1). After incubations, hemocytes were washed twice with cold insect Ringer's solution, then lysed in boiling electrophoresis sample buffer and processed for immunoblot analysis.

The viability of the hemocytes was assessed by exclusion of trypan blue dye (Sigma).

2.6. Phagocytosis assay

200-μl aliquots of hemocytes suspended in Grace's medium (0.5×10^6 cell/ml) were added into the wells of a multiwell glass Lab-TeK chamber (Nunc). Hemocyte monolayers were prepared, followed by osmotic loading of FAK antibodies, c-Src antibodies or rabbit preimmune serum, as described above. After loading and cell recovery, hemocytes were challenged with *E. coli*. The phagocytic activity of *E. coli* was determined by a fluorescence-quenching assay with trypan blue. The assay is a modification of a method originally established for vertebrate blood cells to distinguish ingested from attached bacteria [15]. In brief, 50 μl of fluorescein isothiocyanate-labeled *E. coli* suspension ($\sim 1\times10^8$ *E. coli*) was added into 200 μl of monolayers (ratio *E. coli*:hemocyte, 20:1). After incubation for 2 h at 25°C, the non-adhering *E. coli* was removed by exchanging the supernatants three times by Grace's insect medium. 250 μl of the supernatant in each well was exchanged by a trypan blue solution (2 mg/ml in Grace's medium). 10 min later the cells were washed with culture medium and then fixed with a 4% formaldehyde solution for 10 min. Trypan blue quenches only non-phagocytosed bacteria. Phagocytosis was observed by fluorescent microscopy.

2.7. Cell lysis and immunoprecipitation

Cells were lysed in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 5 mM NaF, 1 mM PMSF, 10 μg/ml leupeptin and 10 U/ml aprotinin) at 4°C. Insoluble material was removed by centrifugation ($16\,000\times g$ for 15 min at 4°C) and supernatants were collected. For immunoprecipitation, 400 μg of protein was incubated with 4 μg anti-FAK for 3 h and then for an additional hour with anti-rabbit IgG-magnetic beads. The immune complexes were washed four times with lysis buffer. Proteins were eluted from the beads by boiling samples for 5 min in 50 μl of electrophoresis sample buffer.

2.8. SDS–PAGE and immunoblot analysis

SDS–PAGE was performed according to Laemmli [16] in 10% acrylamide, 0.10% bisacrylamide slab gels [13]. Equal amounts of proteins in immune complexes or total cell lysates were resolved by SDS–PAGE. After gel separation, the proteins were transferred electrophoretically onto Immobilon P polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA) according to Towbin et al. [17]. Membranes were then incubated in SuperBlock™ Blocking Buffer in Tris-buffered saline (TBS; Pierce) for 1 h at room temperature. Subsequently, the membranes were incubated with primary antibody at the appropriate concentration (0.5 μg/ml for anti-rat ERK1, anti-Y397 and RC20 antibodies or 0.2 μg/ml for FAK antibody) in TBS (10 mM Tris–HCl, pH 7.5, 100 mM NaCl) containing 10% (v/v) SuperBlock™ Blocking Buffer and 0.05% (v/v) Tween 20 for 1 h at room temperature. Membranes were washed three times for 10 min in TBS containing 0.05% (v/v) Tween 20 followed by incubation with the rabbit ABC staining system (Santa Cruz) according to the manufacturer's instructions. Immunoreactivity was detected on X-ray film by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech). The stripping of membranes after ECL detection was performed according to the manufacturer (Amersham Pharmacia Biotech).

3. Results

3.1. Expression of FAK in hemocytes

Recently, a *Drosophila melanogaster* FAK homolog (140 kDa) was demonstrated, which shares strong sequence simi-

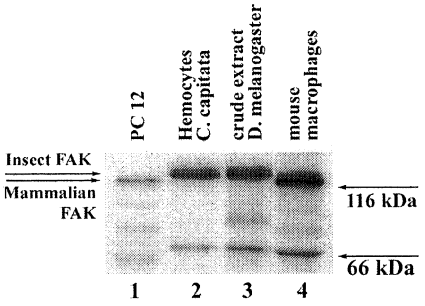

Fig. 1. Identification of the *C. capitata* homolog of the FAK protein. Protein extract of *C. capitata* larval hemocytes was separated by 10% SDS–PAGE and analyzed by immunoblotting with rabbit polyclonal anti-FAK antibody. Lane 1: PC12 cell lysate. Lane 2: hemocyte lysate. Lane 3: protein extract from third instar larvae of *D. melanogaster*. Lane 4: mouse macrophage lysate.

larity not only with mammalian FAK but also with the more recently described mammalian Pyk2 [18]. In situ hybridization and immunostaining data revealed expression of DFak56 in the central nervous system, embryonic brain, epidermis, nerve cord and visceral mesoderm.

In the present study the expression of FAK in the hemocyte lysate of *C. capitata* was demonstrated. Total lysate from hemocytes of third instar larvae of medfly as well as crude extract from third instar larvae of *D. melanogaster* were analyzed on SDS–PAGE followed by immunoblotting for FAK. Fig. 1 clearly shows that mammalian polyclonal anti-FAK recognized a doublet of endogenous FAK at 140 kDa in *C. capitata* hemocyte lysate, indicating the specificity of the immunoreactive band, and a second band at 66 kDa, probably a proteolytic fragment of FAK. The expression of FAK as a doublet was also previously observed [18].

Given the presence of FAK in hemocytes, we initially tested whether at the functional level, hemocyte FAK behaves like DFak56 and vertebrate FAKs. For this purpose suspended hemocytes were treated with Fn and mAbs against β3 integrin. The results in Fig. 2 clearly show that in both cases FAK becomes phosphorylated. In contrast, in untreated hemocytes kept in suspension, FAK displays much less phosphorylation. Therefore, hemocyte FAK appears to be phosphorylated via an integrin-mediated signal.

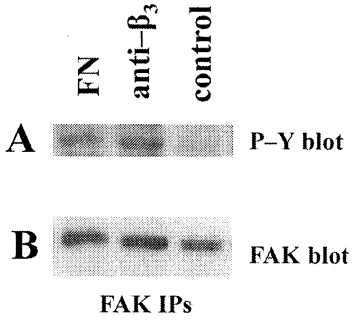

Fig. 2. Stimulation of tyrosine phosphorylation of FAK by Fn and mAbs against β3 integrin. Hemocytes in suspension culture were incubated with 10 μg/ml Fn, 10 μg/ml mAbs against β3 integrin or in Grace's medium (control) for 10 min. The cell lysates were prepared and immunoprecipitated with anti-FAK and then subjected to Western blotting with anti-phosphotyrosine antibody (A). To confirm the presence of equal amounts of FAK protein the membrane was stripped and reprobed with anti-FAK antibodies (B).

3.2. Stimulation of ERK1 and FAK tyrosine phosphorylation by *E. coli* in hemocytes

LPS can cause a strong activation of ERK1 in hemocytes [9]. To examine the changes in the tyrosine phosphorylation status of ERK1 and FAK in response to *E. coli* by insect hemocytes, hemocytes in suspension were treated with *E. coli* cells for several time intervals and lysates were subjected to anti-phosphotyrosine immunoblot analysis. Fig. 3 clearly shows that *E. coli* induced a prominent tyrosine phosphorylation of several hemocyte proteins indicated by arrowheads. To see whether ERK1 and FAK kinases were among the phosphorylated proteins (Fig. 3A), the membrane was sequentially stripped and reprobed with anti-FAK and anti-ERK1 antibodies (Fig. 3B,C). In both cases the bands under consideration were aligned perfectly with those recognized by FAK and ERK1 antibodies, respectively. Furthermore, by comparing Fig. 3A,B, in regard to FAK, it can be concluded that although the quantity of FAK remained rather unchanged (Fig. 3B), the induction of FAK phosphorylation followed *E. coli* stimulation, was rapid and transient. In the same gel (Fig. 3A,C) it can be observed that the phosphorylation of ERK1 in response to *E. coli* also showed a rapid and transient phosphorylation.

To further verify whether the 140-kDa tyrosine-phosphorylated protein in *E. coli*-treated lysates is FAK, we examined cell lysates from hemocytes stimulated with *E. coli* by immunoprecipitation with an anti-FAK antibody followed by immunoblotting with an anti-phosphotyrosine antibody. Cell lysates prepared from non-stimulated hemocytes were used as

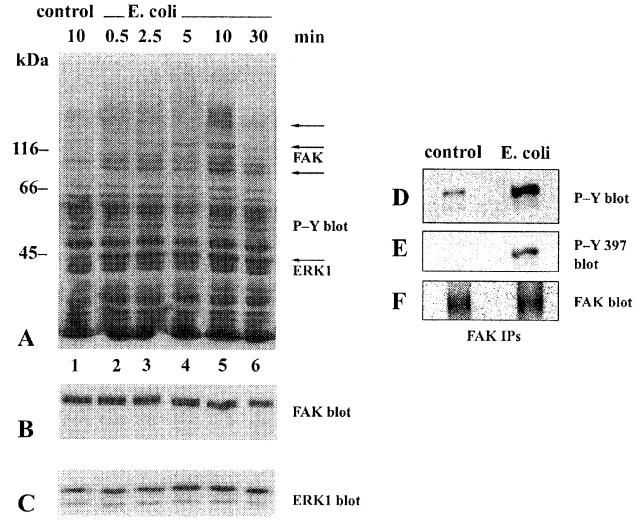

Fig. 3. *E. coli*-induced tyrosine phosphorylation of FAK. A: Hemocytes in suspension culture were stimulated with *E. coli* cells for the indicated times (lanes 2–6) or not (lane 1). Subsequently, the hemocytes were washed and lysed in boiling electrophoresis buffer. Hemocyte lysate proteins were separated by 10% SDS–PAGE and analyzed by immunoblotting with anti-phosphotyrosine mAbs. The migration positions of FAK (B) and ERK1 (C) were revealed after stripping of the membrane and reprobing with anti-FAK and anti-ERK1 antibodies. The molecular mass markers (kDa) are shown on the left. D: Cell lysates obtained after treatment with *E. coli* for 10 min or not (control) were immunoprecipitated with anti-FAK antibody and subjected to Western blotting with anti-phosphotyrosine mAbs. Subsequently the membrane was stripped and reprobed with anti-Y397 (E). The presence of equal amounts of FAK protein was confirmed after stripping the membrane and reprobing with anti-FAK antibody (F).

control. As shown in Fig. 3D, *E. coli* induced tyrosine phosphorylation of FAK as expected. To clarify whether *E. coli* promotes activation of FAK, sequential restripping of the membrane and reprobing with anti-Y397 antibodies demonstrated FAK phosphorylation at the autophosphorylation/SH2-binding site, Y397 (Fig. 3E). Finally, to confirm the presence of equal amounts of FAK protein, the membrane was stripped and reprobed with anti-FAK antibody (Fig. 3F).

3.3. FAK plays an essential role in phagocytosis

To elucidate whether FAK phosphorylation is implicated in the processes for *E. coli* phagocytosis by insect hemocytes, we used osmotic lysis of pinocytic vesicles to load hemocytes with certain antibodies. Initially, we loaded hemocytes with anti-FAK antibodies and then loaded and unloaded hemocytes with anti-FAK were treated with *E. coli* for 10 min and then were subjected to anti-phosphotyrosine immunoblotting analysis. Fig. 4 clearly shows that in hemocytes loaded with anti-FAK antibodies the level of phosphorylation in a number of high molecular weight proteins including FAK was significantly reduced. In addition, in hemocytes loaded with anti-FAK the phagocytic activity of the hemocytes was reduced to about 50% compared with the unloaded hemocytes (Fig. 5A). In order to examine that this effect is not a non-specific consequence of osmotic loading of IgGs, hemocytes were loaded with an antibody directed against an unrelated protein (anti-MCP1) and then stimulated with *E. coli* for 10 min (Fig. 4A). The specificity of the anti-FAK effect was checked by loading the hemocytes with preimmune serum (Fig. 5A) and the successive uptake of anti-FAK IgGs by hemocytes was demonstrated by SDS–PAGE of treated hemocytes (Fig. 5B). The lack of FAK phosphorylation in the hemocytes loaded with anti-FAK in response to *E. coli* (Fig. 4), and the blockage of phagocytosis (Fig. 5), as well as the blockage of phagocytosis

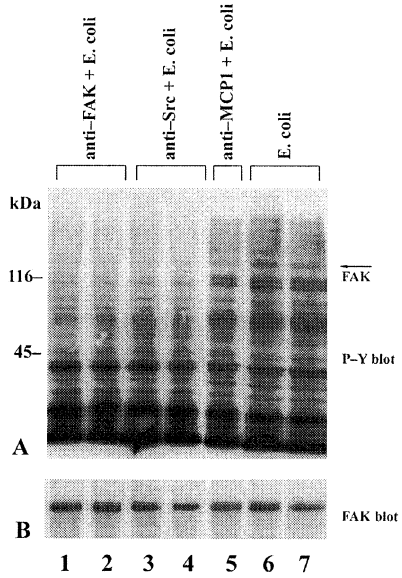

Fig. 4. Inhibition of *E. coli*-induced FAK tyrosine phosphorylation by anti-FAK and anti-Src antibodies loaded into the cytoplasm of the hemocytes. Hemocytes in suspension were loaded with anti-FAK (lanes 1 and 2), anti-Src (lanes 3 and 4) and anti-MCP1 (lane 5) or not (lanes 6 and 7). After cell recovery, hemocytes were challenged with *E. coli* for 10 min (lanes 1, 3, 5 and 6) and 30 min (lanes 2, 4 and 7). Subsequently, the lysates were treated in the assays as described in Fig. 3A.

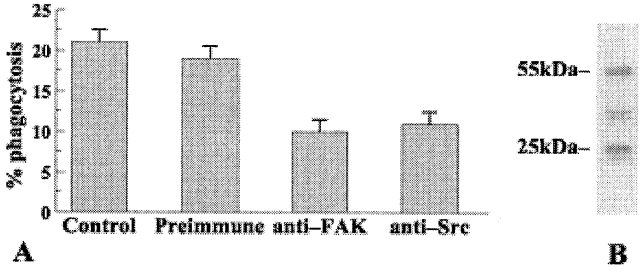

Fig. 5. Inhibition of *E. coli* phagocytosis by FAK and Src antibodies loaded into the cytoplasm of hemocytes. A: Hemocyte monolayers cultured for 3 h after loading with anti-FAK or anti-Src antibodies or rabbit preimmune serum were challenged with *E. coli* (control: unloaded hemocytes). After incubation for 2 h at 25°C, the non-adhering *E. coli* cells were removed and hemocytes were treated with trypan blue solution (2 mg/ml in Grace's medium) and then fixed with a 4% formaldehyde solution. Phagocytosis was observed by fluorescent microscopy. B: Immunoblot analysis of anti-FAK IgGs loaded into the cytoplasm of hemocytes. Hemocyte monolayers cultured for 3 h after loading with anti-FAK IgGs, were lysed in electrophoresis buffer and lysate proteins were separated by SDS–PAGE, electroblotted onto polyvinylidene fluoride membrane and probed directly with rabbit ABC staining system.

by anti-β3 integrin and RGD peptide [9], support the assumption that FAK-associated signaling events promote *E. coli* phagocytosis by insect hemocytes.

To explore whether the observed ability of FAK to promote *E. coli* phagocytosis is attributable to direct phosphorylation by FAK or is possibly a result of the ability of FAK to interact with and activate Src family kinases, we loaded hemocytes with anti-Src, instead of anti-FAK antibodies. As it is well known, activation of vertebrate FAK involves autophosphorylation at Tyr^{397}. The motif surrounding this site is recognized by Src members, which phosphorylate additional tyrosine residues in FAK, which in turn create binding sites for other signaling proteins [10]. The obtained results (Fig. 4), clearly show that FAK kinase showed a low level of phosphorylation in hemocytes loaded with anti-Src, indicating the Src family kinases participation. Furthermore, the phagocytic activity of the hemocytes was reduced to about 50%, compared with the unloaded hemocytes (Fig. 5A), indicating that both autophosphorylation and Src-mediated phosphorylation of FAK are essential for the transmission of a signal downstream to target substrates.

4. Discussion

Phagocytosis of a pathogen is a multiple step process that involves recognition, adherence, activation of pseudopodium formation, invasion and assembly of phagosomes, and it is essential for host defense in higher eukaryotes. Evidently, some of these steps require the activation of signal transduction pathways. Recently, we demonstrated that phagocytosis of *E. coli* by insect hemocytes requires both activation of the Ras/MAPK signal transduction pathway for recognition and attachment and β3 integrin for internalization [9].

In the current paper, we extended our studies concerning the signaling pathways implicated in the processes of bacteria phagocytosis by insect hemocytes. A key question that immediately arises is what signaling molecules link β3 integrin to *E. coli* phagocytosis by insect hemocytes. In this paper, we succeeded in demonstrating FAK in insect hemocytes (Fig. 1). FAK is a family of non-receptor and non-membrane-associ-

ated protein tyrosine kinases, which have been implicated in controlling several cellular functions, including cell spreading and migration and cell survival [10]. In 1999, the expression of a *D. melanogaster* FAK homolog, DFak56, was reported in the central nervous system, embryonic brain, epidermis, nerve cord and visceral mesoderm and appears to be implicated in migration processes during *D. melanogaster* development [18,19]. DFak56 shows a strong homology with mammalian FAK in domains critical for the FAK function, such as autophosphorylation and Src phosphorylation sites [19].

FAK tyrosine phosphorylation is induced by adhesion receptors as well as by a number of bioactive molecules including neuropeptide, immunoglobulin, cytokines and neurotransmitters [20]. The present data clearly show that *E. coli* induce a rapid activation of FAK, increasing its phosphorylation levels (Fig. 3), implying a role for hemocyte FAK in signaling pathways required for bacterial recognition and phagocytosis. Furthermore, antibody-mediated inhibition of FAK results in both blockage of FAK phosphorylation (Fig. 4) and phagocytosis (Fig. 5), indicating that the phosphorylation of FAK is a prerequisite for blockage of *E. coli* phagocytosis by insect hemocytes. In other words, FAK-associated signaling events promote phagocytosis.

FAK possesses several tyrosine-phosphorylated sites, which play an important role in its function. To explore downstream signaling events associated with FAK phosphorylation in relation to the phagocytosis process, we examined the role of Src family kinases. It is well established that Src-kinase family members co-operate with FAK, in order to transduce integrin-mediated signals. FAK autophosphorylation at Tyr^{397} creates binding sites for the SH2 domain of the Src members, which promotes in addition phosphorylation of FAK at Tyr^{925} [21]. This phosphorylated site on FAK recruits Grb2 and stimulates the activation of the Ras/MAPK pathway. Our experiments with osmotic-loaded anti-Src antibodies indicate that this specific inhibition of Src-kinases is able to inhibit both FAK phosphorylation and phagocytosis as well (Figs. 4 and 5), supporting the participation of Src family kinases in the process of phagocytosis. In addition, immunoblotting experiments using anti-Y397 (Fig. 3E) further confirm the involvement of FAK and Src family kinases in the signaling events promoting phagocytosis. Consequently, the signaling for the phagocytosis processes is transmitted from β3 integrin via the FAK/Src complex to downstream target substrates.

The involvement of a FAK/Src complex in the process of pathogen phagocytosis by insect hemocytes expand the known signaling functions of FAK and provide insight into signaling events associated with phagocytosis and hence cell-mediated immunity. Future studies aim at determining the mechanisms by which FAK signaling affects phagocytosis.

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