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Elk-1 Is a Novel Protein-Binding Partner for FAK, Regulating Phagocytosis in Medfly Hemocytes

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Abstract Focal adhesion kinase (FAK) and its downstream signaling targets, mitogen-activated protein kinase (MAPKs), are implicated in the process of phagocytosis by insect hemocytes. The goal of this study was to explore further the signaling pathways underlining the process of phagocytosis. The combination of bioinformatics, biochemical, and immunofluorescence approaches strongly support the expression of Elk-1-like protein in medfly hemocytes. Elk-1 is phosphorylated in *E. coli* or latex beads-challenged hemocytes and osmotic loading experiments as well as flow cytometry analysis demonstrated that Elk-1-like protein regulates the uptake of bacteria. RNA interference (RNAi) and pharmacological inhibitors show that the signaling for Elk-1 phosphorylation is transmitted via FAK/Src and MAPKs pathways. Furthermore, confocal analysis clearly shows that FAK and the phosphorylated FAK at Y397 are localized in the nucleus and cytoplasm, whereas, the phosphorylated Elk-1-like protein is exclusively localized in the nucleus. Finally, co-immunoprecipitation and reciprocal co-immunoprecipitation analysis demonstrated the association of low molecular weight protein bands recognized by FAK antibodies, with Elk-1 or phospho-Elk-1 at ser 383 and confocal microscopy specifies that this association occurs only in the nucleus. These results are strongly supporting that Elk-1-like protein is a novel protein-binding partner for FAK, a finding that significantly broadens the potential functioning of FAK and Elk-1 generally. Evidently, the complex participates in the process of phagocytosis in medfly hemocytes. J. Cell. Biochem. 103: 1895–1911, 2008.

Key words: signaling; innate immunity; phagocytosis; Elk-1; FAK; *E. coli*

In insects, phagocytosis is an important innate immune response against pathogens and parasites. Recently, it has been reported that prophenoloxidase (proPO) activation system and several signal transduction pathways regulate this process [Metheniti et al., 2001; Cerenius and Soderhall, 2004; Mavrouli et al., 2005; Lamprou et al., 2007]. The proPO activation system is composed of proteins recognizing several microbial components (pattern recognition proteins), several serine proteases, and their zymogens, proPO, as well as proteinase inhibitors that function as regulatory factors [Cerenius and Soderhall, 2004]. ProPO is synthesized in the hemocytes and appears to be distributed ubiquitously in the cytoplasm as well as on the surface of hemocytes [Mavrouli et al., 2005; Ling and Yu, 2006]. The proPO activation system is triggered by several microbial components, such as LPS and peptidoglycans, ensuring by this way, that, the system will become active in the presence of potential pathogens.

The documented intracellular signaling pathways in response to pathogen infection involved in the phagocytosis process are focal adhesion kinase (FAK)/Src and mitogen-activated protein kinases (MAPKs) pathways that activate the secretion of prophenoloxidase-activating

Abbreviations used: FAK, focal adhesion kinase; proPO, prophelоloxidase; PAPs, prophenoloxidase-activating proteinases; PO, phenoloxidase; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal regulated kinase; MEK, MAPK/ERK Kinases; RT-PCR, reverse transcription-polymerase chain reaction; RNAi, RNA interference; FITC, fluoroscein isothiocyanate conjugated; HRP, horseradish peroxidase; ELISA, enzyme-linked immunoabsorbent assays; PI, propidium iodide.

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proteinases (PAPs). PAPs are part of proPO activation system and upon their secretion in response to pathogens, activate by partial proteolysis cell-free or cell-surface-associated phenoloxidase (PO), a prerequisite for bacteria engulfment [Metheniti et al., 2001; Lamprou et al., 2005; Lamprou et al., 2007]. These data prompted us to further clarify the role of FAK/Src pathway in the process of phagocytosis by primary insect hemocytes.

FAK is considered to be a component of central importance because it is known to associate with multiple cell surface receptors (integrins, growth factor receptors, and G-proteins-linked receptors) and signaling proteins through which modulates the activity of several intracellular signaling pathways [Schlaepfer et al., 1999; Zhai et al., 2003]. FAK is a family member of nonreceptor and non-membrane-associated tyrosine kinases, which have been implicated in controlling several cellular functions, including cell spreading, migration, apoptosis, phagocytosis, and cell survival [Schlaepfer et al., 1999; Lamprou et al., 2007; Mamali et al., 2007]. Interestingly, N-terminal FAK fragments are present in the nucleus in several different cell lines [Lobo and Zachary, 2000; Jones et al., 2001; Jones and Stewart, 2004], suggesting that FAK may participate in a novel signaling pathway between the cell surface and the nucleus. The emergence of full-length and/or N-terminal fragments of FAK as signaling molecules at both the cell cytoplasm and in the nucleus suggests important implications in these different cell compartments.

The goal of this study is to explore whether in our system, FAK and/or FAK fragments are imported in the nucleus and are interacting with nuclear components, controlling several functions in functional and physical association with these molecules. In this report, we explored the interaction of FAK with Elk-1-like protein in primary medfly hemocytes. Elk-1 belongs to the ETS domain transcription factor family and the ternary complex factor subfamily [Sharrocks, 2002]. Elk-1 is thought to participate in neuronal differentiation [Lobo and Zachary, 2000], cell proliferation [Sharrocks, 2002], tumorigenesis [Chai et al., 2001], and apoptosis [Shao et al., 1998]. Elk-1 is localized both throughout the cytoplasm and in the nucleus, raising the possibility that Elk-1 may have extra-nuclear functions. Indeed, recently, it was demonstrated that Elk-1 associates with the mitochondrial permeability transition pore complex in neurons [Barrett et al., 2006]. In this report, we show the functional and physical association as well as the co-localization of FAK fragments, with Elk-1-like protein. In addition, we demonstrate that Elk-1-like protein is required for bacteria engulfment. This study aimed to clarify further the signal transduction pathways regulating the process of phagocytosis by primary hemocytes from the medfly *Ceratitis capitata*.

MATERIALS AND METHODS

Materials and Antibodies

Polyclonal antibodies against FAK (c-903), p^{Tyr397}FAK, Ets-1/Ets-2, CD14 (M-305), and c-Src were obtained from Santa Cruz, CA. Affinity-purified rabbit polyclonal antibodies to Elk-1 and p^{ser383}Elk-1, as well as mouse monoclonal antibody to p^{ser383}Elk-1, were purchased from Cell Signaling Technology (Beverly, MA). Goat anti-rabbit IgG-horseradish peroxidase (HRP conjugated), while goat anti-mouse IgG-HRP was from BD Transduction Laboratories™, Cy™3-conjugated goat anti-rabbit from Jackson ImmunoResearch Laboratories, Inc., and goat anti-rabbit IgG-fluoroscein isothiocyanate conjugated (FITC) from Molecular probes, Inc. Antibodies against actin and goat anti-mouse FITC as well as carboxy-modified latex beads were obtained from Sigma (St. Louis, MO). U0126 and PD 98059, MAPK/extracellular signal-regulated kinase (ERK) kinases (MEK)1/2 inhibitors, were obtained from Cell Signaling Technology, SB 202190, p38 inhibitor, was obtained from Sigma and SP 600125, JNK inhibitor, was purchased from Calbiochem. Donkey anti-rabbit IgGs (Amerlex-M magnetic separation reagent) were purchased from Amersham Life Science (UK). Other materials were obtained as indicated.

FITC-Labeled *E. coli*

FITC-labeled *E. coli* (DH10B) was prepared after incubation of 10^8 heat-killed bacteria with 1-mg FITC, in 0.5-ml 0.5 M Na_2CO_3/0.5 M $NaHCO_3$ pH 9.5, for 30 min in the dark. FITC-conjugated *E. coli* was rinsed three times with PBS, resuspended in Grace's medium, and stored in aliquots at $-20^{\circ}C$.

Collection of Hemocytes and Cell Viability Test

Ceratitis capitata were reared as described previously [Charalambidis et al., 1996]. Isolation and homogenization of 3rd instar larva hemocytes were performed according to Charalambidis et al. [1995]. In brief, hemolymph was collected and centrifuged at 200*g* for 10 min at 4°C. Sedimented hemocytes were washed three times with Ringer's solution (128 mM NaCl, 18 mM $CaCl_2$, 1.3 mM KCl, and 2.3 mM $NaHCO_3$, pH 7.0). The viability of hemocytes was assessed by exclusion of trypan blue dye (Sigma) under a light microscope.

Nuclear Isolation

After the collection of the hemocytes and the final wash with Ringer's solution, the cells were resuspended in 5 ml of Buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT) and incubated on ice for 5 min. Then, a small drop of the cell suspension was put on a glass slide and checked that the cells were swollen but not burst. Afterwards, the cell suspension was transferred to a pre-cooled 7 ml homogenizer and the cells were homogenized 10 times using a tight pestle, while keeping the homogenizer on ice. The homogenized cells were centrifuged at 200*g* for 5 min at 4°C. The pellet, which contains enriched but not highly pure nuclei, was resuspended with 3 ml S_1 solution (0.25 M sucrose, 10 mM $MgCl_2$) and after that the resuspended pellet was layered over 3 ml of S_2 solution (0.35 M sucrose, 0.5 mM $MgCl_2$). Then the resuspended pellet was centrifuged at 1,430*g* for 5 min at 4°C and resulted to a cleaner nuclear pellet.

Protein Determination

Proteins were determined according to Bradford [1976] with a modified solution containing 10% (w/v) Coomassie G250 (Merck, Germany) in 5% (v/v) ethanol, 10% (v/v) H_3PO_4. O.D. was recorded at 595 nm.

Immunoprecipitation

Hemocytes 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 units/ml aprotinin) at 4°C. Insoluble material was removed by centrifugation (16,000*g* for 15 min at 4°C) and supernatant was collected. For immunoprecipitation, 400 μg of crude extract protein was incubated with 2 μg anti-FAK, -p^{tyr397}FAK, -Elk-1, -p^{ser383}Elk-1, and -Ets-1/Ets-2 polyclonal antibodies for 2 h at 4°C and then for an additional hour at 25°C with an Amerlex-M secondary antibody reagent (Amersham Life Science). The immune complexes were washed four times with TBS. Proteins were eluted from the beads by boiling the samples for 3 min in 50 μl of electrophoresis sample buffer. Immunoprecipitated proteins were analyzed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotted with anti-FAK, -p^{tyr397}FAK, -Elk-1, -p^{ser383}Elk-1, and -Ets-1/Ets-2 polyclonal antibodies polyclonal antibodies.

SDS–PAGE and Immunoblot Analysis

SDS–PAGE was performed on 10% acrylamide and 0.10% bisacrylamide slab gels, according to Laemmli [1970]. Samples with equal amounts of protein were analyzed and electroblotted onto Immobilon P polyvinylidene fluoride membranes (Millipore Corp.). Membranes were incubated in SuperBlock™ blocking buffer (Pierce) for 1 h at room temperature. Subsequently, membranes were incubated overnight at 4°C with primary antibody diluted 1:1,000 in TBS (10 mM Tris–HCl, pH 7.5, 100 mM NaCl), 10% (v/v) SuperBlock™ blocking buffer and 0.05% (v/v) Tween-20. Membranes were washed and incubated with HRP-linked secondary antibody (Transduction Laboratories, Lexington, KY; Santa Cruz Biotechnology, CA) for 1 h at room temperature. Immunoreactive proteins were visualized on X-ray film by enhanced chemiluminescence (ECL) methodology (Amersham). Stripping of membranes was performed according to the manufacturer's instructions (Amersham). Prestained protein markers, broad range, were use to indicate the size of the protein bands (Cell Signaling Technology).

Flow Cytometry Analysis

Larval hemocytes (5×10^5 cells) were incubated in 100 μl Grace's insect medium containing *E. coli*–FITC (10 bacteria per hemocyte) for 10 min at 25°C. Internalized *E. coli*–FITC was measured by quenching surface-exposed FITC–*E. coli* with or without trypan blue 4% in Ringer's solution. Approximately, 10,000 cells from each sample were analyzed by flow cytometry using a Coulter EPICS-XL-MCL

cytometer (Coulter, Miami, FL), and the data were processed using the XL-2 software. The percentage decrease or increase of phagocytosis was calculated, supposing that the phagocytosis in the cells that were incubated only with *E. coli* is 100%.

Fluorescence Microscopy

Isolated hemocytes were suspended in Grace's medium, treated with or without *E. coli,* and allowed to attach on glass slides for 10 min at 25°C. The slides were washed with Ringer's solution to remove non-adherent hemocytes. The resulting monolayers were pre-permeabilized with 0.1% Triton X-100 for 10 sec, washed with TBS, and then fixed in a cold 100% methanol chamber at −20°C for 30 min. Slides were then rinsed with TBS and permeabilized with 0.1% Triton X-100 for 1 min in RT. Hemocytes were treated afterwards with a protein blocking agent for 10 min at RT to reduce non-specific binding. Following saturation, slides were incubated with anti-FAK, -p^{tyr397}FAK, -Elk-1, and -p^{ser383}Elk-1 (diluted 1:100 in TBS) for 1 h at 37°C in a humid atmosphere. Following antibody treatment, the slides were washed with TBS and further incubated for 1 h at 37°C, in a humid atmosphere in the shelter of the light, with a secondary anti-rabbit or anti-mouse antibody coupled to a fluorochrome (FITC or Cy3) diluted 1:100 in TBS. Hemocyte monolayers were then washed with TBS, afterwards, in some cases incubated for 10 min with PI, and then mounted with an aqueous mounting medium (Sigma) and observed under a fluorescence microscope or a confocal fluorescence microscope to examine the distribution and localization of FAK, p^{Tyr397}-FAK, -Elk-1, and -p^{ser383}Elk-1.

Osmotic Loading of FAK and c-Src Antibodies

Intracellular loading of Elk-1 or CD14 or FAK and 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 [Okada and Rechsteiner, 1982]. 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 Elk-1 IgGs or CD14 IgGs or FAK IgGs and c-Src IgGs (30 μg/ml) in Grace's insect medium. Hemocytes were then centrifuged and the pellet was incubated in a hypotonic medium (hypotonic solution of diluted Grace's medium:water (6:4)) for 2 min. Following the incubation, the hemocytes were centrifuged and the pellet was washed three times with normal Grace's medium. Afterwards, the 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 min. *E. coli* cells were suspended in Grace's insect medium ($\sim 10^8$ cells/ml) and then were added into the suspended hemocytes (ratio *E. coli*:hemocyte, 10:1). After incubations, hemocytes were washed twice with cold insect Ringer's solution and then analyzed by flow cytometry. The viability of the hemocytes was assessed by exclusion of trypan blue dye (Sigma).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was carried out using the QIAGEN® OneStep RT-PCR Kit (QIAGEN), according to the supplier's instructions. Total RNA was isolated from adult *Drosophila* flies as described in Holmes and Bonner [1973] and the resulting FAK cDNA (1,011 bp), after the reverse transcription, was amplified using the following primers that include a 5′ T7 RNA polymerase-binding site (TAATACGACTCACTATAGGGAGACCAC): forward FAK primer (5′-TAATACGACTCACTATAGGGAGACCAC<u>AGTCGACCACCTAACCGCGCAGATGACG</u>-3′) and reverse FAK primer (5′-TAATACGACTCACTATAGGGAGACCAC<u>TGACTACATGACGATGTGAGAATGCG</u>-3′). The thermal cycling conditions were as follows: 50°C for 30 min; 95°C for 15 min; followed by 30 cycles of 94°C for 30 s; 57°C for 1 min; and 72°C for 1 min; with a final incubation at 72°C for 10 min. The PCR products (1,011 bp) were electrophoresed on 1% agarose gel in TAE buffer (Tris 0.4 M, EDTA-Na_2-salt 0.01 M, and acetic acid 0.2 M) and visualized by staining with ethidium bromide (0.5 μg/ml).

cDNA Cloning

The purified FAK cDNA (1,011 bp) was cloned into a pCRII plasmid vector, as described in Invitrogen's TA Cloning Kit, and then it was sent for sequencing analysis and sequenced to confirm that the insert is the FAK cDNA.

PCR Amplification

As a template for the PCRs, we used the cloned FAK cDNA. *Taq* polymerase (New England BioLabs) was used according to the manufacturer's recommended protocol with the same as above forward (5′-TAATACGACTCACTATAGGGAGACCACAGTCGACCACCTAACCGCGCAGATGACG-3′) and reverse FAK primers (5′-TAATACGACTCACTATAGGGAGACCACTGACTACATGACGATGTGAGAATGCG-3′), which include a 5′ T7 RNA polymerase-binding site (TAATACGACTCACTATAGGGAGACCAC). Thermocycle conditions were as follows: 95°C for 2 min; and 25 cycles of 94°C for 1 min, 65°C for 45 s, 72°C for 1 min; and a final extension step of 72°C for 10 min. PCR products (1,011 bp) were photographed after agarose gel electrophoresis.

Double Stranded RNA (dsRNA) Production

The purified PCR (1,011 bp) products were used as templates to produce dsRNA by using a MEGAscript RNAi kit (Ambion, Inc.), following the manufacturer's instructions for the synthesis and purification of the dsRNA. The dsRNA products were precipitated with LiCl and resuspended in miliQ-H_20. RNA concentration was measured at A_{260} and in 1% agarose gels. dsRNAs were stored at −70°C.

RNA Interference (RNAi)

Suspended medfly hemocytes (5×10^5/100 μl) were incubated in Grace's medium with 8 μg of FAK dsRNA for up to 6 h and were then either lysed or used for flow cytometry or immunofluoresence microscopy. Long-term incubations were not possible as the viability of the hemocytes decreases considerably.

Enzyme-Linked Immunoabsorbent Assays (ELISA)

Hemocytes (5×10^5/100 μl) were either incubated for 5.30 h in Grace followed by 30 min treatment with *E. coli* at 25°C (10 bacteria per hemocyte)/or latex (10 beads per hemocyte) or incubated in the presence of FAK dsRNA for up to 6 h at 25°C, but the last 30 min *E. coli* (10 bacteria per hemocyte)/or latex (10 beads per hemocyte) was added. Hemocytes incubated in plain medium were used as a control. Cells were then lysed in lysis buffer. Insoluble material was removed by centrifugation (16,000*g* for 15 min at 4°C), supernatant was collected, and total protein was determined. Samples were diluted with 150 mM Na_2CO_3-buffered solution, pH 9.0, to a final concentration of 50 mM Na_2CO_3 and 2–4 μg protein/ml. Aliquots of 100 μl from each sample were added in respective wells of 8-well strips (Costar) and left overnight at 4°C. Strips were washed four times with TBS containing 0.05% (v/v) Tween 20. To avoid non-specific interactions, 250 μl SuperBlock™ (Pierce Rockford, IL) was added to all wells, and the strips were allowed to incubate for 2 h at 37°C. The antigens bound onto the wells were detected with anti-p^{ser383}Ellk-1 polyclonal antibody, (1:1,000) in TBS 10% (v/v) SuperBlock™ and 0.05% (v/v) Tween 20, for 1 h at 37°C. Wells were washed and incubated with 100 μl goat anti-rabbit IgG antibody labeled with HRP for 1 h at 37°C. After washing, peroxidase activity was determined using tetramethylbenzidine (TMB) as a substrate. The enzymic reaction was stopped with 50 μl 1 N H_2SO_4, and color/optical densities were measured at 450 nm with an ELISA reader.

RESULTS

Expression of an ELK-1-Like Protein in Medfly Hemocytes

Detailed molecular and genetic studies, coupled with the sequencing of the fly genome, have demonstrated eight Ets-related genes in *Drosophila*. All show high homology to genes in vertebrates including human [Hsu and Schulz, 2000]. An initial target of this study was to explore whether Elk-1, a member of Ets gene family, is expressed and functions in medfly hemocytes in 3rd instar larvae. The antibodies used for this purpose were polyclonal Ets-1/Ets-2 (C-275) that are recommended for detection of Ets-1 and Ets-2 in human and other vertebrates, in *Drosophila*, *Xenopus*, and *C. elegans*, polyclonal human Elk-1, and p^{ser383}Elk-1. Bioinformatics approach shows that Ets genes are highly conserved in phylogenetically diverged species from insects to man [Albagli et al., 1996]. In addition, the majority of Ets family proteins do not have phosphorylation sites and biochemical approaches show that the Ets members that have phosphorylation sites do not cross-react with MAPKs as is the case for Elk-1.

To investigate the expression of an Ets family member and, in particular, the Elk-1 transcription factor in medfly hemocytes, we performed immunoprecipitation analysis with

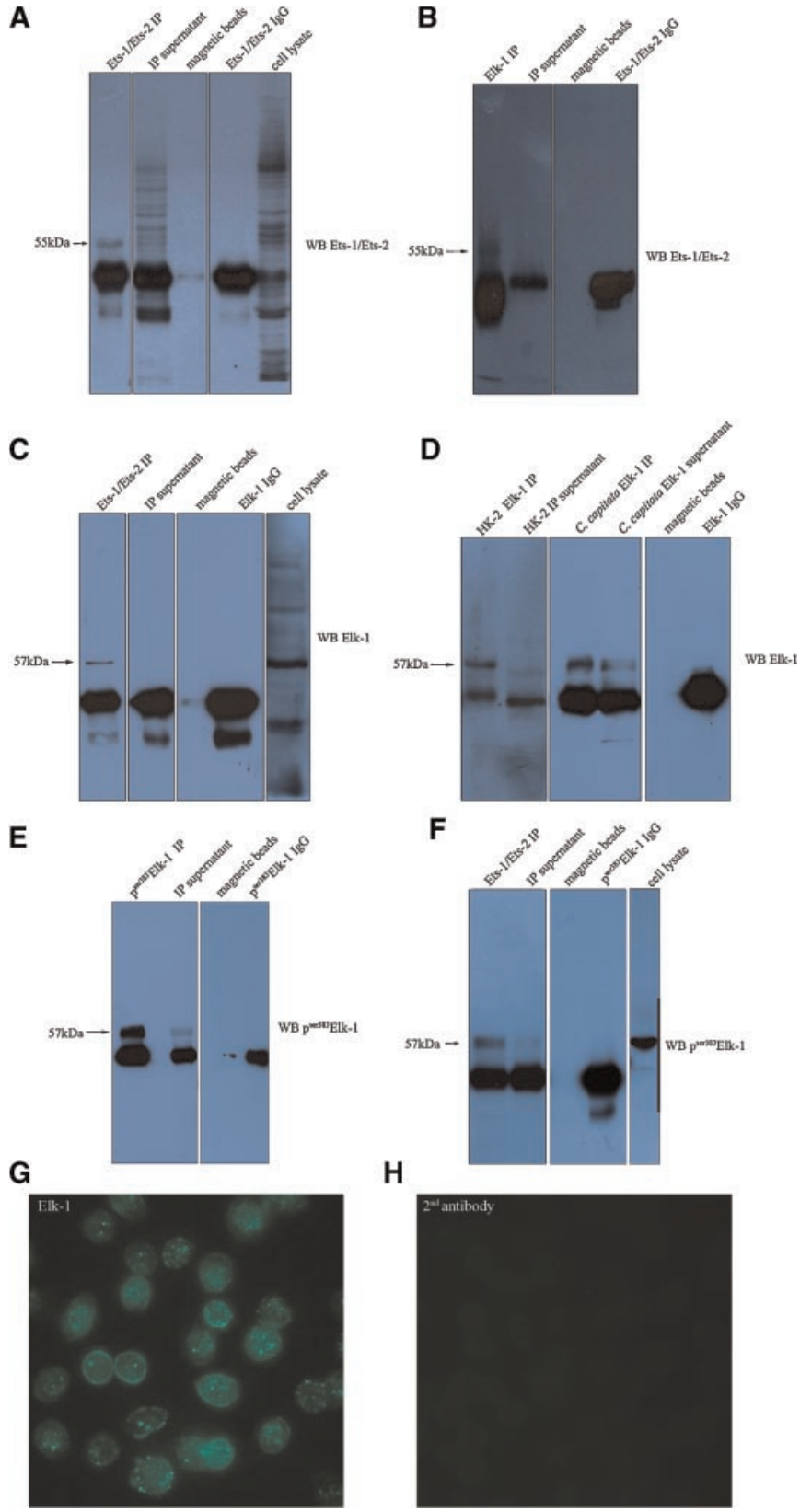
A
Ets-1/Ets-2 IP
IP supernatant
magnetic beads
Ets-1/Ets-2 IgG
cell lysate
55kDa
WB Ets-1/Ets-2
B
Elk-1 IP
IP supernatant
magnetic beads
Ets-1/Ets-2 IgG
55kDa
WB Ets-1/Ets-2
C
Ets-1/Ets-2 IP
IP supernatant
magnetic beads
Elk-1 IgG
cell lysate
57kDa
WB Elk-1
D
HK-2 Elk-1 IP
HK-2 IP supernatant
C. capitata Elk-1 IP
C. capitata Elk-1 supernatant
magnetic beads
Elk-1 IgG
57kDa
WB Elk-1
E
p^ser383Elk-1 IP
IP supernatant
magnetic beads
p^ser383Elk-1 IgG
57kDa
WB p^ser383Elk-1
F
Ets-1/Ets-2 IP
IP supernatant
magnetic beads
p^ser383Elk-1 IgG
cell lysate
57kDa
WB p^ser383Elk-1
G
Elk-1
H
2nd antibody

Fig. 1.

Ets-1/Ets-2, or Elk-1 antibodies, followed by Western blotting with Ets-1/Ets-2 or Elk-1 antibodies. Figure 1A–D clearly shows that the immunoprecipitant in all cases is a single protein band with apparent molecular weight of about 55 kDa. Immunoprecipitation with Elk-1 antibodies in human HK-2 cell extracts also revealed a single protein band with similar MW (Fig. 1D). Immunofluorescence labeling confirmed the expression of Elk-1-like protein in hemocytes (Fig. 1G,H). Further confirmation for the expression of Elk-1 in medfly hemocytes was obtained using p^{ser383}Elk-1 antibodies. Figure 1E,F shows that immunoprecipitation analysis with p^{ser383}Elk-1 (Fig. 1E) or Ets-1/Ets-2 (Fig. 1F) antibodies followed by Western blotting with p^{ser383}Elk-1 antibodies also recognizes the same protein band. The above bioinformatics, biochemical, and immunofluorescence approaches strongly support that an Elk-1-like protein with sequence similarity to human Elk-1 and containing the conserved motif Ets, is encoded in the genome of medfly.

Elk-1-Like Protein and Phagocytosis

The signaling for the engulfment of pathogens in medfly hemocytes is transmitted from RGD-binding receptors via FAK/Src and MAPKs pathways to bacteria-dependent secretion of certain components, such as PAPs that are prerequisites for bacteria uptake [Lamprou et al., 2005, 2007; Mavrouli et al., 2005]. To explore whether *E. coli* also signals for the phosphorylation of Elk-1-like protein, suspended hemocytes in Grace's medium were challenged with *E. coli* and the phosphorylation of Elk-1-like protein was evaluated by Western analysis. Figure 2A clearly shows that *E. coli* increases the phosphorylation of Elk-1-like protein at ser 383 in hemocytes significantly. These results were further confirmed by ELISA (Fig. 2B), and immunofluorescence analysis (Fig. 2C,E). Therefore, *E. coli* induces Elk-1-like protein phosphorylation.

The *E. coli*-dependent phosphorylation of Elk-1-like protein prompted us to elucidate whether Elk-1-like protein is also a good candidate for the regulation of phagocytosis of bacteria. For this purpose, suspended hemocytes in Grace's medium were loaded with either antibodies against Elk-1, or FAK/Src, as positive controls or CD14, an irrelevant antibody, as a negative control, in the presence of bacteria and the level of phagocytosis was monitored by flow cytometry (Fig. 3). The results clearly show a decrease of about 58% in the presence of Elk-1 antibodies (Fig. 3D) and 22% in the presence of FAK and Src antibodies (Fig. 3E), in the uptake of bacteria compared with unloaded hemocytes (Fig. 3C). The specificity of the anti-Elk-1 antibodies was checked by loading the hemocytes with the anti-CD14 antibodies (Fig. 3F). The expression of Elk-1-like protein is therefore an important determinant of bacteria phagocytosis in the medfly hemocytes.

Functional Association of FAK/Src and MAPKs Pathways With Elk-1-Like Protein

The above results encouraged us to explore whether Elk-1-like protein in medfly hemocytes is a downstream target, for FAK/Src and MAPKs pathways that lead in the uptake of bacteria and abiotic components [Lamprou

Fig. 1. Identification of Ets-2, Elk-1-like protein and p^{ser383}Elk-1-like protein in medfly hemocytes. For immunoprecipitation analysis, suspended hemocytes from wandering larval stage were isolated and their lysates were treated with polyclonal antibodies against Ets-1/Ets-2 and Elk-1 (Ets-1/Ets-2 IP and Elk-1 IP) to evaluate the expression of Ets-2 and Elk-1-like protein. The precipitates (Ets-1/Ets-2 IP and Elk-1 IP), the precipitate supernatants (IP supernatant), the secondary antibody conjucated with beads (magnetic beads), the polyclonal antibody for which the SDS–PAGE was blotted (Ets-1/Ets-2 IgG and Elk-1 IgG) and the crude cell lysate (cell lysate) were then resolved on 10% SDS–PAGE and blotted with Ets-1/Ets-2 and Elk-1 polyclonal antibodies, respectively (**A**–**D**). Human kidney proximal tubular epithelial cells (HK-2 cells) were used as a positive control (D). Also, for immunoprecipitation analysis suspended hemocytes from wandering larval stage were isolated and their lysates were treated with polyclonal antibodies against p^{ser383}Elk-1 and Ets-1/Ets-2 (p^{ser383}Elk-1 IP and Ets-1/Ets-2 IP) to evaluate the expression of p^{ser383}Elk-1-like protein and Ets-2. The precipitates (p^{ser383}Elk-1 IP and Ets-1/Ets-2 IP), the precipitate supernatants (IP supernatant), the secondary antibody conjugated with beads (magnetic beads), the polyclonal antibody for which the SDS–PAGE was blotted (p^{ser383}Elk-1 IgG), and the crude cell lysate (cell lysate) were then resolved on 10% SDS–PAGE and blotted with p^{ser383}Elk-1 polyclonal antibody (**E**, **F**). The samples in each of the six panels (A–F) were run in one gel. For immunofluorescence identification of Elk-1-like protein (**G**), suspended hemocytes from wandering larval stage were isolated and then fixed and stained with 1st antibody against Elk-1 and then with a secondary anti-rabbit antibody coupled to a fluorochrome (FITC). Hemocytes which were stained only with the secondary anti-rabbit antibody coupled to a fluorochrome (FITC) were used as a control (**H**). Photomicrographs shown are representing of about 70% hemocytes obtained in three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

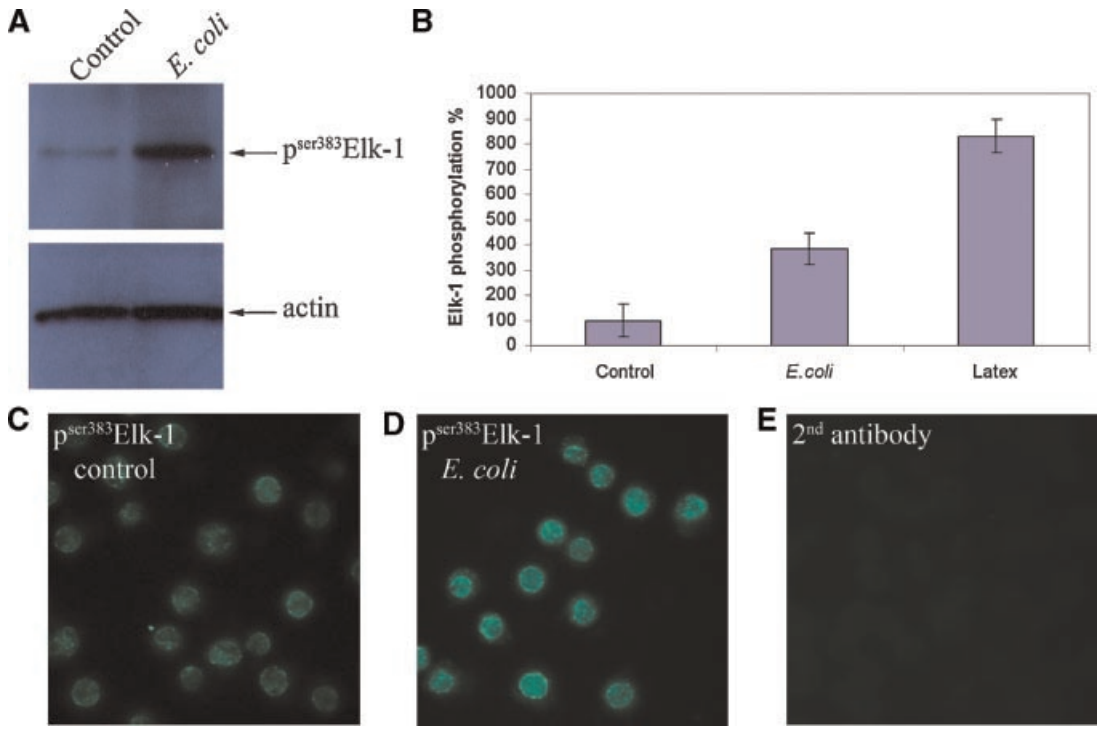

Fig. 2. Elk-1-like protein phosphorylation in *E. coli*-challenged hemocytes. Suspended hemocytes in Grace's medium were challenged with *E. coli* (ratio *E. coli*:hemocyte, 10:1) for 10 min at 25°C and the phosphorylation of Elk-1-like protein was evaluated by Western analysis (**A**). Actin was used as a loading control. For ELISA analysis (**B**), hemocytes were treated with *E. coli* (10 bacteria per hemocyte)/or latex (10 beads per hemocyte) for 30 min at 25°C. Following the incubation, hemocytes were lysed and the lysates were plated in a 96-well assay plate, and analyzed for p^{ser383}Elk-1-like protein, using polyclonal antibody against p^{ser383}Elk-1. Each bar represents the mean and standard error of the mean (n = 6). Each well contained 4 μg/ml hemocyte lysate. For immunofluorescence identification of p^{ser383}Elk-1-like protein (**C**, **D**) in the presence or absence of *E. coli*, suspended hemocytes of wandering stage were isolated and then fixed and stained with 1st antibody against p^{ser383}Elk-1 and then with a secondary anti-rabbit antibody coupled to a fluorochrome (FITC). Hemocytes which were stained only with the secondary anti-rabbit antibody coupled to a fluorochrome (FITC) were used as a control (**E**). Photomicrographs shown are representing of about 70% hemocytes obtained in three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

et al., 2007]. For this purpose, we initially analyzed the effect of FAK dsRNA, on the phosphorylation of Elk-1-like protein. In these experiments, we used dsRNA corresponding to *Drosophila* FAK that works well in medfly hemocytes, bringing about 55% diminution of FAK expression (Fig. 4A–C) [Mamali et al., 2007]. The depletion was only partial, evidently, because of the short-term incubations, as the viability of hemocytes decreases in long-term incubations [Lamprou et al., 2007; Mamali et al., 2007].

To understand FAK silencing in the phosphorylation of Elk-1, medfly hemocytes in suspension were separated into three equal parts. The first part was incubated for 6 h in Grace's medium (control). The second was incubated for 5.5 h in Grace's medium followed by 30 min *E. coli* or latex beads in Grace's medium (*E. coli*/latex) and the third was incubated for 6 h with 8 μg of FAK dsRNA, but for the last 30 min *E. coli* or latex beads were added (FAK dsRNA + *E. coli*/FAK dsRNA + latex). Figure 4 clearly shows that FAK expression silencing in response to FAK dsRNA treatment in *E. coli* or latex beads, challenged hemocytes, decreased the phosphorylation of Elk-1 at ser 383 compared with

Fig. 3. Elk-1-like protein regulates bacteria phagocytosis. Suspended hemocytes (5×10^5 cells/100 ml medium) in Grace's medium were loaded with either antibodies against Elk-1, or FAK/Src, as positive controls or CD14, an irrelevant antibody, as a negative control, in the presence of FITC-labeled *E. coli* (ratio FITC-labeled *E. coli*:hemocyte, 10:1) for 10 min at 25°C and the level of phagocytosis was monitored by flow cytometry after trypan blue quenching (**D**–**F**), compared with unloaded hemocytes (**C**). The uptake of FITC-labeled *E. coli* by hemocytes in normal conditions (no osmotic loading) (**B**) is also a control compared to hemocytes incubated in the absence of bacteria (**A**).

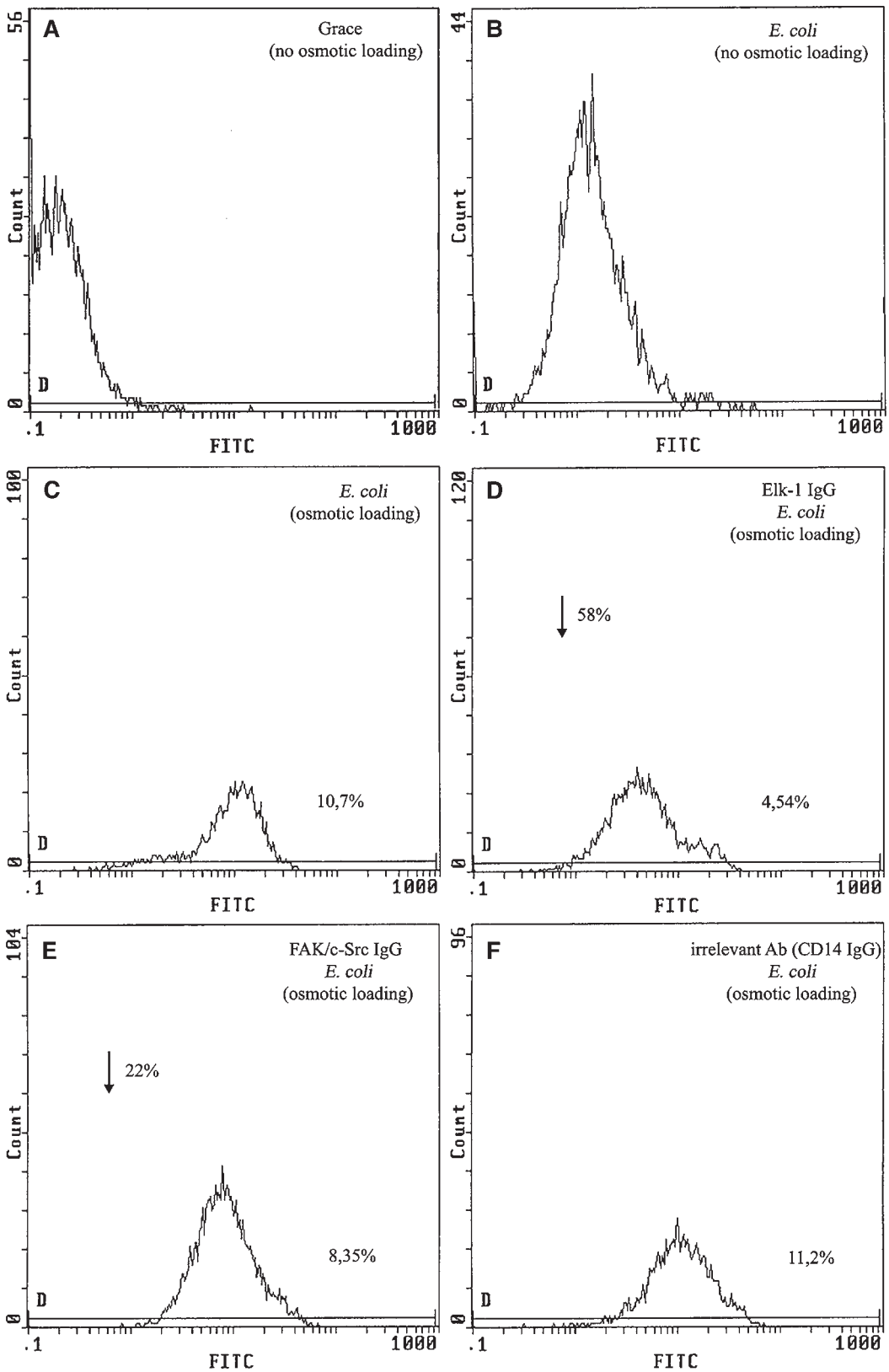
A
Grace
(no osmotic loading)
B
E. coli
(no osmotic loading)
C
E. coli
(osmotic loading)
10,7%
D
Elk-1 IgG
E. coli
(osmotic loading)
58%
4,54%
E
FAK/c-Src IgG
E. coli
(osmotic loading)
22%
8,35%
F
irrelevant Ab (CD14 IgG)
E. coli
(osmotic loading)
11,2%
Count
FITC
.1
1000

Fig. 3.

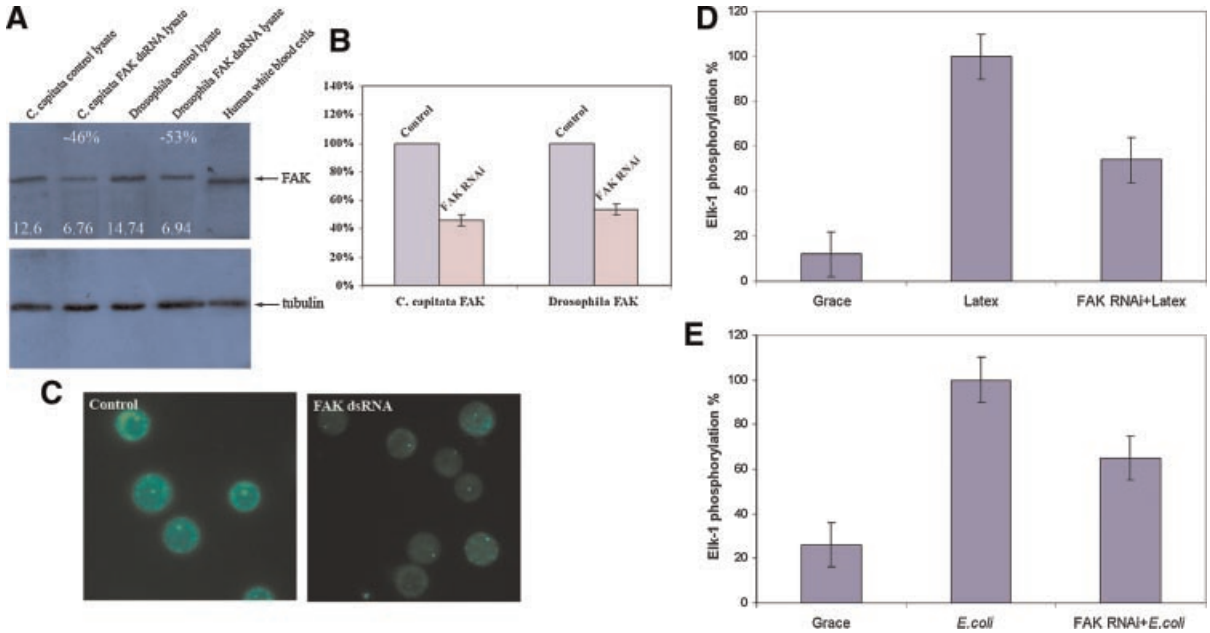

Fig. 4. Effect of FAK silencing on the phosphorylation of Elk-1-like protein. To observe the effect of FAK silencing, suspended hemocytes from wandering larval stage were incubated in the presence or absence of 8 μg of FAK dsRNA for 6 h and its effect on FAK expression was evaluated by immunoblotting analysis and ELISA. For Western blot analysis (**A**), hemocytes were untreated or treated with 8 μg of FAK dsRNA for up to 6 h. Cell lysates (10 μg) were then resolved on 10% SDS–PAGE and blotted with anti-FAK polyclonal Ab. Tubulin was used as a loading control. Human white blood cells lysate was used as a positive control for FAK expression. The data were quantitated with relative to the tubulin numerical quantities listed below and above the second and the forth lane (*C. capitata* FAK dsRNA lysate and *Drosophila* FAK RNA lysate, respectively) is listed the percentage decrease of total FAK protein expression compared to the controls (*C. capitata* control lysate and *Drosophila* control lysate, respectively). For ELISA analysis (**B**), hemocytes were treated with 8 μg of FAK dsRNA for up to 6 h. Hemocytes were lysed and the lysates then plated in a 96-well assay plate, and analyzed for total FAK, using polyclonal antibodies against FAK. Each bar represents the mean and standard error of the mean (n = 6). Each well contained 4 μg/ml hemocyte lysate. Immunofluorescence identification of FAK (**C**) in the presence or absence of 8 μg of FAK dsRNA suspended hemocytes of wandering stage were isolated and then fixed and stained with antibodies against FAK. The data are representative of one experiment of five performed. The effect of FAK silencing on the phosphorylation of Elk-1-like protein was observed in (**D** and **E**) where suspended hemocytes from wandering larval stage were either incubated for 5.30 h in Grace followed by 30 min treatment with *E. coli*/or latex beads or incubated in the presence of 8 μg of FAK dsRNA for up to 6 h, but the last 30 min *E. coli*/or latex beads were added (FAK dsRNA + *E. coli*/or FAK dsRNA + latex beads). Hemocytes incubated in plain medium were used as a control (grace). Afterwards, the hemocytes were lysed and the lysates were plated in a 96-well assay plate and analyzed by ELISA to detect the status of Elk-1-like protein phosphorylation using p^{ser383}Elk-1 polyclonal antibody. Each bar represents the mean and standard error of the mean (n = 6). Each well contained 4 μg/ml hemocyte lysate. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

E. coli and latex beads-challenged hemocytes (Fig. 4D,E).

Next, we explored whether Elk-1 is phosphorylated via activation of MAPKs. To elucidate this hypothesis, hemocytes suspended in Grace's insect medium were supplemented with pharmacological inhibitors for MAPKs designed for use in mammalian systems. The great homology of MAPKs between insect and mammals, as retrieved from Fly base and NCBI HomoloGene base gives us confidence that MAPKs pathways are phylogenetically conserved and suggest that the inhibitor studies had produced biologically relevant results. Figure 5 shows that when hemocytes suspended in Grace's medium were supplemented with either SP600125 (4 μM) for JNK or SB202190 (10 μM) for p38 or U0126 (8 μM) and PD098059 (8 μM) for ERK (specific inhibitors for MAPKs), followed by *E. coli* challenging, a considerable decrease of phosphorylation of Elk-1 was observed. Consequently, FAK/Src and MAPKs pathways signal in *E. coli*-challenged hemocytes through Elk-1-like protein.

Localization and Physical Association of FAK With Elk-1-Like Protein

The subcellular compartmentalization of signaling molecules is critical for cell regulation in response to a variety of stimuli. Therefore, given the above data, we studied the distribution of

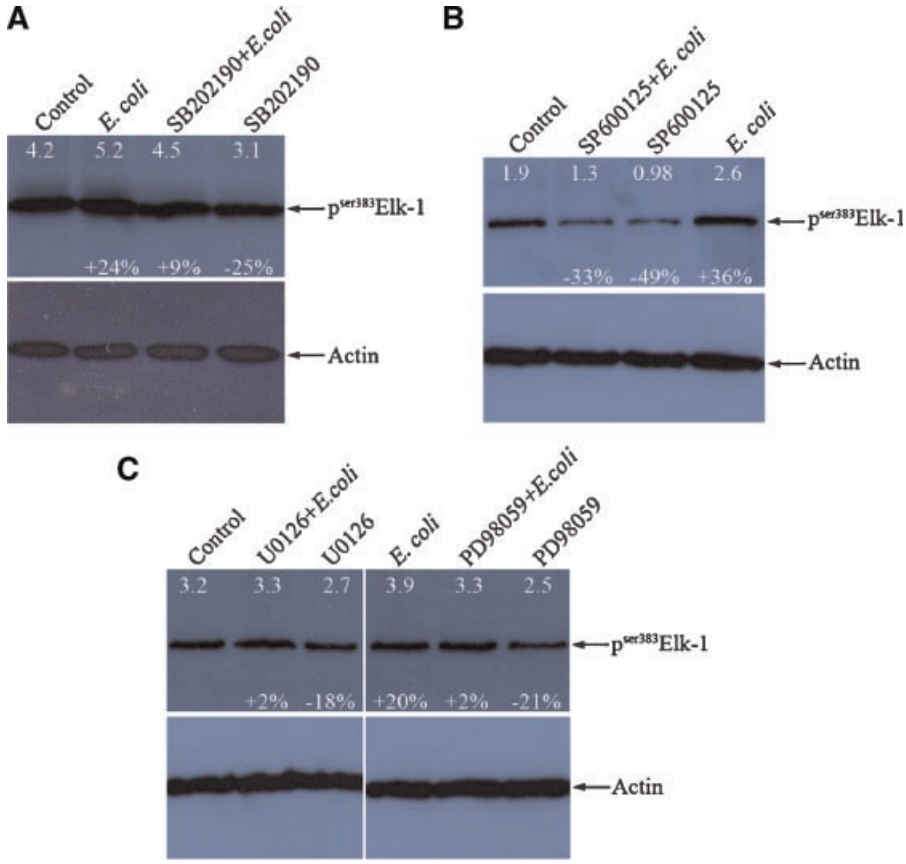

Fig. 5. Elk-1-like protein phosphorylation via activation of MAPKs. Hemocytes suspended in Grace's insect medium were supplemented with pharmacological inhibitors for MAPKs, SB202190 (10 μM) for p38 (**A**), SP600125 (4 μM) for JNK (**B**), U0126 (8 μM) and PD098059 (8 μM) for ERK (specific inhibitors for MAPKs) (**C**) for 30 min at 25°C, followed by *E. coli* challenging (ratio *E. coli*:hemocyte, 10:1) for 10 min at 25°C. Afterwards, the hemocytes were lysed and the cell lysates (10 μg) were resolved on 10% SDS–PAGE and blotted with polyclonal Ab against p^{ser383}Elk-1. Actin was used as a loading control. The samples in each of the three panels (A–C) were run in one gel. The data were quantitated with relative to the actin numerical quantities listed above and below is listed the percentage decrease/or increase of Elk-1-like protein phosphorylation compared to the control (lysate of hemocytes incubated in plain medium). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

FAK, FAK phosphorylated at Y 397, Elk-1, and Elk-1 phosphorylated at ser 383 in medfly hemocytes. Confocal microscopy analysis (full projections and slices via nucleus) shows that FAK and p^{tyr397}FAK are localized in the cytoplasm and nucleus (Fig. 6A–D). These results support that FAK, a signaling molecule at hemocyte membrane, cytoplasm, and in the nucleus must have important implications for the transfer of information from hemocyte surface receptors to the nucleus. Furthermore, biochemical and immunofluorescence data demonstrated that p^{ser383}Elk-1 is rather exclusively localized into the nucleus (Fig. 6E–H), whereas Elk-1-like protein appears to be localized both in the cytoplasm and in the nucleus as is the case for mammalian cells [Barrett et al., 2006] (Fig. 6I,K).

The functional association of FAK with Elk-1 and their localization both in the cytoplasm and in the nucleus of hemocytes encouraged us to explore the physical association of FAK with Elk-1. It is widely accepted that the signaling molecules participating in a pathway are usually in a direct physical association. Recently, in our system we demonstrated in medfly hemocytes in suspension that FAK, Src, and ERK signaling molecules regulated by RGD-binding receptors and participating in phagocytosis, show both a functional and a physical association [Lamprou et al., 2007]. In this study, the physical association of FAK and Elk-1 was explored, using co-immunoprecipitation, reciprocal co-immunoprecipitation, Western blot, and immunofluorescence analysis. Immunoprecipitation experiments

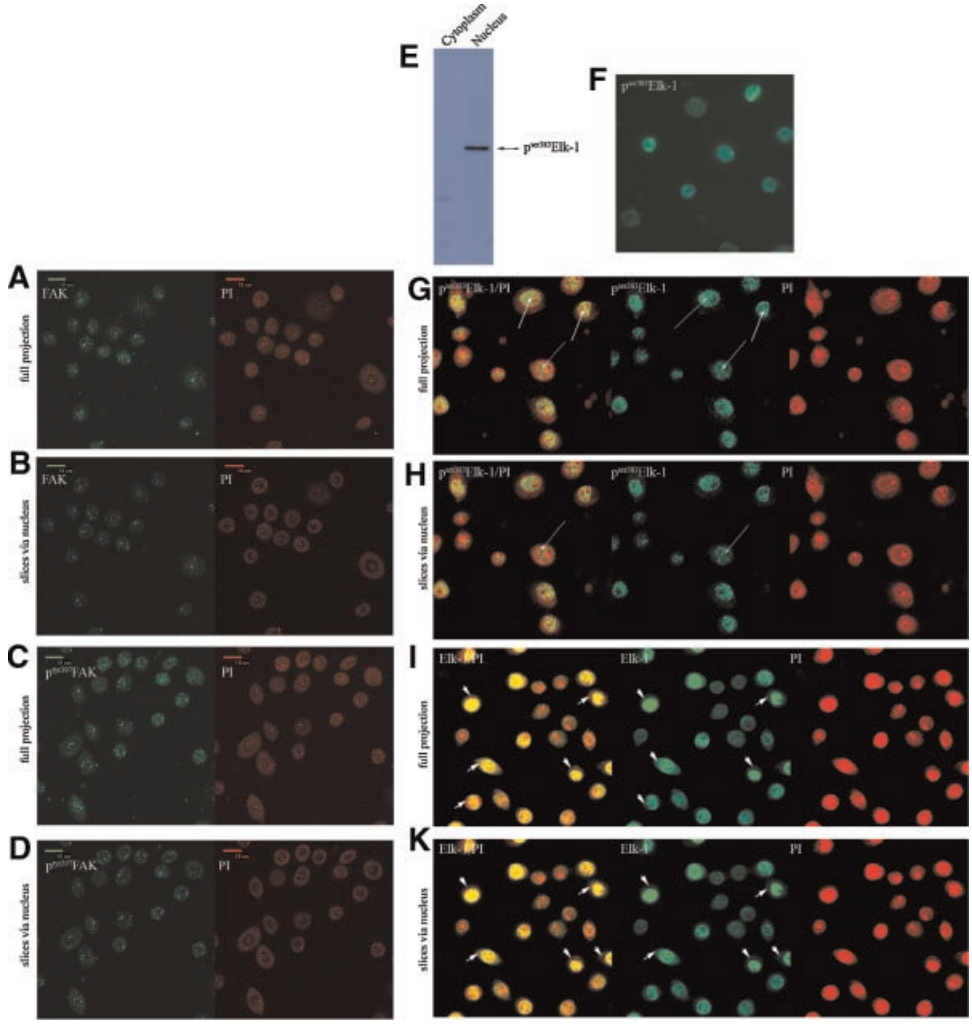

Fig. 6. Localization of FAK, p^{tyr397}FAK, Elk-1-like, and p^{ser383}-Elk-1-like protein in medfly hemocytes. Suspended hemocytes from wandering stage were isolated and then fixed and stained with antibodies against FAK, p^{tyr397}FAK, and propidium iodide. The confocal images shown are full projections (combined all the slices) of the hemocytes stained against FAK (**A**) and p^{tyr397}FAK (**C**) as well as projections of the hemocytes only of the nuclear slices stained against FAK (**B**) and p^{tyr397}FAK (**D**). Photomicrographs shown are representatives of about 80% of hemocytes, obtained in three independent experiments. For Western blot analysis, suspended hemocytes from wandering stage were homogenized and nuclei were isolated from the cytoplasmic substances and then lysed nuclei and cytoplasmic substances (10 μg) were resolved on 10% SDS–PAGE and blotted with polyclonal Ab against p^{ser383}Elk-1 (**E**). Immunofluorescence identification of p^{ser383}Elk-1-like protein in isolated nuclei which were fixed and stained with 1st antibody against p^{ser383}Elk-1 and then with a secondary anti-rabbit antibody coupled to a fluorochrome (FITC) (**F**). The localization of Elk-1-like and p^{ser383}Elk-1-like protein in medfly hemocytes is also demonstrated by confocal analysis in suspended hemocytes from wandering stage which were isolated and then fixed and stained with antibodies against p^{ser383}Elk-1, Elk-1, and propidium iodide (**G**–**K**). White arrows indicate the nuclei and white arrowheads the cytoplasm. The confocal images shown are full projections (combined all the slices) of the hemocytes stained against p^{ser383}Elk-1 (G) and Elk-1 (I) as well as projections of the hemocytes only of the nuclear slices stained against p^{ser383}Elk-1 (H) and Elk-1 (K). Photomicrographs shown are representatives of about 80% of hemocytes, obtained in three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

demonstrated that when FAK was immunoprecipitated from medfly hemocyte lysate in the presence or not of *E. coli* using anti-FAK polyclonal antibody, co-immunoprecipitation of Elk-1 was observed, in Western blot analysis (Fig. 7A, lanes 1 and 3). But, when p^{tyr397}FAK was immunoprecipitated from medfly hemocyte lysate in the presence or not of *E. coli* using anti-p^{TYR397}FAK polyclonal antibody there was no co-immunoprecipitation of Elk-1 observed, in Western blot analysis (Fig. 7A, lanes 5 and 7). The same blots when stained

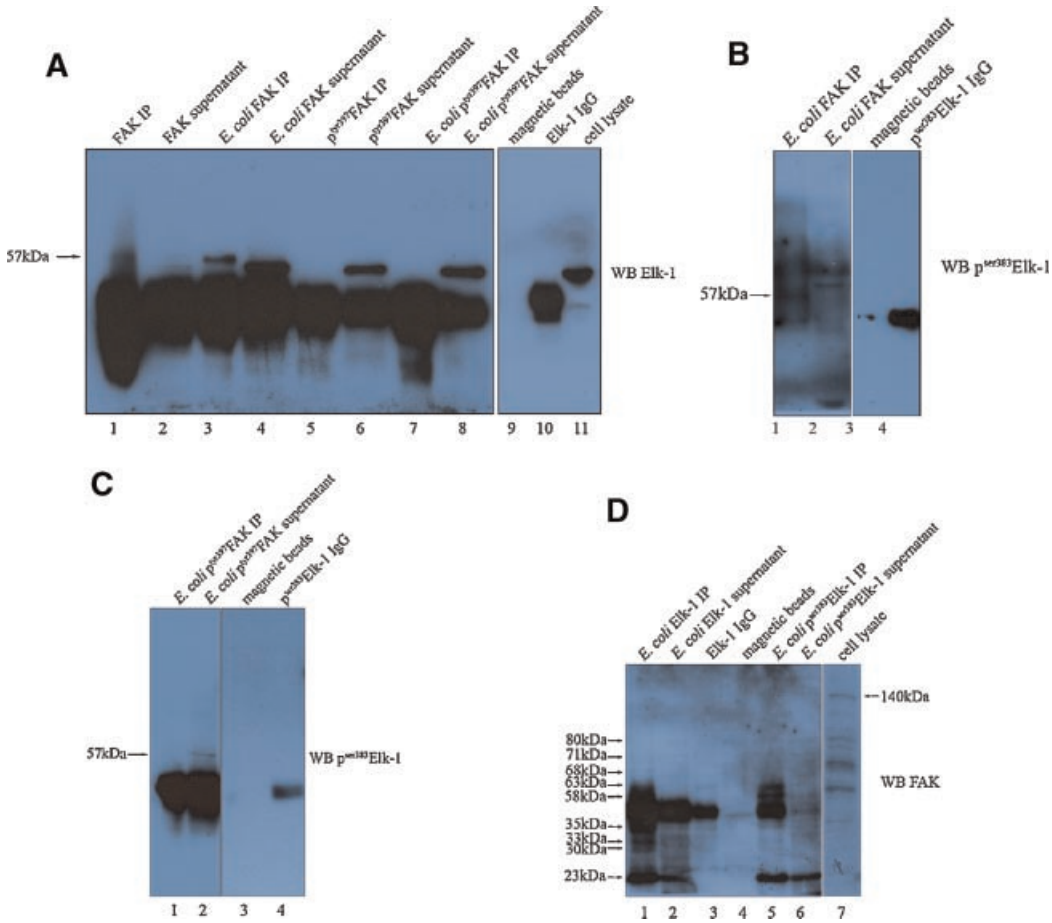

Fig. 7. Physical association of FAK with Elk-1-like protein and p^{ser383}Elk-1-like protein and co-localization of FAK with Elk-1-like protein. **A**: Suspended hemocytes from wandering larval stage were isolated, treated in the presence or absence of bacteria, then lysed, and their lysates were treated with polyclonal antibodies against FAK and p^{tyr397}FAK. The precipitates were then resolved on 10% SDS–PAGE and blotted with Elk-1 polyclonal antibody to evaluate the physical association of FAK and p^{tyr397}FAK with Elk-1-like protein. **B**: Suspended hemocytes from wandering larval stage were isolated, treated in the presence of bacteria, then lysed, and their lysates were treated with polyclonal antibody against FAK. The precipitates were then resolved on 10% SDS–PAGE and blotted with p^{ser383}Elk-1 polyclonal antibody to evaluate the physical association of FAK with p^{ser383}Elk-1-like protein. **C**: Suspended hemocytes from wandering larval stage were isolated, treated in the presence of bacteria, then lysed and their lysates were treated with polyclonal antibody against p^{tyr397}FAK. The precipitates were then resolved on 10% SDS–PAGE and blotted with p^{ser383}Elk-1 polyclonal antibody to evaluate the physical association of p^{tyr397}FAK with p^{ser383}Elk-1-like protein. **D**: Suspended hemocytes from wandering larval stage were isolated, treated in the presence of bacteria, then lysed, and their lysates were treated with polyclonal antibodies against Elk-1 and p^{ser383}Elk-1. The precipitates were then resolved on 10% SDS–PAGE and blotted with FAK polyclonal antibody to evaluate the physical association of Elk-1-like protein and p^{ser383}Elk-1-like protein with FAK. The samples in each of the four panels (A–D) were run in one gel. **E**–**H**: Suspended hemocytes from wandering stage were isolated, treated with *E. coli* or not (control), fixed and stained with 1st antibodies against FAK or p^{tyr397}FAK and p^{ser383}Elk-1 and then with a secondary Cy3-conjugated goat anti-rabbit or an anti-mouse antibody coupled to a fluorochrome (FITC). The confocal images shown are full projections (combined all the slices) of the hemocytes stained against FAK and p^{ser383}Elk-1 (A, B) as well as against p^{tyr397}FAK and p^{ser383}Elk-1 (C, D) and on the lower left corner of each projection there is a high-magnification fluorescence microscopy cell image. The co-localization of FAK with p^{ser383}Elk-1, in *E. coli*-challenged hemocytes, is indicated with yellow color (B). Photomicrographs shown are representatives of about 80% of hemocytes, obtained in three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

with anti-p^{ser383}Elk-1 only FAK was co-immunoprecipitated (Fig. 7B, lane 1).

The interaction of Elk-1-like protein with FAK was confirmed through reciprocal co-immunoprecipitation with antibodies against Elk-1 or p^{ser383}Elk-1 followed by Western blotting with FAK (Fig. 7D). These reciprocal co-immunoprecipitation experiments clearly show some interesting results (Fig. 7D). When Elk-1 or phospho-Elk-1 immunoprecipitants

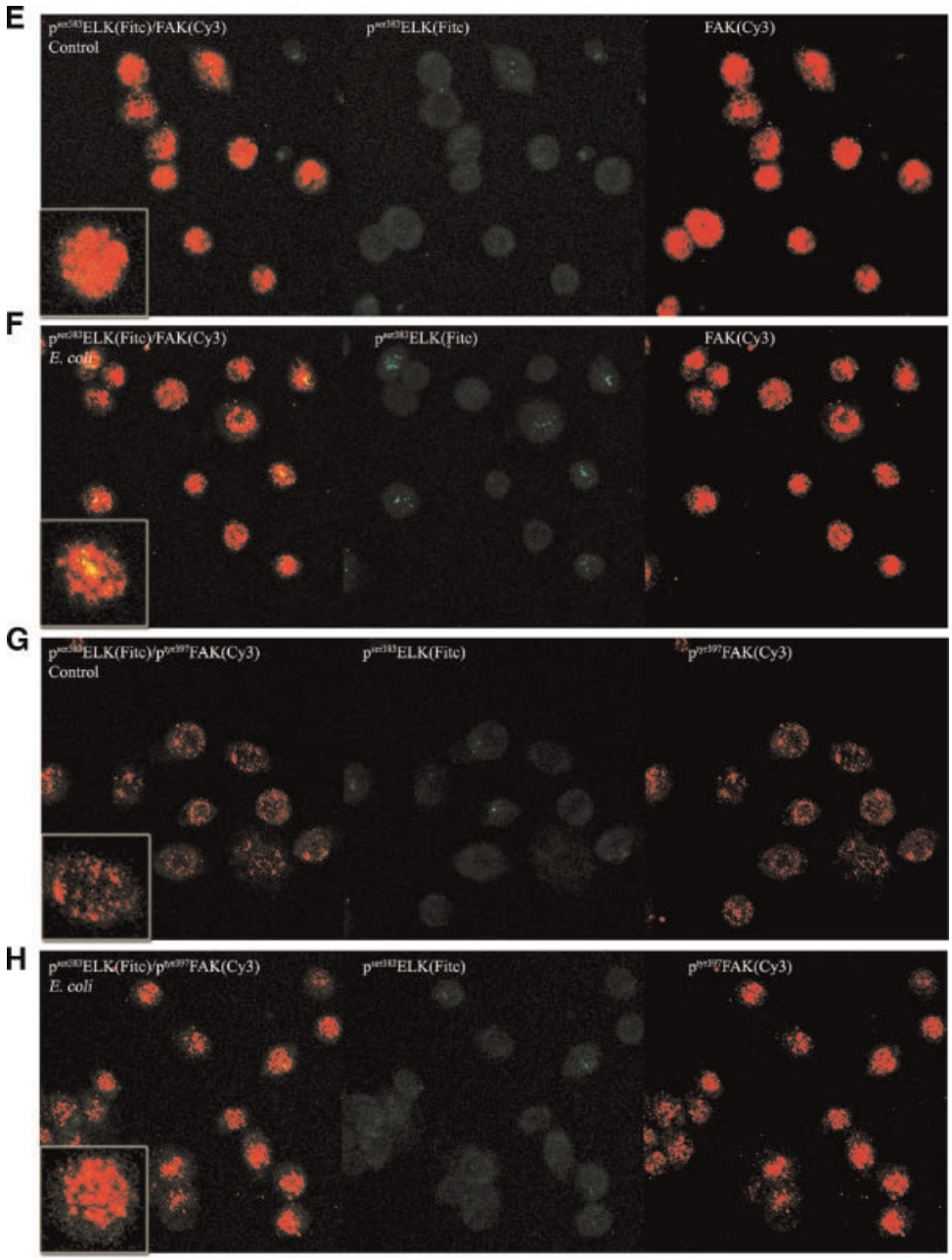

Fig. 7. (*Continued*)

are blotted and stained with FAK polyclonal antibodies, several low molecular weight protein bands (23, 30, 33, 35, 58, 63 kDa) were detected (Fig. 7D), probably due to the high level of endogenous proteolytic activity in these cells [Kanost et al., 2004], as is the case for mammalian cell lines [Jones and Stewart, 2004]. Full-length FAK is not visible. These data show an association between Elk-1 or p^{ser383}Elk-1 and FAK immunoprecipitants but not with Y397 FAK immunoprecipitants.

In light of the above data, confocal microscopy analysis was performed to confirm the association of Elk-1 with FAK and to visualize the subcellular compartments where co-localization of FAK with Elk-1-like protein occurs. These experiments revealed that only FAK and p^{ser383}Elk-1 are co-localized, although it remains unresolved in our cell system whether phosphorylation of Elk-1 occurs in the cytoplasm or in the nucleus (Fig. 7E–H). The biochemically observed interaction of FAK with

Elk-1 cannot be observed in confocal analysis because both antibodies used are rabbit's. Consequently, these results strongly support that Elk-1-like protein is a novel protein-binding partner for FAK, a finding that significantly broadens the potential functioning of FAK and Elk-1-like protein generally.

DISCUSSION

Given that the gross cell biological features of phagocytosis are shared between insects and mammals, it is well accepted that the intracellular signaling pathways underlining phagocytosis also should be similar. Well-characterized signaling events employed by mammalian leucocytes and insect hemocytes are those associated with FAK and MAPKs.

FAK has a key role in integrating signals from growth factor and integrins at the cell membrane [Sieg et al., 2000] as well as propagating signals to other intracellular signaling molecules [Schlaepfer et al., 1994]. Similarly, FAK has been demonstrated in medfly hemocytes to propagate signal to other intracellular signaling molecules during apoptosis and phagocytosis [Lamprou et al., 2007; Mamali et al., 2007]. In addition, activation of MAPKs signaling pathways during phagocytosis is well conserved, having been demonstrated in mammalian systems [Yamamori et al., 2000; Leverrier and Ridley, 2001] as well as in insect hemocytes [Mizutani et al., 2003; Plows et al., 2004; Lamprou et al., 2007]. FAK is usually functionally and physically associated with MAPKs and MAPKs are also functionally associated with members of Ets family transcription factors, including Elk-1 [Chen et al., 1992; Yang et al., 2000; Yordy and Muise-Helmericks, 2000]. We, therefore, targeted these signaling pathways identified in mammals and insects as bases for the design of our study. In short, we selected Elk-1 in relation to FAK to explore their interactions and the possible involvement of Elk-1 in the process of phagocytosis. These studies aimed to further clarify the role of FAK and MAPKs in the process of phagocytosis by primary medfly hemocytes.

Bioinformatics approach shows that Ets genes are highly conserved in phylogenetically diverged species from insects to man [Albagli et al., 1996]. In addition, the majority of Ets family proteins do not have phosphorylation sites and biochemical approaches show that the Ets members that have phosphorylation sites do not cross-react with MAPKs as is the case for Elk-1. These data give us confidence that the expressed Elk-1-like protein in medfly hemocytes (Figs. 1 and 2) is phylogenetically conserved and is very likely to be biologically relevant, particularly given the conservation of signaling mechanisms that are present in innate immune responses across different animal groups.

Given the above information we decided to study whether Elk-1, a member of Ets family, interacting with FAK, regulates the process of phagocytosis. Recently, we demonstrated with knockdown experiments that FAK is a prerequisite for *E. coli* uptake by medfly hemocytes [Lamprou et al., 2007]. In this report, knockdown experiments using dsRNA for FAK in suspended hemocytes resulted in blockage of Elk-1-like protein phosphorylation (Fig. 4) and osmotic loading experiments using Elk-1 antibodies demonstrated the dependence of phagocytosis from Elk-1-like protein (Fig. 3). These findings give us confidence that FAK/Src pathway signals Elk-1 phosphorylation via MAPKs pathways in *E. coli*-challenged hemocytes. In support is the great homology of MAPKs between insect and mammals, as retrieved from Fly base and NCBI HomoloGene base.

In light of the functional association of FAK with Elk-1-like protein, we explored whether a physical association between these two signaling molecules also exists. Confocal microscopy analysis shows that FAK and p^{tyr397}FAK are localized in the cytoplasm and nucleus in medfly hemocytes (Fig. 6A). These data strongly support that FAK, a signaling molecule at hemocyte membrane, cytoplasm, and in the nucleus might have important implications for the transfer of information from hemocyte surface receptors to the nucleus. Furthermore, co-immunoprecipitation and reciprocal co-immunoprecipitation experiments using FAK antibodies demonstrate the physical association of FAK with Elk-1-like protein or p^{ser383}Elk-1-like protein (Fig. 7A,B,D). In contrast, immunoprecipitates with Y397 FAK antibodies are not associated with Elk-1 or pElk-1-like protein. More careful observations on Figure 7D shows that the protein bands immunoprecipitated with Elk-1 or pElk-1 antibodies and recognized by FAK antibodies are of low molecular weight. Furthermore, these protein bands are not recognized by Y397 FAK antibodies (Fig. 7A,C).

These data support that these low molecular weight protein bands might be FAK fragments not including or not phosphorylated at Y397.

Confocal analysis further specifies that FAK and p^{ser383}Elk-1-like protein are co-localized only in the nucleus (Fig. 7F). Unfortunately, we cannot visualize whether FAK and Elk-1-like protein are also co-localized, as both antibodies used are rabbit. Given the above findings as well as that only N-terminal FAK fragments are imported in the nucleus in several different mammalian cell lines [Lobo and Zachary, 2000; Jones et al., 2001; Jones and Stewart, 2004], it can be concluded that FAK fragments not included or not phosphorylated at Y397 are associated with Elk-1 and/or pElk-1, whereas larger FAK fragments including Y397 are not associated with Elk-1 and/or pElk-1-like protein. This is an indication that the associated and the free signaling molecules present in the nucleus might do different functions. Evidently, the complex of FAK fragments with Elk-1-like protein participates in the process of phagocytosis by medfly hemocytes.

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