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Apoptosis in Medfly Hemocytes Is Regulated During Pupariation Through FAK, Src, ERK, PI-3K p85a, and Akt Survival Signaling

Irene Mamali,[1] Marianthi N. Tatari,[1] Ilina Micheva,[2] Maria Lampropoulou,[1] and Vassilis J. Marmaras[1]*

[1]Department of Biology, University of Patras, 26500 Patras, Greece
[2]Department of Internal Medicine, Haematology Division, Patras University Medical School, 26500 Patras, Greece

Abstract Focal adhesion kinase (FAK) and its downstream signaling targets are implicated in the process of apoptosis induced by external stimuli, in several mammalian systems. In this report, we demonstrate, that medfly (*Ceratitis capitata*) hemocytes do undergo apoptosis during larval development. In particular, we show using Western blot, ELISA and flow cytometry analysis, that FAK expression silencing in transfected by FAK double-stranded RNA (dsRNA) hemocytes, enhances twofold hemocyte apoptosis, by signaling through Src, MEK/ERK, and PI-3K/Akt signaling pathways. FAK expression silencing, in response to FAK dsRNA treatment, blocks partially the phosphorylation of its downstream targets. Pre-incubation of hemocytes, with specific inhibitors of FAK downstream signaling molecules, demonstrated that all these inhibitors reduced hemocyte viability and enhanced the magnitude of apoptosis about threefold. This data suggest that these pathways contribute to hemocyte survival and/or death during development. The expression and phosphorylation of FAK, Src, PI-3K p85a, Akt, and ERK signaling molecules appear to be dependent upon developmental stages. The expression and phosphorylation of the above signaling molecules, in annexin-positive and annexin-negative hemocytes is also distinct. The maximum expression and phosphorylation of FAK, Src, PI-3K p85a, Akt, and ERK appeared in annexin-positive hemocytes, in both early and late apoptotic hemocytes. The novel aspect of this report is based on the fact that hemocytes attempt to suppress apoptosis, by increasing the expression/phosphorylation of FAK and, hence its downstream targets signaling molecules Src, ERK, PI-3K p85a, and Akt. Evidently, the basic survival pathways among insects and mammals appear to remain unchanged, during evolution. J. Cell. Biochem. 101: 331–347, 2007.

Key words: apoptosis; FAK/Src; ERK; PI-3K/Akt; insect hemocytes; signaling

Apoptosis is an evolutionary conserved process, used by several animal species, including mammals and insects, to developmentally delete vestigial structures, control cell number, and remodel tissues and organs. The large diversity of regulators of apoptosis and their interactions complicate the analysis of their individual functions, particularly during development [McCarthy, 2003]. In mammals, one of the proteins implicated in the mechanism of apoptosis is the focal adhesion kinase (FAK), a non-receptor tyrosine kinase that is involved in the control of cell–extracellular interactions. In many cell types, inhibition of FAK can induce apoptosis and overexpression of FAK can protect cells from apoptosis, induced in response to several stimuli [Schaller, 2001]. These findings support the role of FAK in transmitting a cell survival signal.

Abbreviations used: FAK, focal adhesion kinase; PI-3K, phosphatidylinositide 3′-kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular-signal regulated kinase; MEK, MAPK/ERK Kinases; GRB2, growth factor receptor-bound protein 2; RT-PCR, reverse transcription-polymerase chain reaction; RNAi, RNA interference; FITC, fluoroscein isothiocyanate conjugated; HRP, horseradish peroxidase; FACS, fluorescence-activated cell sorter; TUNEL, terminal transferase deoxytidyl uridine end labelling; SH2 domain, Src homology domain; Akt, protein kinase B (PKB); ELISA, enzyme-linked immunoabsorbent assays; PI, propidium iodide.

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*Correspondence to: Vassilis J. Marmaras, University of Patras, 26500 Patras, Greece.
E-mail: marmaras@upatras.gr

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The survival signals are mediated by integrin receptors. The interaction between the extracellular signaling molecules and integrin receptors causes phosphorylation and activation of FAK [Lukashev et al., 1994; Schaller and Parsons, 1994; Grigoriou et al., 2005]. Tyrosine 397 is an autophosphorylation site of FAK and plays a critical role in downstream signaling, providing binding sites for SH2 domains of the survival signaling pathway molecules Grb2-Sos, Src, and PI-3K [Schlaepfer et al., 1994; Grigoriou et al., 2005; Mitra et al., 2005]. Akt, one of the downstream targets of PI-3K, plays an essential role in regulating the balance between apoptosis and survival. Given the important role of FAK in the control of the apoptosis, there is considerable interest in the mechanism by which FAK signals, such as the downstream signaling pathways mediating integrin-FAK survival signaling, are diversified. The factors determining which pathway is utilized remain obscure.

Developmental apoptosis in *Drosophila* is regulated by at least five pro-apoptotic genes [Cashio et al., 2005]. Several insect systems do undergo apoptosis during larval-pupal transformation. Hemocytes are the primary mediators of cell-mediated immunity in insects, as monocytes/macrophages are in mammals. Hemocytes are responsible for a number of immune functions, among which phagocytosis, encapsulation, and melanization have been, documented [Meister and Lagueux, 2003; Lamprou et al., 2005; Mavrouli et al., 2005]. To date, hemocyte apoptosis is the best described anti-viral response in insects, hence an important element in insect immunity against viral infections [Clarke and Clem, 2003].

The process of hemocyte apoptosis, however, has not yet been studied, either in response to external stimuli, in vitro, or developmentally, during the process of transformation of larva to pupa. FAK, an important component of apoptosis in mammals, has already been identified and characterized in insects, including hemocytes [Palmer et al., 1999; Metheniti et al., 2001]. *Drosophila* homologue of FAK is a single protein with the domain structure characteristic of mammalian FAK and PYK2. The tyrosine kinase domain is most highly conserved in both DFAK56 and human FAK, exhibiting 63% identity [Fox et al., 1999; Fujimoto et al., 1999; Palmer et al., 1999].

This study is a first attempt to elucidate apoptosis in insect hemocytes. In particular, we wanted to explore the signaling molecules/pathways of apoptosis that occur during the normal developmental process, which is usually the result of a gene-directed cell suicide program and, to compare it with induced apoptosis in vitro. This study presents basic evidence that reveals hemocytes during larval-pupal metamorphosis undergo apoptosis. Moreover, based on FAK RNAi experiments, we present convincing evidence for the involvement of FAK and its downstream targets, Src, ERK, PI-3K, and Akt signaling molecules/pathways, in the process of developmentally regulated apoptosis.

MATERIALS AND METHODS

Materials and Antibodies

Polyclonal antibodies against FAK (c-903), p^{Tyr397}FAK, PI-3K p85a, pPI-3K p85a, ERK, and c-Src were obtained from Santa Cruz, CA. Affinity-purified rabbit polyclonal antibodies to Akt, p^{Ser473}Akt, p^{Thr308}Akt, non-p^{Tyr527}Src, p^{Tyr527}Src, p^{Tyr416}Src, and pERK were purchased from Cell Signaling Technology (Beverly, MA). Affinity-purified mouse polyclonal antibody against non-p^{Tyr416}Src was purchased from Cell Signaling Technology. Goat anti-rabbit IgG-HRP (horseradish peroxidase conjugated), rabbit anti-goat IgG as well as the rabbit ABC staining system were purchased from Santa Cruz Biotechnology, while goat anti-mouse IgG-HRP was from BD Transduction Laboratories™, and goat anti-rabbit IgG-FITC (fluoroscein isothiocyanate conjugated) from Molecular probes, Inc. Antibodies against actin and tubulin were obtained from Sigma (St. Louis, MO).U0126 and PD 98059, MEK1/2 inhibitors as well as LY294002, PI-3-Kinase inhibitor, were obtained from Cell Signaling Technology, Akt inhibitor (a phosphatidylinositol ether analog) and PP2 (a potent and selective inhibitor of the Src family of tyrosine kinases) were purchased from Calbiochem (USA), wortmannin (from *Penicillium funiculosum*, a potent and specific PI3-K inhibitor), cytochalasin D (from *Zygosporium masonii*, potent inhibitor of actin polymerization), demecolcine (depolymerises microtubules; blocks mitosis at metaphase) were purchased from Sigma. Donkey anti-rabbit IgGs (Amerlex-M magnetic separation reagent) were purchased from Amersham

Life Science (UK). Other materials were obtained as indicated.

Collection of Hemocytes and Cell Viability Test

Ceratitis capitata were reared as described previously [Charalambidis et al., 1996]. Isolation and homogenization of third 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.

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. OD 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-Src, -PI-3K p85a, and -Akt 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-Src, -PI-3K p85a, and -Akt polyclonal antibodies.

SDS–PAGE and Immunoblot Analysis

SDS–PAGE was performed on 10% acrylamide and 0.10% bisacrylamide slab gels, according to Laemli [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, IL) 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 horseradish peroxidase-linked secondary antibody (Transduction Laboratories, Lexington, KY, Cell Signaling Technology, Santa Cruz Biotechnology) 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). Pre-stained protein markers, broad range, were used to indicate the size of the protein bands (Cell Signaling Technology).

Annexin V Binding Staining

The analysis of annexin V binding was carried out with an Annexin V-FITC Detection Kit I (PharMingen, San Diego, CA) according to the manufacturer's instructions. Cells were collected, washed twice with cold TBS, and centrifuged at 200*g* for 5 min. Cells were resuspended in 1× binding buffer at a concentration of 1×10^6 cells per ml, 100 μl of the solution were transferred to a 5 ml culture tube, and 5 μl of annexin V-FITC and 5 μl of PI were added. Cells were gently vortexed and incubated for 15 min at room temperature in the dark. Finally, 400 μl of 1× binding buffer was added to each tube, and samples were analyzed by FACScan flow cytometer (Becton Dickinson). For each sample, 10,000 ungated events were acquired. PI (−)/annexin (+) cells represent the early apoptotic populations and PI (+)/annexin (+) cells represent late apoptotic populations.

Fluorescence Microscopy

Isolated hemocytes were suspended in Grace's medium and allowed to attach on glass slides for 10 min at 25°C. The slides were washed with Ringer's solution to remove nonadherent hemocytes. The resulting monolayers were pre-permeabilized with 0.1% Triton X-100 for 10 s, 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^{Y397}FAK, -PI-3K p85a, -pPI-3K p85a (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 antibody coupled to a fluorochrome (FITC) diluted 1:100 in TBS. Hemocyte monolayers were then washed with TBS and mounted with an aqueous mounting medium (Sigma) and observed under a confocal fluorescence microscope to examine the distribution of FAK, p^{Tyr397}FAK, PI-3K p85a, and pPI-3K p85a.

Detection of Apoptosis by TUNEL Staining

To determine apoptosis in *C. capitata* hemocytes, single-strand breaks in high-molecular-weight DNA were detected by labeling free 3-OH termini with modified nucleotides in an enzymatic reaction. This was performed by using an in situ cell-death detection kit (Boehringer Mannheim) that incorporates fluorescein-conjugated nucleotides using terminal deoxynucleotide transferase. Sheep anti-fluorescein Fab fragments conjugated with horseradish peroxidase were detected by light microscopy after incubation with 3,3-diaminobenzidine. The apoptotic index was calculated after counting 500 cells in randomly selected fields by use of a 40× objective and a 10× ocular. Two investigators counted the cells independently on blinded probes.

RT-PCR

Reverse transcription PCR (RT-PCR) was carried out using the QIAGEN® OneStep RT-PCR Kit (QIAGEN), according to 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′-TAATACGACTCACTATAGGGAGACCAC<u>AGTCGACCACCTAACCGCGCAGATGACG</u>-3′) and reverse FAK primers (5′-TAATACGACTCACTATAGGGAGACCAC<u>TGACTACATGACGATGTGAGAATGCG</u>-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.

DsRNA Production

The purified PCR (1,011 bp) products were used as templates to produce double-stranded RNA (dsRNA) by using a MEGAscript RNAi kit (Ambion, Inc.), following manufacturer's instructions for the synthesis and purification of the dsRNA. The dsRNA products were precipitated with LiCl and resuspended in miliQ-H_2O. RNA concentration was measured at A_{260} and in 1% agarose gels. DsRNAs were stored at −70°C.

RNA Interference (RNAi) in Drosophila and Medfly Hemocytes

Suspended drosophila and medfly hemocytes (5×10^5/100 μl) were incubated in Grace's medium with FAK dsRNA for up to 6 h and were then either lysed or used for flow cytometry or immunofluoresence microscopy. Longer

term incubations were not possible as the viability of the hemocytes decreases considerably.

ELISA

Hemocytes (5×10^5/100 μl) were incubated in Grace's medium with FAK dsRNA for 6 h. The control hemocytes' incubation medium did not contain FAK dsRNA. 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) 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-FAK polyclonal antibody (Santa Cruz Biotechnology) (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 or rabbit anti-goat IgG antibodies labeled with horseradish peroxidase (Santa Cruz Biotechnology) 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

To carry out this study we used medfly hemocytes from larvae at different, well distinct developmental stages; larvae at feeding stage (L6), fully developed larvae just emerge from the food and burrowed into the sand (WS, wandering stage), where after approximately 2 h they pupate, initially as white pupae (WP) and after about 2 h into brown pupae (BP).

Apoptosis of Hemocytes During Larval-Pupal Transformation

Apoptosis is a key component of developmental processes in several animal species, including insects. To test the hypothesis, that during larval-pupal transformation, insect hemocytes do undergo apoptosis, suspended hemocytes of white pupal stage were treated with annexin (Fig. 1). Flow cytometry analysis shows that hemocytes from third instar larvae constitute a rather homogeneous population—the plasmatocytes (Fig. 1A). A rather large portion of these hemocytes do indeed undergo apoptosis (Fig. 1B, PI (−)/annexin (+) hemocytes—early apoptotic cells, PI (+)/annexin (+) hemocytes—late apoptotic cells). PI (−)/annexin (−) hemocytes are classified as live cells and PI (+)/annexin (−) cells as dead cells (Fig. 1B). Apoptotic hemocytes were also observed by TUNEL method. As shown in Figure 1E, a portion of hemocytes were TUNEL positive.

Flow cytometry analysis of hemocytes in each phase (live, apoptotic, or dead) during larval-pupal transformation indicated that, the kinetic of early apoptotic hemocytes is quite different from the late apoptotic hemocytes. Whereas, early apoptotic hemocytes occurred rapidly and peaked at the white pupal stage, late apoptotic hemocytes were not developed until the maximal level of apoptosis and peaked 1 h later (Fig. 2). It is a matter of interest that, the data presented in Fig. 2 indicated that a rather large portion of hemocytes, die through developmentally regulated apoptosis. Hemocytes also undergo apoptosis in response to external chemical or physical stimuli (camptothesin and heat shock—data not shown).

These results are in accordance with mammals, where approximately 75% of immature B lymphocytes and 95% of immature T lymphocytes die during this process [Egerton et al., 1990; Surh and Sprent, 1994]. Similar results were observed in LPS-induced apoptosis and necrosis of BAL cells [Medan et al., 2002]. Consequently, hemocytes during larval-pupal stage are a good model system to study apoptosis during development, and, to compare results with those of in vitro induced apoptosis.

The Involvement of FAK in Developmentally Regulated Apoptosis

The functional significance of FAK and other mammalian signaling molecules for apoptosis remains undefined in many instances, and, evidently in developmentally regulated apoptosis in insects. Previously, we demonstrated the presence of total and Y397 phosphorylated FAK in medfly hemocytes, during larval-pupal transformation [Metheniti et al., 2001].

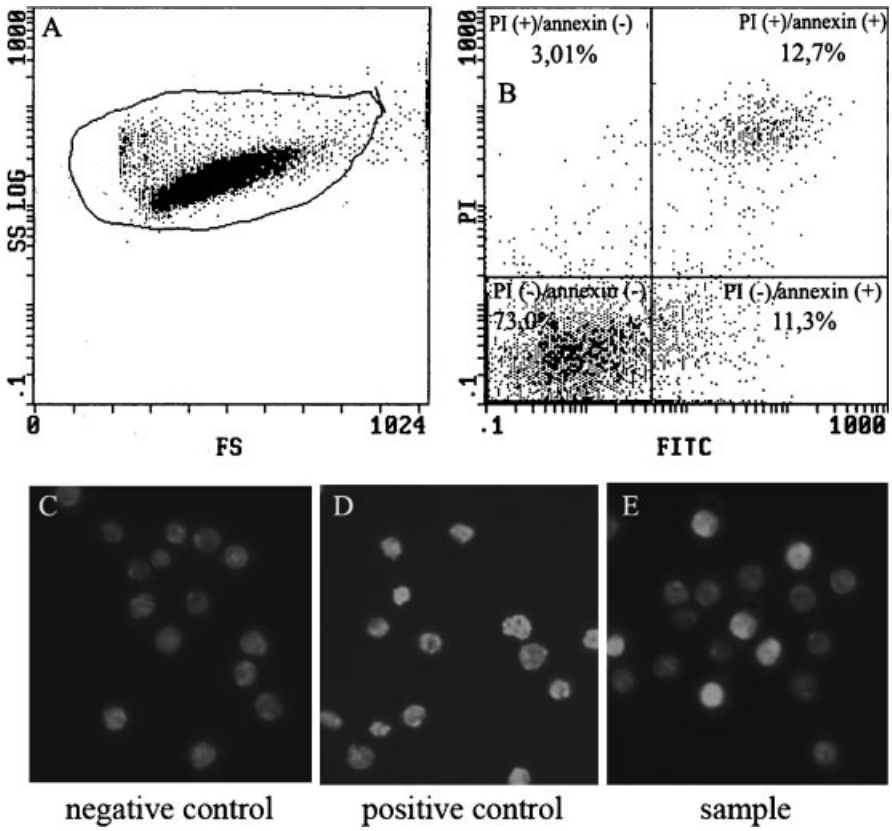

Fig. 1. Identification of apoptosis in medfly hemocytes of third instar larvae. Suspended hemocytes from white pupae were used to evaluate apoptosis. Apoptosis (**A** and **B**) assayed by flow cytometry, using annexin V-FITC and propidium iodide (PI), as a dead cell discriminator. In the dot blot analysis of upgated hemocytes, the x-axis represents annexin V staining and the y-axis represents PI uptake. The percentages of cells in the quadrants are shown. The data are representative of one experiment of five performed. **C**, **D**, **E**: Apoptosis assayed by TUNEL (FITC-labeled nucleotides incorporated into apoptotic nuclei). The cells with green fluorescence represent apoptotic hemocytes. DNAase-treated hemocytes were used as a positive control and untreated cells as a negative control.

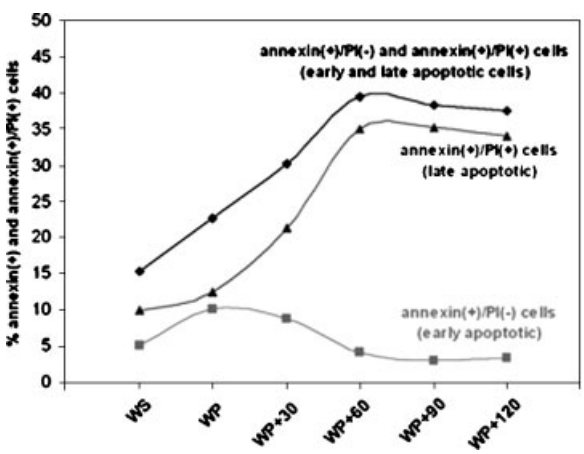

Fig. 2. Apoptosis in medfly hemocytes during larval development. Suspended hemocytes from wandering stage (WS), white pupae (WP), WP plus 30 min, WP plus 60 min, WP plus 90 min, and WP plus 120 min (brown pupae), were used. Apoptosis was evaluated by quantification of flow cytometry determination by annexin-V FITC assay and dead cells by PI assay. Three separate experiments were performed.

To explore whether FAK signaling pathway might play a role in developmentally regulated apoptosis in medfly hemocytes, FAK dsRNA was transfected into hemocytes of wandering stage larvae, to silence FAK expression. As total RNA from *Drosophila* adults was used as a template for RT-PCRs, we initially explored whether the final produced FAK dsRNA, corresponding to *Drosophila* FAK, silences the FAK expression in medfly hemocytes. Immunoblot analysis clearly shows that total FAK protein expression in medfly hemocytes transfected with FAK dsRNA was decreased about 46% compared with control experiments (Fig. 3A). Similarly, *Drosophila* suspended hemocytes transfected with FAK dsRNA also decreased total FAK protein expression about 53% (Fig. 3A). Further confirmation of the effect of FAK dsRNA on the expression of FAK was obtained by ELISA (Fig. 3B). As shown in Figure 3B, FAK protein expression decreases

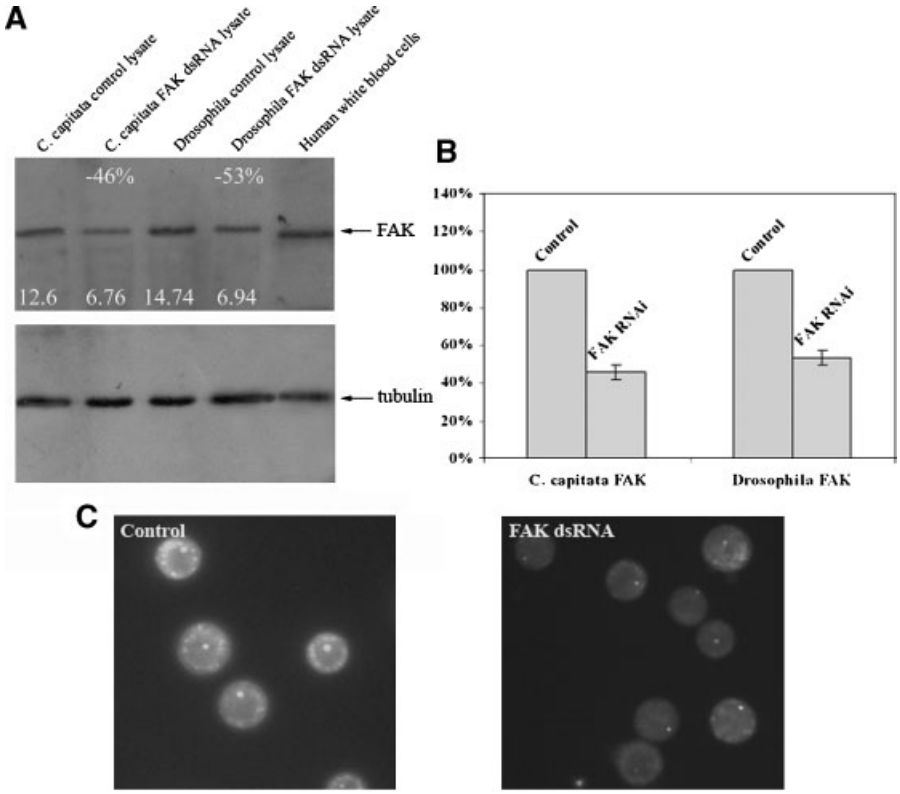

Fig. 3. FAK silencing by FAK dsRNA in medfly hemocytes. Suspended hemocytes from wandering larval stage were incubated in the presence or absence of FAK dsRNA for 6 h and its effect on FAK expression was evaluated by immunoblotting analysis and ELISA. For Western blot analysis (**A**) hemocytes were untreated or treated with 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 the 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 % 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 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 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.

by about 55% in the presence of FAK dsRNA, in medfly hemocytes and by about 50% in *Drosophila* hemocytes. The depletion was only partial, evidently, due to the short-term incubations, as the viability of hemocytes decreases in long-term incubations. The partial depletion of FAK was also confirmed by immunofluorescent experiments (Fig. 3C).

To understand FAK silencing in the developmentally regulated hemocyte apoptosis, flow cytometry experiments were performed, using FAK dsRNA to transfect hemocytes or hemocytes transfected with paxillin dsRNA and untreated hemocytes, as controls (Fig. 4A,B,C). The kinetic studies in response to FAK dsRNA concentration, clearly show an increase in early apoptotic cells (PI (−)/annexin (+)) with a maximum in panel E in Figure 4 (19%), which is a nearly twofold increase compared to the control in panel A in the same figure (11.3%). Similarly, an increase in late apoptotic hemocytes (PI (+)/annexin (+)) was also observed with a maximum in panel F in Figure 4 (23.3%), which is a nearly twofold increase compared to the control in panel A in the same figure (12.7%). Paxillin dsRNA transfected hemocytes or untransfected hemocytes with FAK dsRNA, demonstrated not only the specificity of FAK dsRNA function, but also that dsRNA does not interfere in the process of apoptosis (Fig. 4). Consequently, the expression of FAK modulates hemocyte developmentally regulated apoptosis.

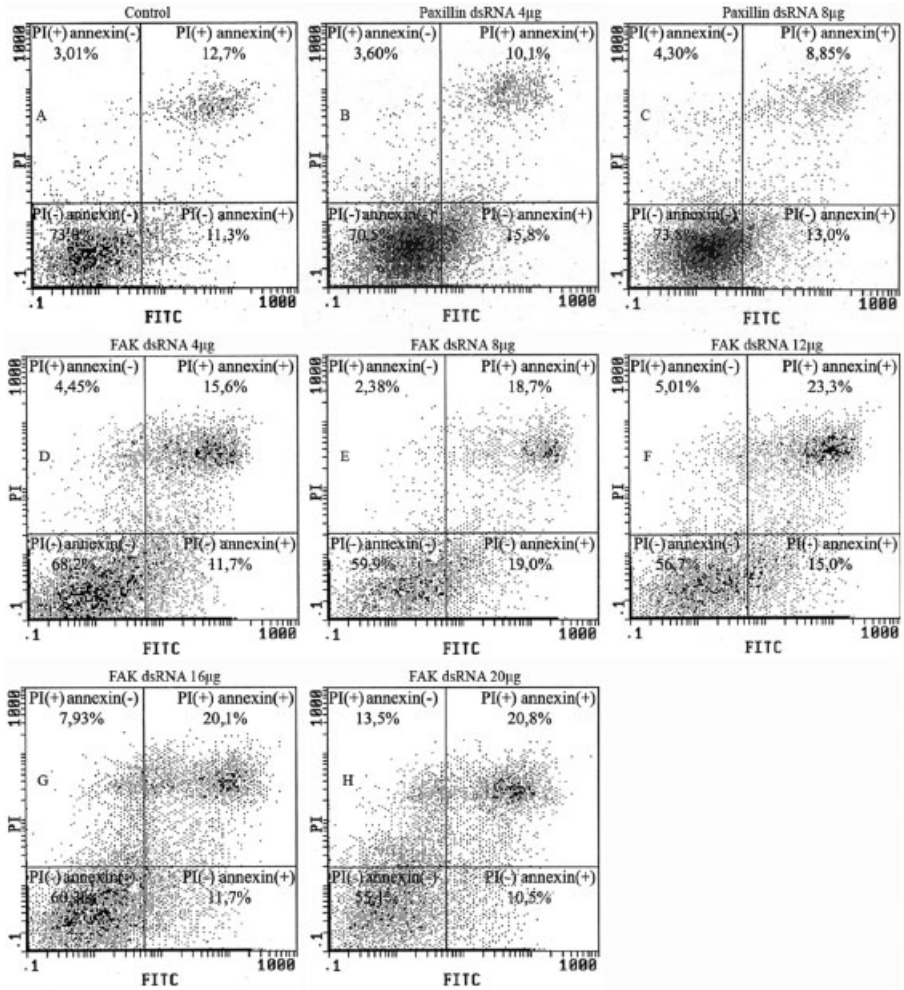

Fig. 4. Kinetic of apoptosis in relation to increasing concentration of FAK dsRNA. Suspended hemocytes of wandering larval stage were treated with 4, 8, 12, 16, and 20 μg (**D**, **E**, **F**, **G**, and **H**) dsRNA for up to 6 h and the evaluation of apoptosis was monitored by flow cytometry. As controls were used hemocytes without FAK dsRNA treatment (**A**) and hemocytes treated with paxillin dsRNA (**B** and **C**). Details for FACs analysis see Figure 1. The data are representative of one experiment of three performed.

FAK Downstream Targets and Apoptosis

The possible involvement of FAK in the modulation of apoptosis, prompted us to elucidate the downstream signaling molecules mediating in apoptosis. As it is well appreciated, FAK uses multiple downstream effectors, to mediate its diverse biological actions. In this study, we focused our interest on the potential roles of Src, p85a regulating subunit of PI-3K, Akt, and ERK signaling molecules. The expression of FAK and ERK like proteins in medfly hemocytes has already been identified [Metheniti et al., 2001; Soldatos et al., 2003]. Therefore, in this report, only the expression of Src, p85a regulating subunit of PI-3K, and Akt homolog in the medfly hemocytes of third instar larvae was explored.

Human Src, p85a regulating subunit of PI-3K, and Akt signaling molecules are closely related to insect homologs. In *Drosophila*, Src64B and Akt1 signaling molecules are 51.3 and 63.7% and in *A. gambiae*, Src (gene ENSANGG00000007531), p85a regulating subunit of PI-3K (gene ENSANGP00000017664), and Akt (gene ENSANGG00000016859) are 54.4, 34.4, and 65.2%, identical in amino acid sequence to their corresponding human homologs respectively (Fly Base, NCBI, HomoloGene base). Furthermore, amino acid sequence surrounding the phosphorylation sites in human p^{Y397}FAK, pPI-3K p85a, p^{Ser473}Akt, p^{Thr308}Akt,

non-p^{Tyr527}Src, p^{Tyr527}Src, p^{Tyr416}Src, and pERK are closely related to *Drosophila* homologs (as retrieved from NCBI). Therefore, polyclonal antibodies which specifically recognize total and activated (phosphorylated) mammalian Src, p85a regulating subunit of PI-3K, and Akt were used to analyze the expression and phosphorylation of Src, p85a regulating subunit of PI-3K, and Akt medfly homologs in hemocytes.

Immunoprecipitation analysis with anti-Src, anti-PI-3K p85a, and anti-Akt antibodies recognizes only a major band in medfly hemocyte, which differs, as expected, in MW from human leukocyte counterparts (Fig. 5A). The expression of FAK and ERK like proteins in medfly hemocytes has already been identified [Metheniti et al., 2001; Soldatos et al., 2003]. The large difference in the medfly p85a homolog should be explained having in mind that *Drosophila* p85

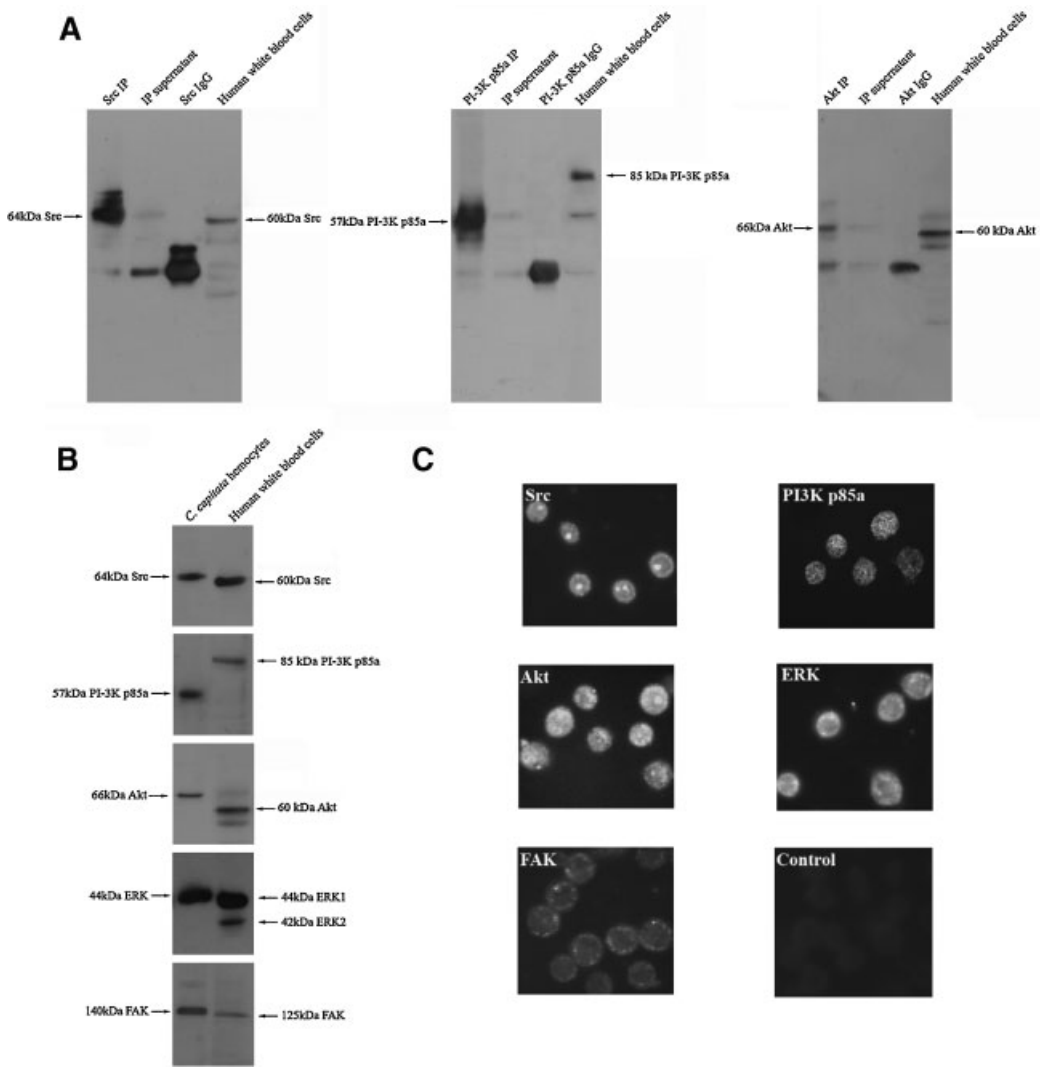

Fig. 5. Identification of Src, PI-3K p85a, Akt, ERK, and FAK in medfly hemocytes. For immunoprecipitation analysis suspended hemocytes from white pupal stage were isolated and their lysates were treated with polyclonal antibodies against Src, PI-3K p85a, and Akt to evaluate the expression of Src, PI-3K p85a, and Akt, respectively. The precipitates were then resolved on 10% SDS–PAGE and blotted with Src, PI-3K p85a, and Akt antibodies, respectively. Immunoprecipitation analysis for FAK and ERK like proteins in medfly hemocytes has already been published [14, 25]. Human white blood cells were used as a positive control (**A**). For Western blot analysis (**B**) cell lysates (10 μg) were resolved on 10% SDS–PAGE and blotted with polyclonal Abs against Src, PI-3K p85a, Akt, ERK, and FAK polyclonal antibodies. Immunofluorescence identification of Src, PI-3K p85a, Akt, ERK, and FAK (**C**) in suspended hemocytes from white pupal stage were isolated and then fixed and stained with 1st antibodies against Src, PI-3K p85a, Akt, ERK, and FAK respectively 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. Photomicrographs shown are representing of about 70% hemocytes obtained in three independent experiments.

homolog is only 60 kDa rather than 85 kDa [Weinkove et al., 1997] and human p85a regulating subunit of PI-3K consists of 724aa, whereas *A gambiae* homolog consists from only 406aa (Fly Base, NCBI, HomoloGene base). Immunoblotting analysis confirmed the above experiments (Fig. 5B). Finally, direct evidence that Src, PI-3K p85a, Akt, ERK, and FAK signaling molecules are localized in medfly hemocytes was obtained from immunofluorescence experiments (Fig. 5C).

To elucidate whether these signaling molecules are downstream targets of FAK in our system, we analyzed the effect of FAK dsRNA, on the phosphorylation of Src, PI-3K p85a, and ERK. Hemocytes in suspension were incubated for 6 h in the presence or absence of dsRNA corresponding to FAK. Hemocyte lysate was then separated into two halves. Half of the lysate was used for ELISA analysis and the other half was used for immunoblotting analysis. Both procedures clearly show that FAK expression silencing in response to FAK dsRNA treatment of hemocytes, decreased the phosphorylation of active p^{416}Src, pERK, and p85a regulating subunit of pPI-3K about 30, 12, and 42%, respectively (Fig. 6A). ELISA results were confirmed by immunoblotting analysis (Fig. 6B). Consequently, FAK appears to modulate medfly hemocytes apoptosis by signaling, at least, through Src, ERK PI-3K p85a, and Akt survival signaling pathways.

To clarify whether the FAK downstream targets, Akt, ERK, Src, and PI-3K p85a are involved in the developmental regulated hemocyte apoptosis, suspended hemocytes of WP were treated with either PD 098059 (8 μM) or U0126 (8 μM) for MEK1/2, wortmannin

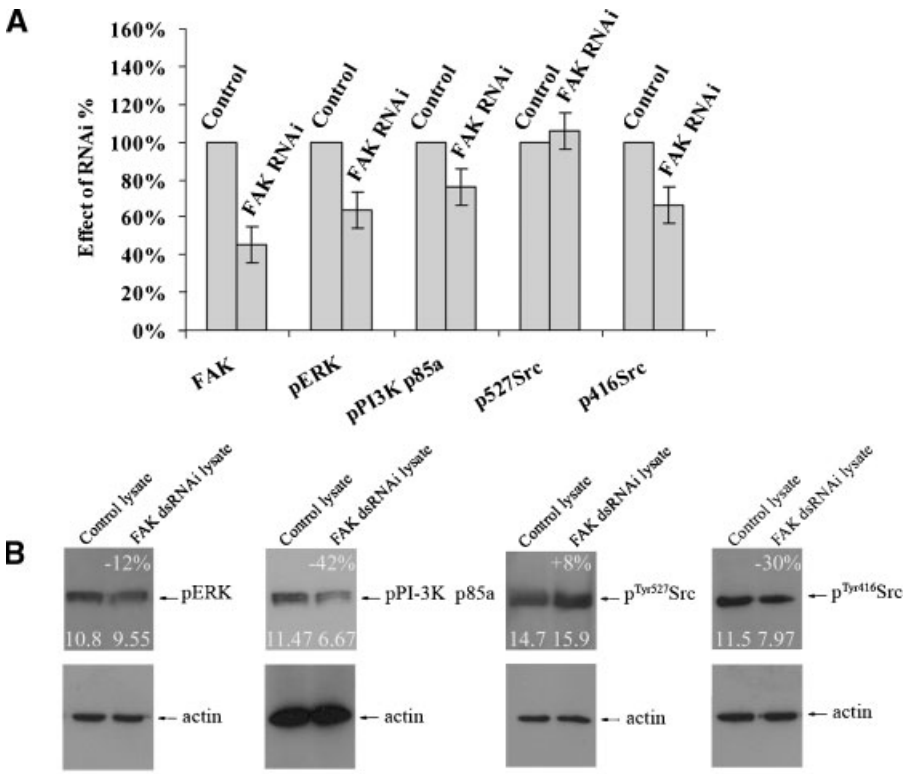

Fig. 6. Effect of FAK silencing on the phosphorylation of ERK, Src, and PI-3K p85a. Suspended hemocytes from wandering larval stage were incubated in the presence or absence of FAK dsRNA for up to 6 h and then the cell lysates were analyzed by ELISA and immunoblotting, to detect the status of ERK, Src, and PI-3K p85a phosphorylation. For ELISA analysis (**A**) hemocyte lysates were plated in a 96-well assay plate, and analyzed for pERK, p^{Tyr527}Src, p^{Tyr416}Src, and pPI-3K p85a, using polyclonal antibodies against pERK, p^{Tyr527}Src, p^{Tyr416}Src, and pPI-3K p85a, respectively. Each well contained 4 μg/ml hemocyte lysate. For Western blot analysis (**B**) cell lysates (10 μg) were resolved on 10% SDS–PAGE and blotted with polyclonal Abs against pERK, p^{Tyr527}Src, p^{Tyr416}Src, and pPI-3K p85a. Each bar represents the mean and standard error of the mean of three independent experiments. Relative to the actin numerical quantities are listed below each lane. Above each lane is listed the % variation of the levels of ERK, PI-3K p85a, and Src phosphorylation compared to the controls.

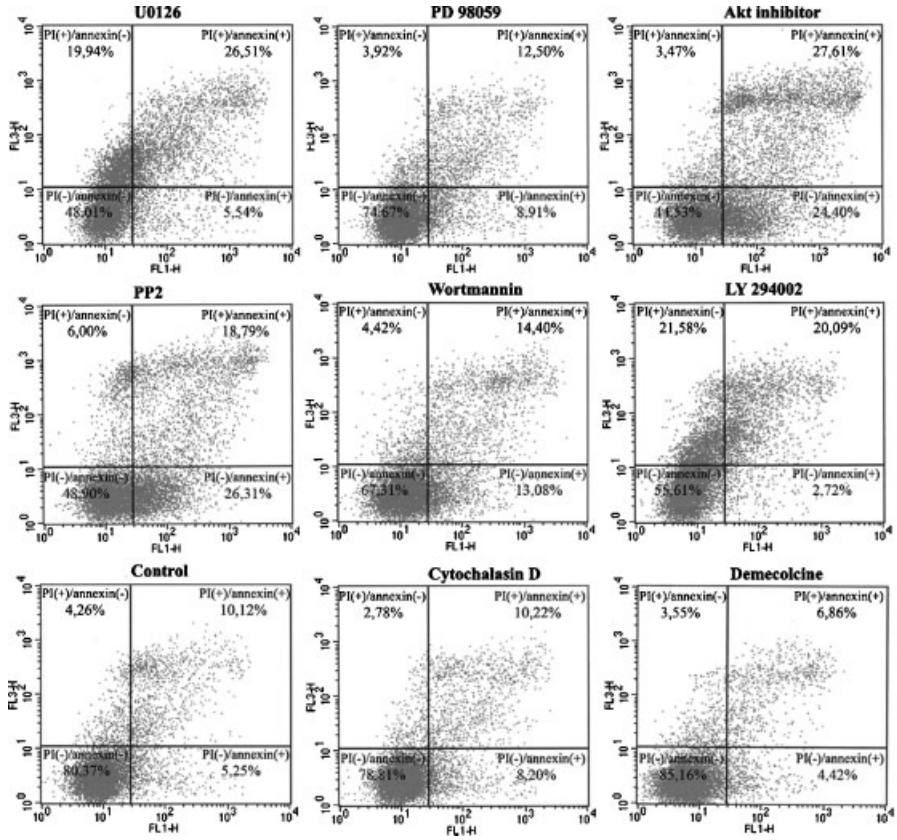

Fig. 7. Effect of downstream targets ERK, Src, PI-3K p85a, and Akt on the developmentally regulated apoptosis. Suspended hemocytes were incubated for 1 h in the presence or absence of ERK, Src, PI-3K, and Akt-specific inhibitors, as well as of specific inhibitors of actin (cytochalasin D) and tubulin (demecolcine). As controls were used hemocytes without any inhibitor treatment. Hemocyte apoptosis was monitored by flow cytometry. Details for the dot blot analysis see Figure 1. The data are representative of one experiment of three performed.

(100 nM) and LY 294002 (10 μM) for PI-3K, PP2 (5 μM) for Src, or Akt inhibitor (5 μM), and their effects on hemocyte apoptosis were monitored by flow cytometry (Fig. 7). As can be seen in this figure, the inhibitors of Akt (Akt inhibitor panel), MEK1/2 (PD 098059 and U0126 panels), p85a regulating subunit of PI-3K (Wortmannin and LY 294002 panels) and Src (PP2 panel) increase the percentages of apoptotic cells (early and late apoptotic cells) observed as follows: 52.01, 21.41, 32.05, 27.48, 22.81, and 45.1%, respectively. In control experiments the percentages of apoptotic cells (early and late apoptotic cells) observed were: 15.37% in the absence of inhibitors (control panel), 18.42% in the presence of cytochalasin D (cytochalasin D panel), and 11.28% in the presence of demecolcine (demecolcine panel). Consequently, the final increase in apoptotic hemocytes observed, compared to the control, was 3.38-fold for Akt inhibitor, 1.39-fold for PD 098059, 2.09-fold for U0126, 1.79-fold for wortmannin, 1.48-fold for LY 294002, and 2.93-fold for PP2, indicating that when the above signaling pathways are blocked, the protection provided by internal factor(s) is lost and hemocytes become susceptible to apoptosis, as in the case of other cell types [Marte and Downward, 1997; Frisch and Screaton, 2001; Gu et al., 2003]. In addition, the inhibitors of cytoskeleton organization (cytochalasin D and demecolcine) did not appear to modulate apoptosis, at least not at the early stages, which were studied (Fig. 7). The very high viability of hemocytes indicates that the chemicals used are not toxic at the concentrations used (Fig. 7, PI (−)/annexin (−) hemocytes). Therefore, these results further support the hypothesis that the activity of these molecules is important for the prevention of apoptosis in hemocytes during development.

Expression and Phosphorylation of FAK, Src, ERK, p85a Regulating Subunit of PI-3K, and Akt Homologs During Development and in Annexin (+) and Annexin (−) Hemocytes

Based on the above data, it is important to explore whether the expression and phosphorylation of signaling molecules under consideration are also regulated developmentally.

Developmental studies showed a rapid increase of total FAK, Src, ERK, p85a regulating subunit of PI-3K and Akt signaling molecules in hemocytes, at larval development (Fig. 8). In other words, all signaling proteins studied appear to enhance their expression abruptly at white pupal stage and remain also high just after the white pupal stage (BP). Based on the relative to the tubulin numerical quantities listed below each lane in Figure 8, the observed increase from L6 to BP in the above signaling molecules was 2.74-fold for FAK, 19.08-fold for ERK, 22.93-fold for Src, 2.61-fold for p85a regulating subunit of PI-3K, and 7.23-fold for Akt. It is interesting to note that, the enhancement in phospho-signaling molecules, during the same developmental period was directly proportional to the increase in total signaling molecule protein expression (Fig. 8). In particular, based on the relative to the tubulin numerical quantities listed below each lane in Figure 8, the observed increase from L6 to BP in p^{Y397}FAK was 3.28-fold, in pERK was 19-fold, in p^{Tyr527}Src was 27.37-fold, in p-PI-3K p85a was 2.92-fold, and in p^{ser473}Akt was 12.63-fold. The obtained biochemical results were further confirmed for FAK and p85a regulating subunit of PI-3K homologs by immunofluorescent experiments (Fig. 9). Similar results were obtained with the remaining signaling molecules (data not shown).

The above observations prompted us to explore the expression and phosphorylation

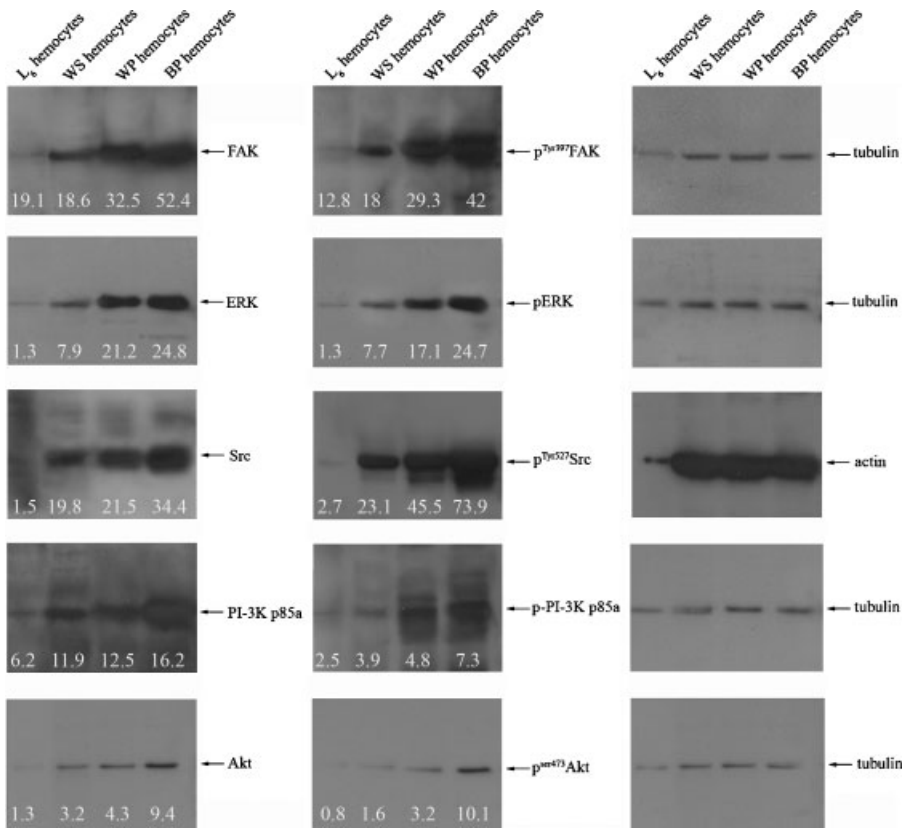

Fig. 8. Expression and phosphorylation of FAK, ERK, Src, PI-3K p85a, and Akt during larval development, as evaluated by Western blot analysis. For Western blot analysis hemocytes of different developmental stages, were lysed, centrifuged, and the lysates (10 μg) were then resolved on 10% SDS–PAGE and blotted with polyclonal Abs against FAK, ERK, Src, PI-3K p85a, and Akt, or their phospho-FAK, ERK, Src, PI-3K p85a, and Akt antibodies. Tubulin/or actin were used as loading controls. Relative to the tubulin/or actin numerical quantities are listed below each lane.

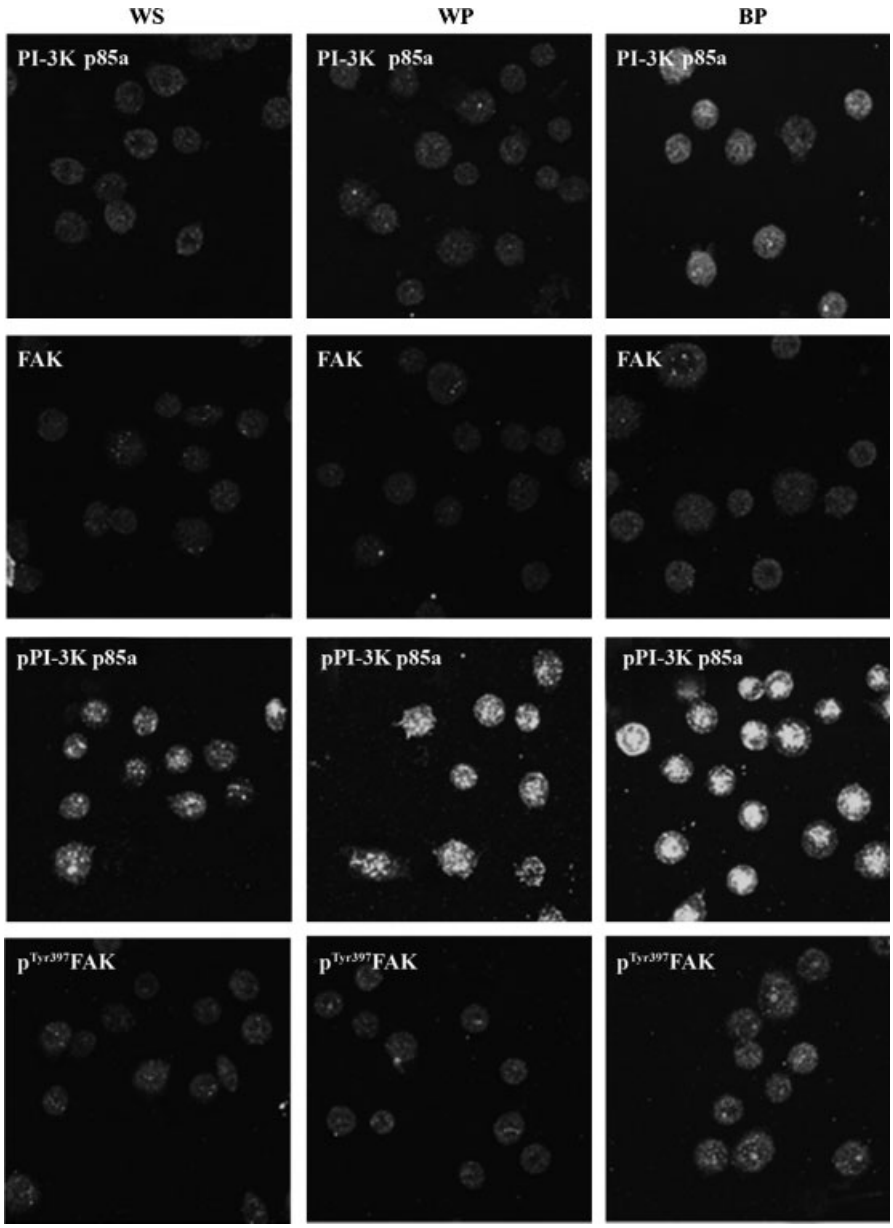

Fig. 9. Immunofluorescent identification of FAK and PI-3K p85a in developing hemocytes. Suspended hemocytes from wandering stage, white pupae, and brown pupae were isolated and then fixed and stained with antibodies against PI-3K p85a, FAK, pPI-3K p85a, and p^{Tyr397}FAK. Photomicrographs shown are representatives of about 80% of hemocytes, obtained in three independent experiments.

of FAK, Src, ERK, p85a regulating subunit of PI-3K, and Akt in the developmentally regulated apoptotic (annexin-positive) and non-apoptotic (annexin-negative) hemocytes. For this purpose, hemocytes of WP were isolated by cell sorter. We collected annexin-positive hemocytes, which can be discriminated as PI (−)/annexin (+) and PI (+)/annexin (+) cells (early and late apoptotic hemocytes) and annexin-negative hemocytes. It is clear that both annexin (+) and annexin (−) hemocytes express these signaling molecules. The expression of FAK, p85a regulating subunit of PI-3K, Src, Akt, and ERK homologs appears to increase in annexin (+) hemocytes (Fig. 10). In particular, based on the relative to the tubulin numerical quantities listed below each lane in Figure 10, the observed increase in PI (−)/annexin (+) compared to PI (−)/annexin (−) cell lysates was 2.37-fold for ERK, 1.61-fold for p85a

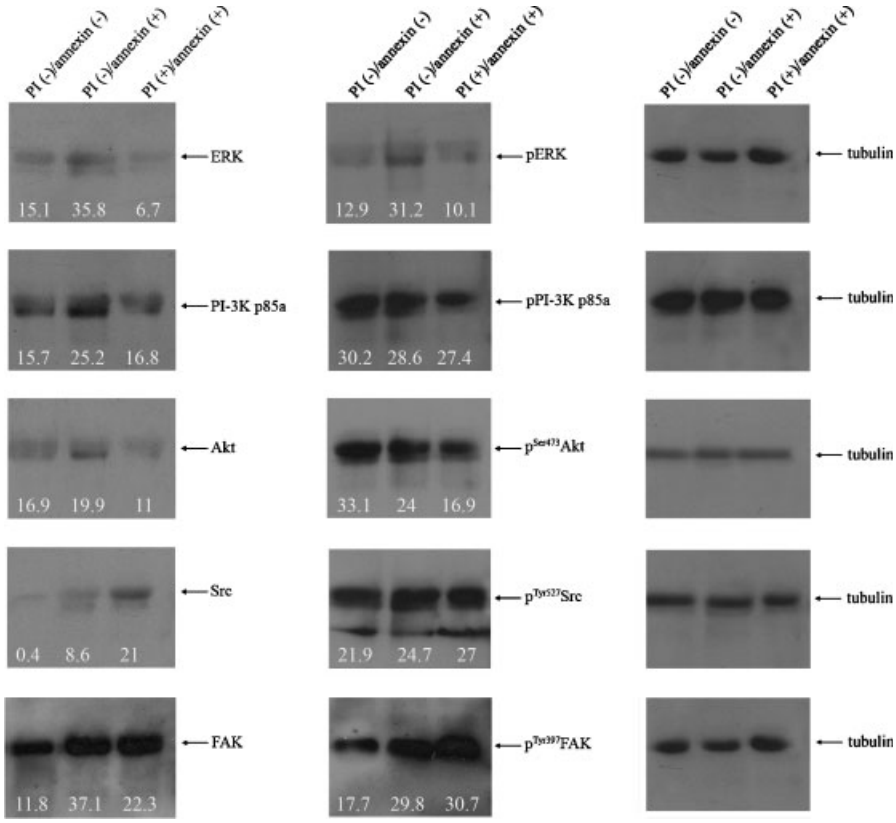

Fig. 10. Expression and phosphorylation of FAK, ERK, Src, PI-3K p85a, and Akt, in annexin (+) and annexin (−) hemocytes. Annexin (+) and annexin (−) hemocytes were isolated by cell sorter and then were subjected for Western blot analysis. For Western blot analysis hemocytes from white pupal stage, were lysed, centrifuged, and the lysates (10 μg) were then resolved on 10% SDS–PAGE and blotted with polyclonal Abs against FAK, ERK, Src, PI-3K p85a, and Akt, or their phospho-FAK, ERK, Src, PI-3K p85a, and Akt antibodies. Tubulin was used as a loading control. Relative to the tubulin numerical quantities are listed below each lane.

regulating subunit of PI-3K, 1.18-fold for Akt, 21.5-fold for Src, and 3.14-fold for FAK. Furthermore, we compared the ratios of the relative numerical quantities of PI (−)/annexin (−) to PI (−)/annexin (+) between pERK (0.41) and ERK (0.42); p-PI-3K p85a (1.06) and PI-3K p85a (0.62); p^{ser473}Akt (1.38) and Akt (0.85); p^{Tyr527}Src (0.89) and Src (0.05); and p^{Y397}FAK (0.59) and FAK (0.32). We observed that the ratios between PI (−)/annexin (−) and PI (−)/annexin (+) in p-PI-3K, p^{ser473}Akt, p^{Tyr527}Src, and p^{Y397}FAK are higher than the ratios between PI (−)/annexin (−) and PI (−)/annexin (+) in PI-3K, Akt, Src, and FAK; possibly to overcome the limited expression of PI-3K, Akt, Src, and FAK in PI (−)/annexin (−) cells (Fig. 10), a situation analogous of that in FAK dsRNA transfected hemocytes (Fig. 6). Tubulin expression remains rather constant in both annexin (+) and annexin (−) hemocytes.

DISCUSSION

In this study, we report that medfly hemocytes do undergo apoptosis at the onset of pupariation, in vivo. Interestingly, at this stage of development, a significant explosion of circulating hemocytes was also observed, which correlate with the rise of ecdysone titer. This steroid hormone has been reported to induce differentiation of hemocytes [Rizki, 1978] and cell death in several larval tissues, including salivary glands and midgut [Twomey and McCarthy, 2005]. Therefore, the apoptosis observed at the onset of pupariation in medfly hemocytes (Figs. 1 and 2) may probably be due to ecdysone titer. Since it has been well documented that at the same stage of development, ecdysone also regulates hemocytes proliferation and differentiation, it appears that this regulation may be due to hierarchical

transcriptional regulation of a number of genes [Twomey and McCarthy, 2005].

In addition, it remains unclear why at this very short developmental time hemocytes increase in number abruptly, differentiate, and then undergo apoptosis. A possible explanation is that during this developmental stage, fully developed medfly larvae emerge from the food and borrowed into the earth, where they pupate. Self-understanding is critical for numerous infections of larvae at this stage and hemocytes contribute drastically to the immunity of larvae, mainly via phagocytosis [Lamprou et al., 2005]. Furthermore, apoptosis is an evolutionary conserved type of programmed cell death that is essential for self-defense against virus infection.

The biological function(s) of signal transduction mechanisms mediated via FAK are increasingly being understood. A key role for FAK is the promotion of survival in several mammalian cells, as well as the protection of cells from apoptosis induced in response to several stimuli. Harnois et al. [2004] reported that FAK participates in the regulation of survival in human HIEC-6 cells and Xia et al. [2004] reported that FAK functioning upstream of PI-3K/Akt pathway, in mediating a β1 integrin viability signals in collagen matrices. Additionally, several studies have been reported that describe the anti-apoptotic roles of FAK in various apoptosis-inducing systems in mammals. Sonoda et al. [2000] reported that overexpression of FAK has an anti-apoptotic role in the apoptosis induced by oxidative stress as well as etoposide in anchorage-independent HL-60 cells. Similarly, Hungerford et al. [1996] reported anchorage-dependent cells that become apoptotic when cells were micro-injected with anti-FAK antibodies.

To test the hypothesis that in the developmentally regulated apoptosis of medfly hemocytes, FAK has also a key survival/anti-apoptotic role, we treated suspended hemocytes with FAK dsRNA to silence FAK expression. We observed that a decrease in FAK expression in FAK dsRNA treated suspended hemocytes has a consequent twofold increase in the magnitude of suspended hemocytes apoptosis (Figs. 3, 4). Therefore, the observed increase of FAK expression and phosphorylation, during the early stage of apoptosis is a strong indication that hemocytes still try to remain alive (Figs. 8 and 9). In other words, hemocytes have survival signals operative through FAK activity at pupariation. As survival pathways require the expression of new genes, our data, concerning developmental studies, show a rapid and considerable increase of total FAK expression in white and brown pupa (Fig. 8), and strongly support that FAK may act as survival signal in medfly hemocytes during larval-pupal development. It must be noted here that FAK also has a regulatory role in the phagocytosis process, both in mammals and insects. The potential role of FAK in apoptosis and phagocytosis in medfly hemocytes during the same developmental stage [Metheniti et al., 2001; Lamprou et al., 2005], strongly support the great complexity of cell signaling pathways and their crosstalk.

Given the important role for FAK in the control of apoptosis, there is considerable interest for the pathways by which FAK signals. Several reports describe the involvement of downstream targets of FAK, such as PI-3K, Akt, and ERK in the process of apoptosis in several mammalian systems. For example, Harnois et al. [2004] reported that the activation of FAK, PI-3K/Akt, and MEK/ERK pathways appear to have distinct roles in mammalian cell survival and/or death. Cooray et al. [2005] reported that PI-3K/Akt and MEK/ERK signaling cascades during virus infection are thought to play an important role, not only in cellular growth and survival, but also in viral replication and apoptosis. Grigoriou et al. [2005] reported that PI-3K/Akt pathway is involved in the regulation of apoptosis and survival of osteoblast-like cells by surface attachment.

Our in vivo data, based on ELISA and Western blot analyses, clearly shows a considerable decrease of FAK protein expression in transfected with FAK dsRNA hemocytes, compared to non-transfected hemocytes (Fig. 3). This decrease of FAK protein expression also decreases the phosphorylation status of Src, p85a regulating subunit of PI-3K, and ERK signaling molecules, demonstrating that these signaling molecules are downstream targets of FAK in hemocytes undergoing apoptosis in vivo (Fig. 6). This data, as well as the increase of apoptosis in response to ERK, Src, PI-3K, and Akt inhibitors (Fig. 7), strongly support that hemocyte survival signals operate through FAK to Src, p85a regulating subunit of PI-3K, Akt, and ERK, suggesting that the combination of these signals may contribute to the resistance to apoptosis in our system. Consequently, the data

reported here, concerning developmentally regulated apoptosis, is in accordance to the data reported for in vitro mammalian systems, such as for HIEC-6 human cells [Xia et al., 2004] and for osteoblast-like cells [Grigoriou et al., 2005].

In conclusion, the novel aspect of this work, concerns the finding that, in our system (developmentally regulated apoptosis in insect hemocytes), hemocytes have survival signals operative through FAK activity, a key survival/anti-apoptotic molecule, at pupariation. At the same stage of development, FAK is also a key molecule for phagocytosis, a cell-mediated innate immune process [Metheniti et al., 2001]. This fact clearly demonstrates the great complexity of the regulatory mechanisms taking place at pupariation. As demonstrated at this stage of development, FAK and its downstream targets increase their expression, evidently to overcome the increased rate of apoptosis (Fig. 8). Likewise, silencing of FAK expression by FAK dsRNA decreases the phosphorylation of FAK downstream targets. In addition, in our system, the signaling pathways involved, may be among others the FAK/Src/ERK and FAK/PI-3K/Akt. These pathways also function in mammalian systems, suggesting that the survival signaling pathways among insects and mammals have probably remained unchanged during evolution.

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