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Echistatin inhibits pp125^{FAK} autophosphorylation, paxillin phosphorylation and pp125^{FAK}–paxillin interaction in fibronectin-adherent melanoma cells

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Echistatin, a snake-venom RGD-containing protein, was previously shown to disrupt cell-matrix adhesion by a mechanism that involves the reduction of pp125^{FAK} tyrosine phosphorylation levels. The aim of this study was to establish the sequence of events downstream pp125^{FAK} dephosphorylation that could be responsible for echistatin-induced disassembly of actin cytoskeleton and focal adhesions in fibronectin-adherent B16-BL6 melanoma cells. The results obtained show that echistatin induces a decrease of both autophosphorylation and kinase activity of pp125^{FAK}. One hour of cell exposure to echistatin caused a 39% decrease of pp125^{FAK} Tyr397 phosphorylation and a 31% reduction of pp125^{FAK} autophosphorylation activity as measured by immune-complex kinase assay. Furthermore, 1 h of cell treatment by echistatin produced a 63% decrease of paxillin phosphorylation, as well as a reduction in the amount of paxillin bound to pp125^{FAK}. Immunofluorescence analysis of echistatin treated cells showed the concomitant disappearance of both paxillin and pp125^{FAK} from focal adhesions. The reduction of paxillin phosphorylation may represent a critical step in the pathway by which disintegrins exert their biological activity, including the inhibition of experimental metastasis *in vivo*.

Keywords: echistatin; B16-BL6 melanoma cells; pp125^{FAK}; paxillin; focal adhesions.

Echistatin belongs to a family of low molecular mass, Arg-Gly-Asp (RGD)-containing proteins isolated from the venom of various snakes [1–4]. These proteins are termed disintegrins for their ability to bind with high affinity integrin receptors on platelets and cell surfaces. Inhibition of platelet aggregation and of cell adhesion has been the first biological activity reported for disintegrins [5]. The ability of some disintegrins to inhibit experimental metastasis *in vivo* has also been proved [6–10]. The exact molecular mechanism by which disintegrins act is still unclear; however, the finding that disintegrins affect integrin-dependent cell signaling provided some new insights into it [11–13].

We previously demonstrated that echistatin, a small-size disintegrin, inhibits B16-BL6 murine melanoma cell adhesion to extracellular matrix (ECM) components and detaches fibronectin-adherent melanoma cells from the substratum [13,14]. Echistatin-induced disruption of cell–matrix interactions in fibronectin-adherent B16-BL6 cells appears to involve an early reduction of pp125^{FAK} phosphorylation that correlates with the disassembly of actin cytoskeleton and of focal adhesions. However, the functional relationship between these events has not been established.

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Abbreviations: ECM, extracellular matrix; MEM, Eagle's minimal essential medium; PTKase, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; FAT, focal adhesion-targeting; TRITC, tetramethyl-rhodamine isothiocyanate; FITC, fluorescein isothiocyanate.

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The 68-kDa protein paxillin, a component of focal adhesions, is a substrate of pp125^{FAK} or of a kinase activated by pp125^{FAK} [15]. The integrin-dependent phosphorylation of pp125^{FAK} and paxillin has been observed in many cell types [16]. Elevated content of tyrosine phosphorylated pp125^{FAK} and paxillin has been found in cells transformed by Rous sarcoma virus [17] and in cells during embryogenesis [18]. Different stimuli, including lysophosphatidic acid [19], platelet-derived growth factor [20] and the neuropeptides bombesin, endothelin and vasopressin [21] are able to induce the phosphorylation of both pp125^{FAK} and paxillin in cultured cells.

pp125^{FAK} directly associates with paxillin and a region of pp125^{FAK} that overlaps the focal adhesion-targeting (FAT) sequence [22–25] mediates this association. However, the targeting of pp125^{FAK} to the focal adhesions and its binding to paxillin seem to involve different structural interactions, as a mutation of the distal C-terminal sequence of pp125^{FAK} inhibits binding without blocking its translocation to focal adhesions [23]. A correct subcellular localization and autophosphorylation of pp125^{FAK} are required for the tyrosine phosphorylation of paxillin *in vivo* [15]; phosphorylation of paxillin creates binding sites for SH-2 domain-containing signaling molecules such as Crk and Csk [25]. Whether pp125^{FAK}-mediated tyrosine phosphorylation of paxillin is important for its recruitment or function at focal adhesions is not yet completely established. Cultured cells from pp125^{FAK}-deficient mouse embryos showed no differences compared to wild-type cells in the levels of tyrosine phosphorylated tensin, paxillin, vinculin and talin, or in their localization to focal adhesions [26]. Further, mutations of Tyr118, the major site of tyrosine phosphorylation in the paxillin molecule, didn't affect its localization to focal adhesions [27].

In order to better understand the sequence of events underlying echistatin-induced disruption of cell–matrix interactions in fibronectin-adherent B16-BL6 melanoma cells we evaluated whether echistatin affects: (a) phosphorylation of pp125^{FAK} Tyr397 residue; (b) pp125^{FAK} autophosphorylation activity; (c) pp125^{FAK}-pp60^{src} binding; (d) paxillin tyrosine phosphorylation; (e) paxillin–pp125^{FAK} association; (f) the localization of the two proteins in focal adhesions.

MATERIALS AND METHODS

Antibodies

Monoclonal anti-phosphotyrosine (PT66) IgG, horseradish peroxidase conjugated goat anti-(mouse IgG) Ig, horseradish peroxidase conjugated goat anti-(rabbit IgG) Ig, tetramethylrhodamine isothiocyanate (TRITC) conjugated goat anti-(rabbit IgG) Ig and fluorescein isothiocyanate (FITC) conjugated rabbit anti-(mouse IgG) Ig were purchased from Sigma (St Louis, MO, USA); rabbit polyclonal anti-(chicken focal adhesion kinase) Ig (BC3) and polyclonal rabbit anti-(human focal adhesion kinase) Ig from Upstate Biotechnology (Lake Placid, NY, USA); monoclonal mouse anti-paxillin IgG from Transduction Laboratories (Lexington, KY, USA); monoclonal mouse anti-pp60^{src} IgG (clone 327) from Calbiochem (Cambridge, MA, USA). Anti-pp125^{FAK} serum was also a kind gift of J. T. Parsons (University of Virginia, Charlottesville, VA, USA). Anti-(phosphotyrosine 397) Ig (number 625857) was obtained by the described procedure [28]. This antibody reacted with the autophosphorylated form of pp125^{FAK} but not with other phosphoproteins.

Chemicals

Aprotinin, bovine serum albumin (BSA), echistatin, human plasma fibronectin, leupeptin, nonessential amino acids, orthovanadate, pepstatin and sodium pyruvate were purchased from Sigma; Eagle's minimal essential medium (MEM), glutamine and vitamine solution were from ICN Biomedicals Inc. (Aurora, OH, USA); fetal bovine serum from Hyclone Laboratories Inc. (Logan, UT, USA); protein A–agarose or protein G–agarose from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The adhesive protein fibronectin appeared greater than 95% homogeneous by SDS/PAGE analysis and Coomassie blue staining.

Cell culture and adhesion

B16-BL6 mouse melanoma cells were grown in MEM supplemented with 10% fetal bovine serum, 1% nonessential amino acid solution, 2% vitamin solution, 1% sodium pyruvate solution and 1% glutamine. Cells were harvested for propagation or cell attachment studies by treatment with 0.25% trypsin/0.02% EDTA in NaCl/P$_i$, pH 7.2, and in 5 mM EDTA in NaCl/P$_i$, respectively. Cells were then washed with MEM and resuspended in complete MEM for propagation or in serum free MEM for adhesion studies. For gel and blot analysis cells were plated at 1×10^6 cells per 6-cm culture dish. Coverslips or tissue culture dishes were coated overnight at 4 °C by incubation with 0.1 mL human plasma fibronectin (10 $\mu g \cdot mL^{-1}$) diluted in NaCl/P$_i$ with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. After coating, coverslips or dishes were treated with 1% BSA in NaCl/P$_i$ for 30 min at 37 °C to block free binding sites on the plastic. Before plating cells, coverslips or dishes were rinsed twice in NaCl/P$_i$.

Fluorescence microscopy

Cells were plated onto fibronectin-coated coverslips and incubated for 3 h in serum-free medium at 37 °C. Non-adherent cells were removed by washing with serum free medium and fibronectin-adherent cells were exposed to 100 $\mu g \cdot mL^{-1}$ echistatin for 30 min. At the indicated time, the cells were washed, fixed in 3% paraformaldehyde, 60 mM sucrose in NaCl/P$_i$ for 20 min at room temperature, permeabilized with 0.2% Triton X-100 in NaCl/P$_i$ for 2 min and blocked by the addition of 1% BSA in NaCl/P$_i$ for 30 min. Cells were incubated for 1 h at room temperature with primary antibodies, washed three times in NaCl/P$_i$, exposed to a 1 : 20 dilution of rhodamine-conjugated goat anti-(rabbit IgG) Ig for 1 h, washed three times in NaCl/P$_i$ and exposed to a 1 : 20 dilution of fluorescein-conjugated rabbit anti-(mouse IgG) Ig for 1 h. After final washes with NaCl/P$_i$, the coverslips were mounted on a microscope slide using a 50% solution of glycerol in NaCl/P$_i$ and the stained cells were examined with a Zeiss Axiophot microscope.

Immunoprecipitation, immunoblotting and immune-complex kinase assay

Cells were kept in suspension for 30 min or plated on substrate-coated dishes and allowed to adhere at 37 °C for different time intervals. In the inhibition studies, cells were allowed to adhere for 3 h, washed with serum-free medium and exposed to 100 $\mu g \cdot mL^{-1}$ of echistatin for the indicated time. At the end of the incubation time, cells were lysed in ice-cold Tris/NaCl/P$_i$ containing 1% NP-40, 150 mM NaCl, 50 mM Tris HCl (pH 8), 5 mM EDTA, 10 mM NaF, 10 mM $Na_4P_2O_7$, 0.4 mM Na_3VO_4, 10 $\mu g \cdot mL^{-1}$ leupeptin, 4 μM pepstatin and 0.1 U trypsin per mL aprotinin. The lysates were clarified by centrifugation at 12 000 ***g*** for 10 min at 4 °C. The amount of proteins in the samples was determined by Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Lysates containing equal amounts of proteins were incubated with swelled protein A–agarose or protein G–agarose for 2 h at 4 °C. After centrifugation at 12 000 *g* for 10 min, supernatant fractions were incubated with anti-pp125^{FAK} serum (BC3), anti-paxillin Ig or anti-pp60^{src} Ig (1 μg per 200 μg protein) for 6 h at 4 °C. Protein A–agarose or G–agarose was then added to the samples and incubated overnight at 4 °C. Beads were sedimented by brief centrifugation after immunoprecipitation and washed extensively with lysis buffer. Proteins were resuspended in SDS sample buffer, boiled for 5 min in Laemmli buffer and run on 10% SDS/polyacrylamide gels. Following electrophoresis, the samples were transferred to nitrocellulose using a Mini Trans-Blot apparatus (Bio-Rad) according to manufacturer's instructions, blocked for 1 h at 42 °C in Tris/NaCl/P$_i$ containing 5% BSA, and washed with Tris/NaCl/P$_i$/Tween (150 mM NaCl, 20 mM Tris HCl, pH = 7.4, 0.3% Tween 20). To probe for phosphotyrosine-containing proteins the filters were incubated overnight with a 1 : 10 000 dilution of antibody PT66 diluted in Tris/NaCl/P$_i$, 1% BSA. Control blots using anti-pp125^{FAK} or anti-paxillin Ig instead of anti-phosphotyrosine Ig PT66 were always run to confirm equal loading of pp125^{FAK} or of paxillin in the lysates. The blots were washed three times with Tris/NaCl/P$_i$/Tween, incubated 2 h with peroxidase conjugated anti-(mouse IgG) Ig diluted 1 : 3000 in Tris/NaCl/P$_i$, 1% BSA, and washed three times with Tris/NaCl/P$_i$/Tween. The proteins were visualized by an ECL chemiluminescence kit (Amersham Corp., Little Chalfont, UK). Quantitation of tyrosine phosphorylated proteins was performed by densitometry using a

Discover Pharmacia scanner equipped with a sun spark classic densitometric workstation.

For kinase assay pp125^{FAK} was immunoprecipitated from 500 μg of lysates as described above. The immunoprecipitates were washed four times in lysis buffer and twice in kinase buffer (50 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol). After the final wash, the pellets were resuspended in 20 μL of kinase buffer and the reaction initiated by the addition of 15 μCi [γ-^{32}P]ATP (3000 Ci·mmol^{-1}, Amersham Corp.). After 10 min the reactions were terminated by the addition of 20 μL of SDS sample buffer, and boiling for 5 min. Samples were resolved on 10% acrylamide gels by SDS/PAGE and then visualized by autoradiography.

RESULTS

Tyrosine phosphorylation of both pp125^{FAK} and paxillin coordinately occurs when cells are plated on fibronectin coated dishes [16]. Adhesion of B16-BL6 melanoma cells to immobilized fibronectin led to a time-dependent increase of tyrosine phosphorylation of pp125^{FAK} and paxillin. The maximum level of phosphorylation of pp125^{FAK} was observed after 30 min of cell adhesion to fibronectin. Paxillin peak of phosphorylation appeared to occur slightly later than that of pp125^{FAK}. High phosphorylated levels of both proteins persisted up to 3 h (data not shown).

We previously observed that echistatin reduces pp125^{FAK} tyrosine phosphorylation in fibronectin-adherent B16-BL6 cells [13]. At least six tyrosines of pp125^{FAK} can be phosphorylated subsequent to integrin engagement: two sites within pp125^{FAK} N-terminal domain, two sites within kinase domain activation loop, and two sites within the C-terminal domain are phosphorylated *in vivo* [29]. Here, we evaluated whether the reduction of pp125^{FAK} phosphorylation induced by echistatin occurs at Tyr397 that is the site of pp125^{FAK} autophosphorylation activity both *in vitro* and *in vivo* [30]. Lysates containing equal amounts of proteins from fibronectin-adherent B16-BL6 cells exposed for different time intervals to 100 μg·mL^{-1} of echistatin were subjected to SDS/PAGE electrophoresis and to Western blotting by using a specific antibody (no. 625857) which recognizes Tyr397 phosphorylated pp125^{FAK} [28]. Fifteen min of echistatin treatment promoted a 69% decrease of Tyr397 phosphorylated pp125^{FAK} levels as compared to control (untreated) cells (Fig. 1A). Prolonged cell exposure to echistatin for 30 and 60 min resulted in a lower decrease of Tyr397 phosphorylation with respect to control cells than that observed at 15 min of echistatin treatment. However, when pp125^{FAK} immune-complexes from the same lysates were also blotted against anti-phosphotyrosine Ig PT66, the expected time-dependent decrease of pp125^{FAK} phosphorylation induced by echistatin was observed (data not shown). This might indicate that echistatin-induced inhibition of pp125^{FAK} Tyr397 phosphorylation persists for a shorter time with respect to other pp125^{FAK} tyrosines.

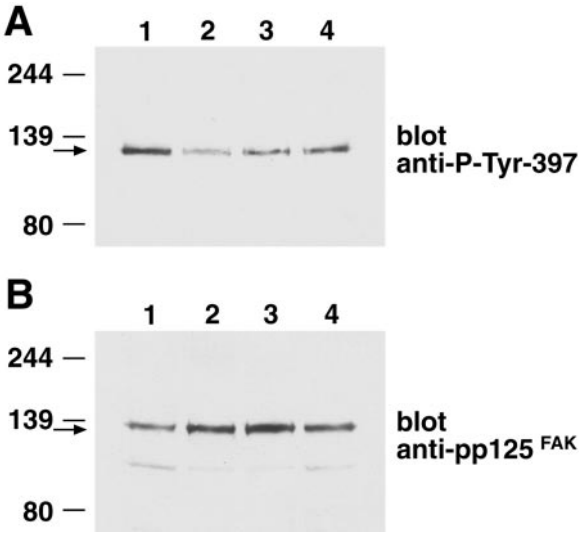

Fig. 1. Echistatin reduces pp125^{FAK} Tyr397 phosphorylation. B16-BL6 cells were plated onto fibronectin-coated dishes for 3 h (lane 1) and then exposed to 100 μg·mL^{-1} of echistatin for different time intervals: 15 min (lane 2); 30 min (lane 3); 1 h (lane 4). Detergent extracted proteins were directly run on SDS/PAGE and blotted with a specific affinity-purified antibody 625857 [blot: anti-(P-Tyr397) Ig] (A). The blot in A was stripped and reprobed against anti-pp125^{FAK} Ig (B). Molecular mass markers are indicated on the left. The position of Tyr397 phosphorylated pp125^{FAK} and of total pp125^{FAK} are indicated by the arrows on the left. Similar results were obtained in at least three experiments of identical design.

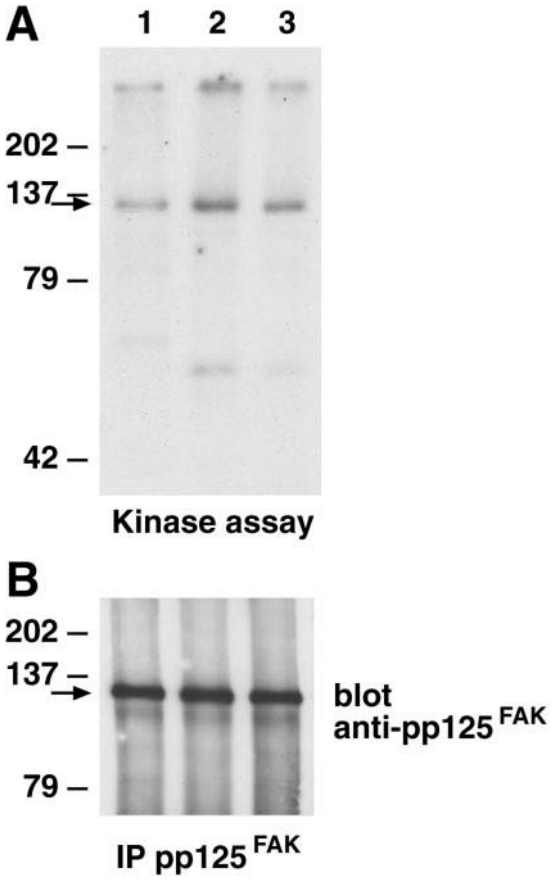

Fig. 2. Echistatin inhibits pp125^{FAK} autophosphorylation activity. B16-BL6 cells held in suspension (lane 1) or plated onto fibronectin-coated dishes for 3 h (lane 2) and treated with 100 μg·mL^{-1} of echistatin for 1 h (lane 3) were lysed and subjected to immunoprecipitation with anti-pp125^{FAK} Ig BC3. Immune-complexes were either incubated with [γ-^{32}P]ATP as described in the text and the products analyzed by autoradiography (A) or transferred onto nitrocellulose filters and blotted against anti-pp125^{FAK} serum (B). Molecular mass markers are indicated on the left. The position of pp125^{FAK} is indicated by the arrows on the left. Similar results were obtained from three separate experiments of identical design.

As the phosphorylation of Tyr397 is required for pp125^{FAK} autophosphorylation activity, we also measured the effect of echistatin on this activity by immune-complex kinase assay and autoradiography (Fig. 2A). pp125^{FAK} autophosphorylation activity was very low in cells held in suspension (lane 1), whereas it increased following cell adhesion to fibronectin (lane 2). Echistatin exposure to fibronectin-adherent cells for 1 h reduced pp125^{FAK} autophosphorylation activity by 31% (lane 3) as compared to control (untreated) cells (lane 2). pp125^{FAK} immune complexes obtained from the same lysates and blotted against anti-pp125^{FAK} Ig showed equal amounts of the protein in all samples (Fig. 2B). Interestingly, 1 h of cell exposure to echistatin caused a comparable extent of reduction of both pp125^{FAK} Tyr397 phosphorylation (39%) (Fig. 1A, lane 3) and pp125^{FAK} autophosphorylation activity (31%) (Fig. 2A, lane 3).

In order to assess the effect of echistatin-induced reduction of pp125^{FAK} phosphorylation on paxillin tyrosine phosphorylation, B16-BL6 cells were allowed to adhere to fibronectin for 3 h and then exposed to the disintegrin for different time intervals. Immunoprecipitation of cell lysates with anti-paxillin Ig followed by immunoblotting with anti-phosphotyrosine Ig showed that echistatin causes a decrease of paxillin phosphorylation as a function of the time of cell exposure to the disintegrin (Fig. 3A). Within 15 min of cell exposure to echistatin, paxillin phosphorylation decreased already by 42% as compared to control (untreated) cells; 1 h and 3 h of cell treatment by the disintegrin caused a 63% and 68% decrease of paxillin phosphorylation, respectively.

As either activated pp125^{FAK} or the pp125^{FAK}-pp60^{src} complex may be responsible of paxillin phosphorylation [15], we evaluated the effect of echistatin on the pp125^{FAK}–pp60^{src} association in fibronectin-adherent B16-BL6 cells. Cells were allowed to adhere to fibronectin-coated dishes for 3 h and then exposed to echistatin for 15 min. After lysis, cell extracts were immunoprecipitated with anti-pp60^{src} Ig and immunoblotted with anti-pp125^{FAK} serum. Echistatin cell treatment caused an about 20% decrease of the amount of pp125^{FAK} coprecipitating with pp60^{src}, as compared to control (untreated) cells (Fig. 4A).

We previously demonstrated that fibronectin-adherent B16-BL6 cells develop focal contacts where β_1-containing integrins, possibly $\alpha_5\beta_1$, are clustered as determined by immunofluorescence techniques [13]. We show now that pp125^{FAK} and paxillin colocalize in these structures (Fig. 5A,B). However, immunofluorescence staining of cells with anti-pp125^{FAK} serum and anti-paxillin Ig suggests that only a fraction of these proteins is concentrated in focal contacts, a significant fluorescence signal being localized in the cytoplasm, in the perinuclear region (Fig. 5A,B). Echistatin treatment of fibronectin-adherent cells induced a progressive albeit rapid detachment of cells from its substrata (Fig. 5C,D). It is likely that adhesion plaques get disassembled upon echistatin interaction with cell membranes. Adhesion plaques are always

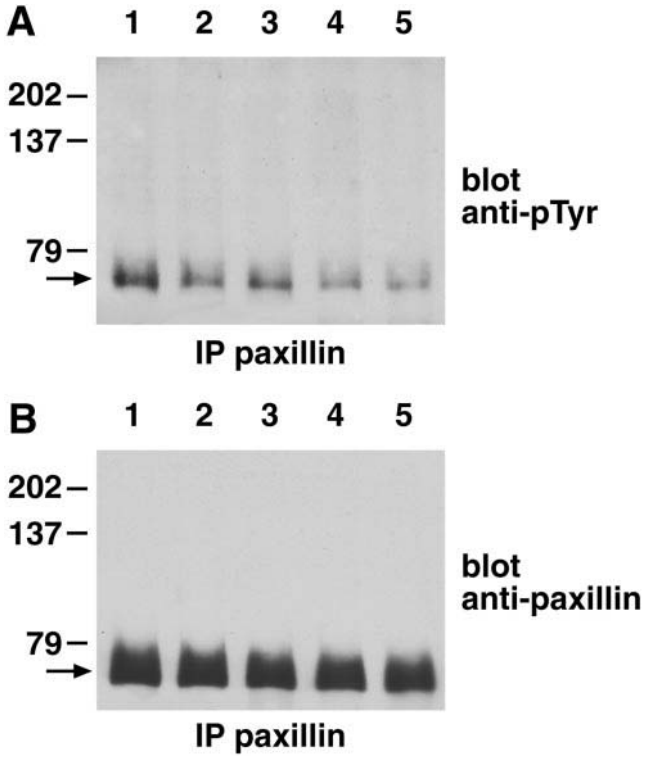

Fig. 3. Echistatin reduces paxillin phosphorylation in fibronectin-adherent B16-BL6 cells. B16-BL6 cells were allowed to adhere to fibronectin-coated dishes for 3 h (lane 1) and then exposed to 100 $\mu g \cdot mL^{-1}$ of echistatin for different time intervals: 15 min (lane 2), 30 min (lane 3), 60 min (lane 4) and 3 h (lane 5). Cell lysates were immunoprecipitated with anti-paxillin Ig and subsequently separated by electrophoresis. After Western blotting, tyrosine phosphorylated proteins were visualized by mAb PT66 (A). The blot in (A) was stripped and reprobed with anti-paxillin (B). Molecular mass markers are indicated on the left. The position of paxillin is indicated by the arrows on the left. Similar results were obtained from five separate experiments of identical design.

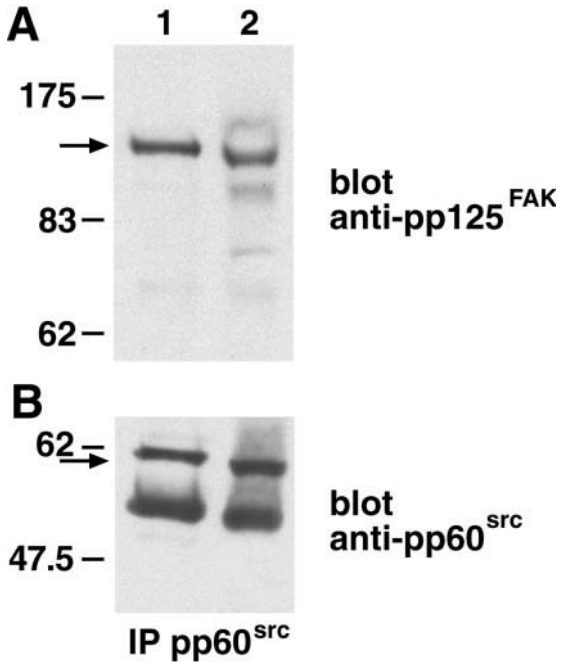

Fig. 4. Echistatin causes a decrease of pp125^{FAK}-pp60^{src} binding. B16-BL6 cells were allowed to adhere to fibronectin-coated dishes for 3 h (lane 1) and then were treated with 100 $\mu g \cdot mL^{-1}$ of echistatin for 15 min (lane 2). Cells were lysed and subjected to immunoprecipitation with anti-pp60^{src} Ig (clone 327). Immune-complexes were transferred on nitrocellulose and blotted against anti-pp125^{FAK} serum (A). The same membrane used in A was reprobed with anti-pp60^{src} Ig to ensure that the same amount of pp60^{src} was immunoprecipitated from the samples (B). Molecular mass markers are indicated on the left. The position of pp125^{FAK} and pp60^{src} are indicated by the arrows on the left. Similar results were obtained from three separate experiments of identical design.

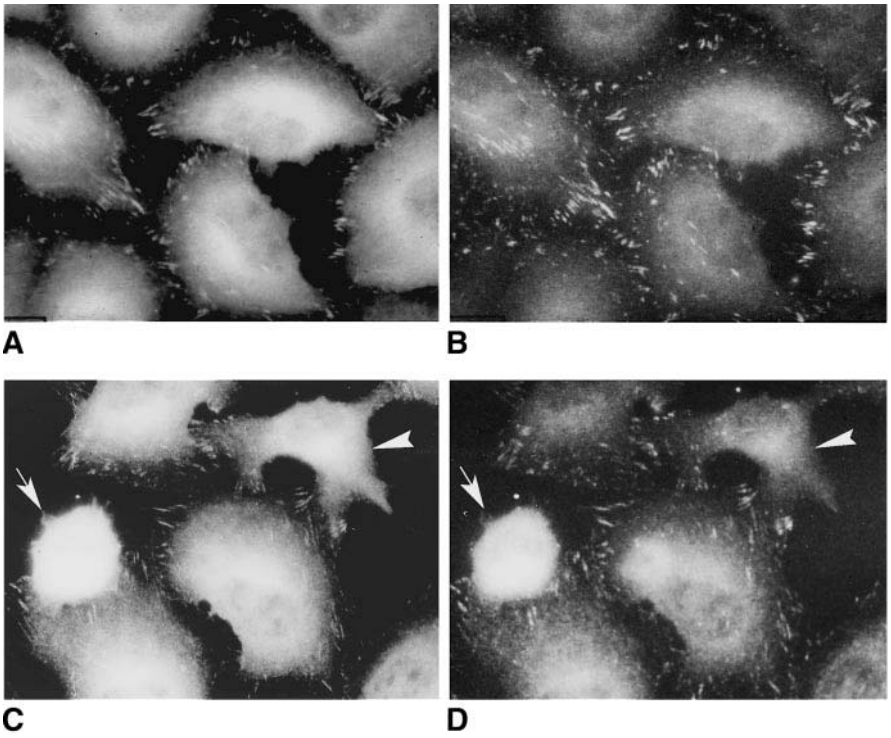

Fig. 5. Loss of pp125^{FAK} and paxillin-containing focal adhesions by echistatin treatment. Cells were double immunostained after fixation with anti-paxillin Ig (A, C) and anti-pp125^{FAK} Ig (B, D). In untreated cells (A, B) pp125^{FAK} and paxillin colocalized in focal adhesion structures. A diffuse cytoplasmic staining was also evident for both proteins mostly in the perinuclear region. Treatment of the cells with echistatin (C, D) induced a progressive cell detachment from the fibronectin substrata (arrowhead) and rounding of the cells (arrow). pp125^{FAK} and paxillin always colocalized in all adhesion plaques suggesting that both proteins act as a complex and/or that the distribution of these proteins is coordinately influenced by echistatin.

positive to both anti-pp125^{FAK} serum and anti-paxillin Ig staining suggesting that the two proteins colocalize and that both coordinately respond to echistatin treatment.

We studied the association of pp125^{FAK} with paxillin to evaluate the effect of echistatin on it. pp125^{FAK} immune complexes prepared from lysates of B16-BL6 cells held in suspension or allowed to adhere to fibronectin for different time intervals were probed with anti-paxillin Ig in order to determine the presence of coprecipitating paxillin. The association of some paxillin with pp125^{FAK} was observed in cells held in suspension (Fig. 6A, lane 1, upper blot); however, an increase of the amount of paxillin coprecipitating with pp125^{FAK} was observed following cell adhesion to fibronectin (Fig. 6A, lanes 2–5, upper blot). The amount of total paxillin was also determined by sequential rounds of immunoprecipitations with anti-paxillin Ig (data not shown). In this way we could calculate the amount of paxillin coprecipitating with pp125^{FAK} which resulted to be approximately 20% of the total paxillin present in

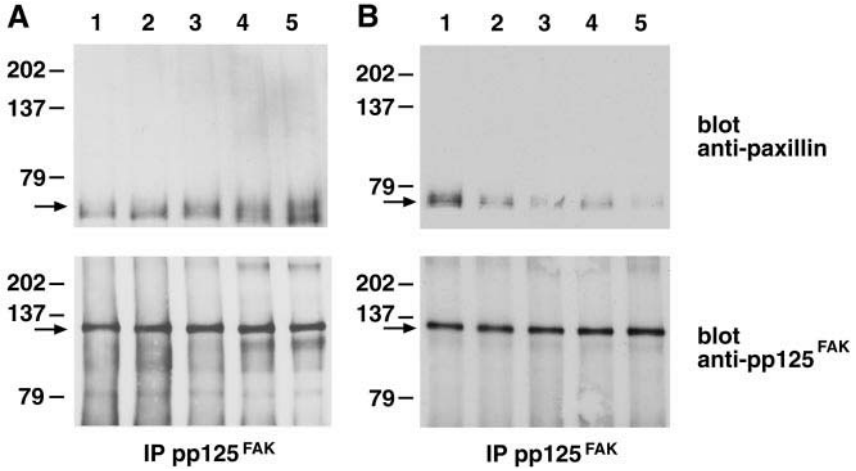

Fig. 6. Echistatin affects pp125^{FAK}–paxillin association in fibronectin-adherent B16-BL6 cells. (A) Time-course of pp125^{FAK}–paxillin association in B16-BL6 cells adhering to fibronectin. Cells were held in suspension (lane 1) or were allowed to adhere to fibronectin-coated dishes for different time intervals: 15 min (lane 2); 30 min (lane 3); 1 h (lane 4); 3 h (lane 5). (B) Time-course of echistatin effect on pp125^{FAK}–paxillin association. Cells were allowed to adhere to fibronectin-coated dishes for 3 h (lane 1) and then were treated with 100 $\mu g \cdot mL^{-1}$ of echistatin for different time intervals: 15 min (lane 2); 30 min (lane 3); 1 h (lane 4); 3 h (lane 5). At the indicated time, cells were lysed and subjected to immunoprecipitation with anti-pp125^{FAK} Ig BC3. Immune-complexes were transferred on nitrocellulose and blotted against anti-paxillin Ig to detect coprecipitating paxillin (upper blots). The same membranes used in upper blots of (A) and (B) were reprobed with anti-pp125^{FAK} serum to ensure that the same amount of pp125^{FAK} was immunoprecipitated from all samples (lower blots). Molecular mass markers are indicated on the left. The position of pp125^{FAK} and paxillin are indicated by the arrows on the left. Similar results were obtained from five separate experiments of identical design.

the cell lysates, when cells adhered for 3 h to immobilized fibronectin. The addition of echistatin to fibronectin-adherent cells caused a time-dependent decrease of the amount of paxillin coprecipitating with pp125^{FAK} (Fig. 6B, upper blot). Fifteen min and 1 h of cell exposure to the disintegrin caused, respectively, 60% and 69% decrease of coprecipitating paxillin as compared to control cells.

DISCUSSION

We recently demonstrated that the interaction of echistatin with integrin receptors on B16-BL6 melanoma cells causes a reduction of pp125^{FAK} tyrosine phosphorylation, which is accompanied by the disassembly of actin cytoskeleton and of focal adhesions [13]. Here we show that echistatin molecular mechanism of action involves: (a) the reduction of pp125^{FAK} phosphorylation at Tyr397 residue, that affects pp125^{FAK} autophosphorylation and kinase activity; (b) the reduction of paxillin phosphorylation; (c) the simultaneous disappearance of pp125^{FAK} and paxillin from focal adhesions; and (d) the inhibition of pp125^{FAK}–paxillin association.

Both pp125^{FAK} and paxillin become phosphorylated following B16-BL6 melanoma cell adhesion to immobilized fibronectin. However, paxillin phosphorylation occurrs slightly later than pp125^{FAK} phosphorylation. Thus paxillin might be a direct substrate for phosphorylation by pp125^{FAK}, or might serve as a substrate for a second protein kinase (PTK) that can be potentially activated by pp125^{FAK}, i.e. pp60^{src} or Csk [15,31].

The phosphorylation of pp125^{FAK} most likely represents an autophosphorylation event that is required for the phosphorylation of downstream targets, including paxillin [32,33]. A model of pp125^{FAK} activation proposed by Giancotti [34] suggests that the interaction of amino-terminal domain of pp125^{FAK}, which plays a negative autoregulatory role by folding back onto the catalytic domain, with the integrin β subunit tail results in a conformational change and oligomerization of pp125^{FAK} molecules. These become activated by a trans-autophosphorylation mechanism similar to that established for receptor tyrosine kinases. Echistatin is likely to directly interact with integrins [3,35,36]. It might promote a conformational change of integrin molecules that could affect the interaction of their β-cytoplasmic tails with the amino terminus domain of pp125^{FAK} causing the subsequent block of pp125^{FAK} activation.

Activated pp125^{FAK} undergoes autophosphorylation at tyrosine residue 397 which creates a binding site for the SH2 domain of Src (and Fyn), possibly resulting in the formation of a bipartite kinase complex composed of pp125^{FAK} and Src (or Fyn) [32,37]. The formation of this bipartite complex is an early and critical event in the assembly of focal adhesion complexes and in the activation of integrin signaling pathways. A likely *in vivo* substrate for the complex has been suggested to be paxillin [29]. We demonstrate here that echistatin treatment results in a decrease of pp125^{FAK} Tyr397 phosphorylation and of its autophosphorylation activity, as measured by immune-complex kinase assay. We also demonstrate that a decrease of paxillin phosphorylation occurs. Even though paxillin is considered a target for pp125^{FAK} transphosphorylation, caution is needed in making a direct correlation between pp125^{FAK} tyrosine phosphorylation levels and pp125^{FAK} kinase activation. For example, in serum-starved adherent fibroblasts pp125^{FAK} can exists in a state where Tyr397/Src SH2 binding site is highly phosphorylated, but under these conditions Src-family PTKs are not significantly associated with pp125^{FAK} and pp125^{FAK} exhibits only low levels of *in vitro* kinase activity [38]. Furthermore, Phe-397 pp125^{FAK} exhibits significant kinase activity *in vitro* even though does not associate with Src-family PTKs [29,32].

As paxillin is a good substrate for activated Src [39], it is possible that the phosphorylation of paxillin may be mediated in part by FAK-associated Src-family PTK activity. Here we show that echistatin treatment of fibronectin-adherent B16-BL6 cells causes a reduction of pp125^{FAK}–pp60^{src} association. Thus, our data suggest that echistatin, by causing the reduction of autophosphorylation activity of pp125^{FAK}, determines a decreased ability of pp125^{FAK} to bind and activate Src kinases, thereby reducing paxillin tyrosine phosphorylation.

The role of tyrosine phosphorylation of paxillin in integrin-directed signaling is still undetermined. Paxillin forms complexes with other cytoskeletal proteins including vinculin [25] and perhaps talin [40], in addition to forming complexes with the three PTKs pp125^{FAK} [23,40], pp60^{src} [31] and Csk [41], the negative regulator of pp60^{src}, and with the adapter protein p47$^{gag-Crk}$ [42]. Therefore, paxillin may serve as an adapter protein itself, a protein that tethers other proteins to a multicomponent complex. Tyrosine phosphorylation of paxillin may regulate protein–protein interactions. It has been suggested that it might also regulate the activation of GTP-binding proteins in a very specific location within the cell [43]. Once active the GTP-binding proteins could transmit a signal to the nucleus and/or a signal inducing structural changes within the cytoskeleton. Thus, echistatin induced reduction of paxillin phosphorylation may represent the event downstream pp125^{FAK} dephosphorylation by which the disintegrin causes the disassembly of actin cytoskeleton and of focal adhesions.

It is known that many PTKs, including pp125^{FAK}, and their substrates, such as talin, paxillin and tensin, accumulate at focal adhesions organized by β_1-containing integrins [44]. These structures seem to represent the privileged site of action of echistatin [13,36]. Some evidence indicates that tyrosine phosphorylation is involved in the regulation of focal adhesion and stress fiber formation [16,19,44], although the microinjection of a dominant-negative pp125^{FAK} protein suggested that tyrosine phosphorylation may not be critically involved in the assembly of focal adhesions [26]. Immunofluorescence analysis demonstrates that pp125^{FAK} and paxillin colocalize in focal adhesions of fibronectin-adherent B16-BL6 cells. However, only a small amount of these proteins is apparently concentrated in these structures, while the largest fraction is in the cytoplasm, in the perinuclear region. The preferential localization of tyrosine phosphorylated paxillin to focal adhesions may represent a general mechanism to compartmentalize focal adhesion components from large nonphosphorylated cytosolic pools [45]. Cell exposure to echistatin inhibits paxillin phosphorylation and causes a dramatic disassembly of focal adhesions, with the simultaneous disappearance of both pp125^{FAK} and paxillin.

The organization and function of focal adhesions is also regulated by protein tyrosine phosphatases (PTPases) [46]. Inhibitors of PTPases prevented most of decrease in protein phosphorylation brought about by cell treatment with trypsin, thus suggesting that PTPases are activated when cell-substratum adhesion is disrupted by trypsin in chicken embryo fibroblasts [47]. However, we previously demonstrated that echistatin acts by inhibiting PTKase activity rather than activating PTPases [13]. Three cellular and one bacterial PTPases have been recently implicated as potential regulators of tyrosine phosphorylation of focal adhesion proteins [46,48]. PTPase activity was found to specifically associate with pp125^{FAK}, both *in vitro* and *in vivo*: however, this interaction appeared to be indirect and was in part mediated by paxillin [49]. Thus, the finding that

paxillin associates with both a PTK and a PTPase suggests a novel model for the regulation of tyrosine phosphorylation by the assembly of complexes containing both enzymes. Paxillin could play a role in coordinating the level of tyrosine phosphorylation of focal adhesion-associated proteins by recruiting PTP and/or pp125^{FAK} to their substrates.

It has been shown that paxillin is directly associated with pp125^{FAK}, yet their tyrosine phosphorylation does not seem to be necessary for this association [23,24]. pp125^{FAK} and paxillin appear to be associated also in the B16-BL6 melanoma cells system either in suspension or after cell adhesion to immobilized fibronectin. However, the amount of paxillin bound to pp125^{FAK} increases by prolonging the time of cell adhesion. Echistatin treatment of fibronectin-adherent B16-BL6 cells causes a time-dependent decrease of the extent of pp125^{FAK}-paxillin binding. The disassembly of focal adhesions induced by echistatin involves the simultaneous disappearance of pp125^{FAK} and paxillin; however, only a small amount of the two proteins remains bound in cells in suspension.

The question regarding the role of the interaction between paxillin and pp125^{FAK} remains unclear. The carboxy terminus of pp125^{FAK} supports both paxillin binding and focal adhesion localization. pp125^{FAK} has been involved in the recruitment of paxillin to focal adhesions [23] but it has also been suggested that pp125^{FAK} does not target, but is targeted by paxillin to focal adhesions [24]. Further evidence demonstrated that paxillin localizes to focal adhesions independent of interactions with either vinculin and/or pp125^{FAK} [44]. Even though pp125^{FAK}-paxillin binding is not necessary or sufficient for focal adhesion localization, it is likely to be necessary for stabilization and maintenance of this subcellular distribution. Further, this interaction may modulate the capacity of these cytoskeletal proteins to interact with many other constituents of the focal adhesion [22], structurally or enzymatically, thereby modulating the assembly and disassembly of these structures and other signaling pathways associated with integrin functions [50].

The importance of pp125^{FAK} in mediating cellular events that govern cell motility has been explored [26,51]. It has been ascertained that pp125^{FAK} controls cell spreading, the rate of focal adhesion formation, and cell migration [26,52,53]. The forced overexpression of pp125^{FAK} in CHO cells results in an increase in cell migration [51]. Furthermore, the increased expression of pp125^{FAK} noted in melanoma cell lines correlates with increased cell motility [54] and the elevated pp125^{FAK} expression observed in colonic and breast tumors appears to be restricted to invasive as opposed to noninvasive tumors [55,56]. Thus, pp125^{FAK} seems to regulate the motility of both normal and malignant cells. Moreover, the recruitment of pp60^{src} and paxillin by pp125^{FAK} and the tyrosine phosphorylation of paxillin appear to be limiting events in cell spreading [57]. Disintegrins as well as synthetic RGD-peptides have been shown to inhibit experimental metastasis *in vivo* [6–10]. The mechanism by which they act still requires further elucidation. However, on the basis of our results we propose that disintegrins exert their antimetastatic activity through the modulation of pp125^{FAK} autophosphorylation and kinase activity which in turn decrease tyrosine phosphorylation of paxillin and focal adhesion organization, thereby affecting cell adhesion and motility.

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