JCMM
Available online at
www.jcmm.ro
www.jcmm.org

J. Cell. Mol. Med. Vol 9, No 2, 2005 pp. 387-397

Activation of focal adhesion kinase in human lung cancer cells involves multiple and potentially parallel signaling events

Nishit K. Mukhopadhyay [a, b], Gavin J. Gordon [a], Chang-Jie Chen [c], Raphael Bueno [a], David J. Sugarbaker [a], Michael T. Jaklitsch [a] *

[a] *Division of Thoracic Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA*
[b] *Division of Urology/Surgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA*
[c] *Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA*

Received: February 24, 2005; Accepted: May 10, 2005

Abstract

Integrins are adhesion receptors that transmit signals bidirectionally across the plasma membrane. In our previous report we have shown that the squamous lung cancer cell line, Calu-1, binds to collagen type IV (Coll IV) through β_1-integrin and results in phosphorylation of focal adhesion kinase (FAK) (Ann Thorac Surg 2004; 78:450–7). Considering the critical role of FAK in cell migration, proliferation, and survival, here we investigated potential mechanisms of its activation and regulation in Calu-1 cells. We observed the phosphorylation of Tyr ^{397} of FAK (the autophosphorylation site of FAK) and paxillin, the immediate downstream substrate of FAK following the adhesion of Calu-1 cells to Coll IV. FAK remains phosphorylated during proliferation either on Coll IV or on uncoated plates for 72 h, as determined by peroxivanadate treatment. Exposure of Calu-1 cells with 60 µM genistein, reduces FAK phosphorylation (7.6 fold) and cell proliferation. Extracellular signal regulated kinases (ERKs) were also phosphorylated after Coll IV attachment. Disruption of Calu-1 cell cytoskeleton integrity by 1-5 µM Cytochalasin D resulted in the inhibition of cell adhesion (50% to 75%, $p<0.19 - 6.6\times10^{-7}$) and ERKs phosphorylation (2 fold) without any effect on FAK phosphorylation. Protein Kinase C inhibitor, Calphostin C at 100 and 250 nM concentrations did not block Coll IV induced FAK phosphorylation, but activated the ERKs in a dose dependent manner. β_1-integrin is essential for Coll IV induced FAK activation, but it is not physically associated with FAK as determined by immunodetection assay. Collectively, this report defines the existence of multiple and potentially parallel Coll IV/β_1-integrin mediated signaling events in Calu-1 cells, which involve FAK, ERKs, and PKC.

Keywords: lung cancer • metastasis • collagen type IV • focal adhesion kinase • extracellular signal regulated kinases • protein kinase C

Introduction

Integrin receptors lack catalytic activity, and must therefore recruit and activate other signaling molecules. One such molecule is focal adhesion kinase (FAK), a non-receptor tyrosine kinase that is enriched in focal adhesions and is ubiquitously expressed throughout development [1, 2]. FAK phosphorylation and kinase activity are closely regulated by integrin mediated and matrix dependent cell adhesion in many cancer cells [3, 4] suggesting that FAK may be an important mediator

* Correspondence to: Michael T JAKLITSCH,
Division of Thoracic Surgery, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street,
Boston MA 02115, USA.
Tel.: 617-732-6988, Fax: 617-730-2898
E-mail: mjaklitsch@partners.org

Reprinted from:
Journal of Cellular and Molecular Medicine

Published by:
the CMM Foundation

of integrin signaling. Furthermore, overexpression and phosphorylation of FAK correlates with increased motility, invasion and alteration in the cytoskeleton. Adhesion and spreading of cells on a variety of extracellular matrix (ECM) proteins, including collagen type IV (Coll IV), leads to an increase in tyrosine phosphorylation and activation of FAK. In melanoma cells, the increased expression of FAK correlates with increased cell motility [5]. The overexpression of FAK in colonic and breast tumors appears to be restricted to invasive as opposed to noninvasive tumors. Overexpression of FAK has also been reported in breast cancer and sarcoma [6, 7]. Therefore, considerable evidence points to a role of FAK participating in cancer cell-ECM interactions. Previous literature supports the conclusion that the phosphorylation and activation of FAK that localized at the focal adhesion contacts, is involved in the reorganization of the cytoskeleton and the regulation of the cell shape. Suppression of adhesion induced tyrosine phosphorylation of FAK may interrupt cancer cell-ECM interactions and affect the invasive and metastatic potential of cancer cells. Activation of the Tyr^{397} residue of FAK following cell adhesion results in association of cytoskeletal proteins like tansin and paxillin to the focal adhesion complex [8]. Moreover, the residues surrounding Tyr^{397} constitute a sequence that binds to the Src-homology domain2 (SH2) motif of another tyrosine kinase, pp60Src. Binding of FAK with Src also links FAK to the adaptor protein Grb2 and then to the Ras signaling pathway [9, 10]. The downstream targets of the Ras signaling pathway are ERK1/2, which have been found to be regulated by activation of FAK with respect to different matrix components [4, 9]. Thus, integrin binding to the ECM creates and activates a bipartite kinase complex and transduces external stimuli from the ECM to the nucleus.

It has also been reported in some cell lines that integrin stimulated FAK activation is dependent upon the integrity of the actin cytoskeleton [11, 12]. Moreover, several studies indicate that down-regulation of phorbol ester-sensitive isoforms of protein kinase C (PKC) and pharmacological PKC inhibitors, such as Calphostin C, result in reduced integrin-stimulated phosphorylation and activation of FAK [13].

In our previous work [14], we have shown an adhesion advantage of a non-small-cell lung cancer (NSCLC) line, Calu-1, to extracellular matrix protein Coll IV which depends on β_1-integrin. The present studies were designed to investigate the mechanism of activation and regulation of FAK and it's downstream targets ERK1/2 following adhesion to Coll IV. Understanding the regulation of FAK and FAK signaling pathway may enable us to identify molecular targets to block the metastatic process in lung cancer.

Materials and methods

Materials

DMEM (Dulbecco's modified Eagle's medium), RPMI 1640 medium, fetal bovine serum (FBS), trypsin, Dulbecco's phosphate-buffered saline (PBS), penicillin-streptomycin and glutamate were purchased from Invitrogen Life Technologies (Carlsbad, CA). Collagen type IV (human) was purchased from Research Diagnostics Inc. (Flanders, NJ). Myelin basic protein (MBP), Hydrogen peroxide (H_2O_2), sodium vanadate (orthovanadate), and nitrocellulose membranes were obtained from Fisher Scientific Co (www.fishersci.ca, Atlanta, GA). Bovine serum albumin (BSA) and soybean trypsin inhibitor, dithiothretol (DTT), β-mercaptoethanol, phenyl-methylsulphonyl fluoride (PMSF), glycerol, $MgCl_2$, Nonidate P-40 (NP-40), leupeptin, aprotinin, adenosine triphosphate (ATP), Calphostin C, Cytochalasin D and horseradish peroxidase secondary antibody were from Sigma Chemical Co. (St. Louis, MO). Proto-gel was from National Diagnostic (Atlanta, GA). Phosphotyrosine inhibitor (Genistein), anti-phosphotyrosine monoclonal antibody (4G10), Paxillin antibody, polyclonal focal adhesion kinase (FAK) antibody, FAK tyr $(p)^{397}$, and β -actin antibody were from our collaborators, but also available commercially from Transduction Laboratories (San Diego, CA). Monoclonal phospho-ERK1/2 antibody is from Cell Signaling Technology (Beverly, MA) and ERK1/2 antibody was obtained from Zymed Laboratories, Inc (San Francisco, CA). β_1-integrin Ab (141720) and p130 Cas Ab used for Western blotting and activation experiment were from Transduction Laboratories (San Diego, CA). ($\gamma^{32}P$)-ATP was purchased from New England Nuclear (Boston, MA).

Cell culture

The human non-small-cell lung cancer line used in this study (Calu-1) was purchased through American Type Culture Collection (www.atcc.org). The cell line was grown in RPMI supplemented with 10% heat inactivated fetal bovine serum, 1% penicillin-streptomycin and 2% glutamine at 37°C and 5% CO_2. Cells were passaged not more than eight generations and then replaced from frozen stocks of earlier passaged cells.

Cell adhesion and proliferation assay

Six well polystyrene plates (9.6 cm^2/well) were coated either with Coll IV (5 µg per well) or BSA (3% BSA in PBS) suspended in 1 ml coating buffer containing 100 mM tris (pH 8.0) and incubated at 4°C for 16 h. The plates were washed with PBS and incubated with 3% BSA for 2 h at 37°C in order to block non-specific binding sites. The plates were then washed twice more with PBS and kept at 4°C until needed.

Adhesion assays were performed with serum starved (14-16 h) cells, detached with 0.25% trypsin/EDTA followed by suspension in medium containing DMEM, 20 mM hepes and 2% BSA. One ml suspension containing 1-2 $x10^5$ cells was added to each well and incubated at 37°C and 5% CO_2. At the end of each incubation time point (15, 30, or 60 min), the plates were washed twice with PBS to remove the non-adherent cells, trypsinized and counted. The data were means of triplicate wells and were expressed as a percentage of deposited cells.

Cell proliferation assay was performed by suspending $1x10^5$ cells/ml in regular medium. One ml of suspension was added to coated and uncoated wells and incubated at 37°C for up to 72 h. Triplicate wells were trypsinized and counted at each time point.

Tyrosine kinase activator and inhibitor treatment

At each data time point, the Calu-1 cells were trypsinised, washed twice with serum free medium containing Soya bin trypsin inhibitor and preincubated for 10 min at 37°C before the addition of peroxivanadate, a generalized tyrosine phosphatase inhibitor (100 µM final, prepared from 10 mM vanadate with 1µl of 500 µM hydrogen peroxide). After incubation for 10 min at 37°C, the cells were washed with PBS, serum free medium, and lysed with NP-40 lysis buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP-40, 200 µM sodium orthovanadate, 1mM dithiothretol, 1 mM phenylmethylsulfonyl fluoride, 2 µg /ml leupeptin and 5 µg/ml approtinin.

Genistein (a generalized tyrosine kinase inhibitor) was added to Calu-1 cells (60 µM final) in normal growth medium for 3 days. The flasks were light protected and incubated at 37°C with the addition of 60 µM genistein every 24 h. After 72 h, the cells were trypsinised, washed, and the adhesion assay was performed as described before.

Immunoprecipitation and Western blotting

Normal (BSA coated) or activated (exposed to Coll IV) cells were lysed at 4°C in the NP-40 lysis buffer. The lysates were centrifuged at 14,000 rpm for 15 min at 4°C and the supernatants (500 µg -1 mg of protein) were used for immunoprecipitation with either anti-phosphotyrosine, anti-FAK, anti-Paxillin or anti-p130Cas antibody at 4°C overnight. After incubation for 1 h with antibody, either protein A- or protein G- sepharose was added to the lysates and incubated overnight at 4°C. The bead bound complexes were pelleted, washed several times with lysis buffer in PBS and boiled with SDS sample buffer for 3-5 min before loading on SDS -PAGE (7.5 to 10%).

For Western blot analysis, the proteins were transferred to nitrocellulose membranes after SDS-PAGE and blocked with 5% dry milk in TBST (25 mM Tris, 2.7 mM KCl, 137 mM NaCl and 0.1% Tween-20, pH 7.4). The blots were incubated with specific primary antibody from 1 h at room temperature to overnight at 4°C. Membranes were washed briefly with TBST and incubated with the horseradish peroxidase conjugated secondary antibody for 1 h at room temperature (1:5000 dilution). Following extensive washing, immunoreactive bands were visualized by chemiluminescence (ECL reagent, New England Nuclear). When required, the membranes were stripped in 62.5 mM Tris-HCL (pH 6.8), 2% SDS and 1.0 mM β-mercaptoethanol for 30 min. at 50°C and reblotted again.

Kinase assay

The Calu-1 cells attached to Coll IV matrix for different time periods were lysed and lysates equivalent to 400 µg of proteins were immunoprecipitated with MAP kinase

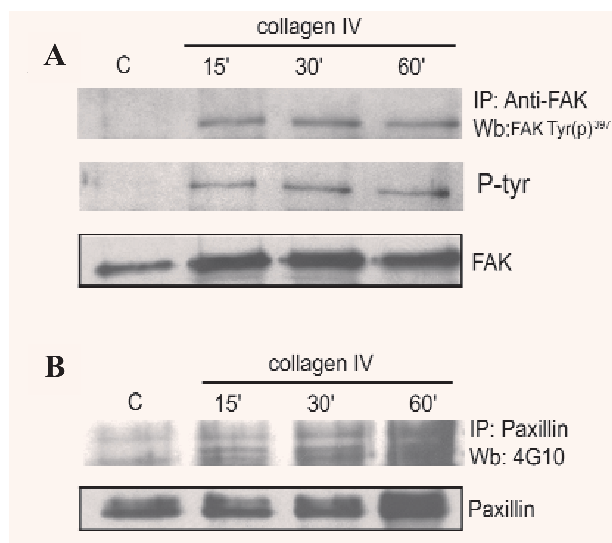

Fig. 1 Immunoprecipitation and immunoblotting analysis of Calu-1 whole cell lysates following adhesion to collagen IV matrix. A Increased FAK activity is implemented by a corresponding increase in the blot using FAK Tyr(p)397 (lane 1); FAK tyrosine phosphorylation increased within 15 min of exposure to Coll IV matrix, and remained elevated for 60 min (lane 2). B Tyrosine phosphorylation of the FAK substrate paxillin follows a profile similar to that of FAK. Control (C) represents either the level of FAK (Fig.1A) or paxillin (Fig.1B) on uncoated plates

(ERK1/2) specific antibody and protein A-sepharose. The immunoprecipitate was washed with lysis buffer, cold PBS and finally with kinase buffer (25 mM Hepes of Ph 7.4, 1 mM DTT, 10 mM $MgCl_2$, 100 µM ATP) before suspending into kinase buffer. Kinase reaction was initiated by adding 1.0 µCi of (γ-^{32}P)ATP and 2 µg of myelin basic protein (MBP) in a 30 µl incubation mixture containing the kinase and was incubated for 20 min at 30°C. The reaction was stopped by adding sample buffer, boiled and was separated in a 10% SDS-PAGE. The gels were stained, dried and exposed to film. After the exposure, the MBP bands were excised and counted by adding scintillation fluid.

Disruption of cytoskeleton

Serum starved Calu-1 cells ($0.5x10^6$) either in presence or absence of Cytochalasin D (1-5 µM), were plated on Coll IV coated plates for 30 minutes. Plates were washed with cold PBS twice and the attached cells were counted in triplicate. For the detection of FAK, 600 µg of proteins from Cytochalasin D treated or untreated cells attached to Coll IV treated plates were immunoprecipitated with FAK antibody and blotted with 4G10 and FAK antibody respectively. For the detection of ERKs, whole cell extracts (50 µg total protein) from Cytochalasin D treated or untreated cells were run on a 10% polyacrylamide gel and were blotted first for phospho Erk1/2 antibody followed by ERK1/2 antibody.

Inhibition of protein kinase C

Serum starved cells were added to either BSA coated or Coll IV coated plates either in presence or absence of Calphostin C (100 and 250 nM). Cell lysates equivalent to 600 µg protein was immunoprecipitated with FAK antibody and blotted for 4G10 and FAK antibody respectively. Whole cell lysates (50 µg total protein) from Calphostin C treated or untreated cells were blotted first with phospho ERK1/2 antibody followed by ERK1/2 antibody after stripping.

Densitometry and statistical analysis

Quantitation of the Western blots was accomplished by using a densitometric-based analysis performed on scanned fluorograms using Scion Image Beta 4.02 software from Scion Corporation (Frederick, MD, info@scioncorp.com). GraphPad Prism v.3.02 (GraphPad Software, San Diego, CA) was used to assess quantified differences in the number of adherent cells to Coll IV using a two-tailed students (parametric) t-test. All differences were determined to be statistically significant if $p<0.05$.

Results

Adhesion to collagen IV matrix leads to increased tyrosine phosphorylation of FAK

We observed tyrosine phosphorylation of proteins in the 60-70 kDa and 100-140 kDa ranges in crude whole cell lysates after 15 min of adhesion to Coll IV matrix (data not shown). Therefore, lysates of

adherent cells were further analyzed by immunoprecipitation with antibodies to FAK followed by Western blotting with an antibody which is specific for FAK Tyr(P)^{397} (the FAK autophosphorylation site) and 4G10 (phosphotyrosine antibody). FAK activity, as judged by autophosphorylation and total tyrosine phosphorylation, is observed within 15 min of adhesion to Coll IV and remained high for 60 min. (Fig. 1A, lane 1&2). Tyrosine phosphorylation of paxillin, a putative FAK substrate, followed a profile similar to the tyrosine phosphorylation of FAK (Fig. 1B) but the phosphorylation of p130^{Cas} was not detected in response to adhesion (data not shown).

Phosphorylation of FAK during proliferation

In order to verify whether FAK remains phosphorylated during proliferation, we did two independent experiments. First, we determined the phosphorylation status of FAK during proliferation on Coll IV matrix. As indicated in Fig. 2A, FAK remains phosphorylated during 72 h of growth on Coll IV matrix compared to uncoated matrix. Secondly, we tested the FAK phosphorylation status of Calu-1 cells during proliferation on uncoated culture discs by blocking the dephosphorylation of FAK by peroxivanadate treatment. The presence of phosphorylated FAK during several days of growth on uncoated discs is visible by treating the Calu-1 cells with 100 µM of peroxivanadate, an inhibitor of tyrosine phosphatase (Fig. 2B).

Effect of tyrosine kinase inhibitor on FAK phosphorylation

We have discussed previously [14] that the potent tyrosine kinase inhibitor genistein drastically inhibits Calu-1 cell proliferation. We treated the Calu-1 cells with genistein (60 µM) for 72 h and determined the tyrosine phosphorylation status of FAK by immunoprecipitation and Western blotting with phosphotyrosine specific monoclonal antibody (4G10) using only the viable cells attached to the plates (~ 30%). As indicated in Fig. 3, treatment of Calu-1 cells with genistein for 3 days inhibited FAK phosphorylation 7.6 fold.

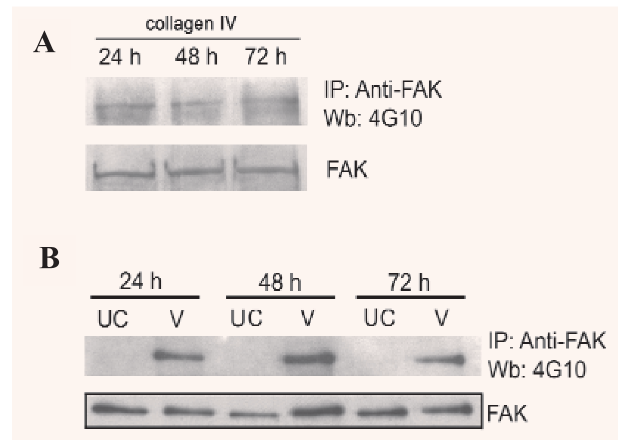

Fig. 2 Phosphorylation of FAK during Calu-1 cell proliferation. A Phosphorylation of FAK on Coll IV coated plates was observed for several days. Cells from each day were collected and lysed at the same time by NP-40 lysis buffer and immunoprecipated with FAK antibody as described in Methods and materials. B FAK tyrosine phosphorylation for several days on uncoated plates as judged by peroxivanadate treatment. Each day the cells were treated with 10 nM peroxivanadate for 10 min, and lysates were made as described in Materials and Methods. UC = Calu-1 cells grown on uncoated plates. V = Calu-1 cells grown on uncoated plates for specified time, then treated with peroxyvanadate for 10 min and lysed. IP, immunoprecipitation; Wb, Western blot.

Activation of MAP kinase after collagen IV adhesion

We investigated the activation of MAP kinase (ERK1/2), a downstream target of FAK in various cell lines, following adhesion of Calu-1 cells to Coll IV for 15, 30, or 60 min, using an ERK1/2 specific antibody. Using MBP as an exogenous ERK's substrate, we followed the same time course of activation as for FAK. It appears that ERK1/2's activity increased 2-fold after Coll IV adhesion and followed the time course of FAK activation with a peak at 30 min. (Fig. 4). As a negative control, we did examine the phosphorylation of MBP using the cells from BSA coated plates, which shows no MAP kinase activation (data not shown).

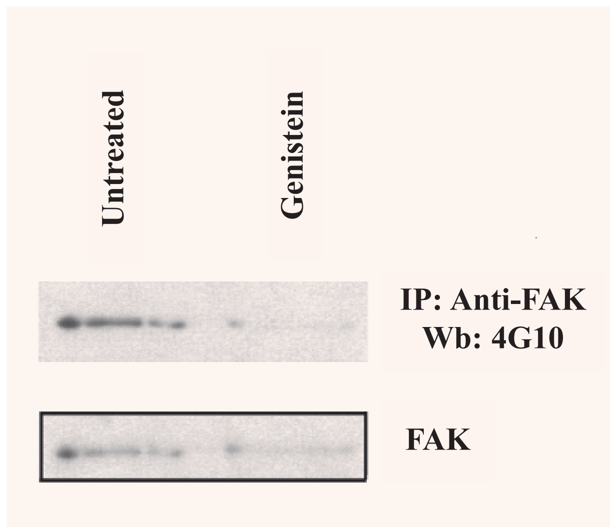

Fig. 3 Effect of genistein, a protein tyrosine kinase inhibitor, on type IV collagen mediated phosphorylation of FAK. Phosphotyrosine blot of FAK from genistein treated (72 h) and untreated cells after immunoprecipitation and Western transfer. Moderate decrease in FAK phosphorylation is seen. IP, immunoprecipitation; Wb, Western blot.

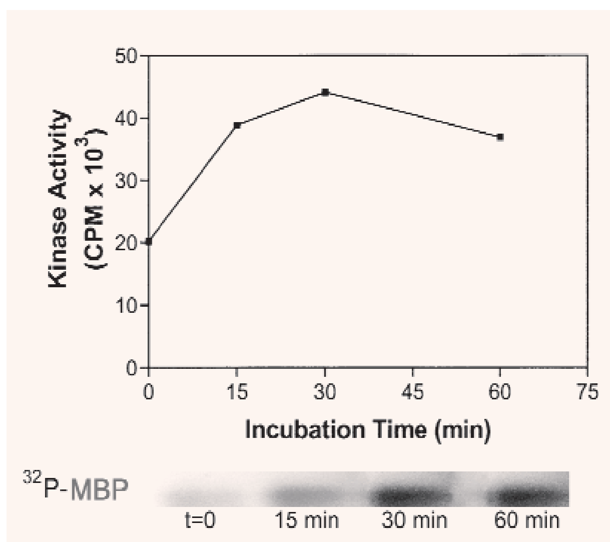

Fig. 4 Activation of MAP kinase. Lysates of Coll IV adherent cells were immunoprecipitated by ERK1 specific antibodies and MBP phosphorylation was assayed by using (γ^{32}p)ATP. The upper panel indicates the fold activation of kinase activity on Coll IV compared to cells on BSA control. The lower panel is the actual autoradiograph of the phosphorylated MBP band after autoradiography. The whole procedures have been described in Materials and Methods in detail.

Effect of cytoskeleton integrity on FAK activation and its downstream signaling

In order to investigate whether the integrity of the actin cytoskeleton is required for integrin dependent FAK activation of Calu-1 cells attached *in vitro* to Coll IV, we used Cytochalasin D, which binds to the barbed ends of actin filaments and prevents actin polymerization. We found that concentrations of Cytochalasin D ranging from 1-5 µM were sufficient to disrupt the adhesion affinity of Calu-1 cells for Coll IV (Fig. 5A). Compared to untreated controls, Cytochalasin D concentrations of 1.0 µM, 2.5 µM, and 5.0 µM lowered the number of Calu-1 cells able to attach to Coll IV by 57% ($p = 1.9 \times 10^{-6}$), 72% ($p = 2.2 \times 10^{-6}$) and 75% ($p = 6.6 \times 10^{-7}$), respectively. Interestingly, this inhibition of adhesion did not change the phosphorylation status of FAK (Fig. 5B), but this treatment disrupted significantly (4.0 to 2.2 fold) the Coll IV stimulated ERK1 and ERK2 phosphorylation in a dose dependent manner (Fig. 5C). Cytochalasin D alone does not have any effect on either FAK or ERK1/2 phosphorylation when added to uncoated plates (data not shown).

The protein kinase C inhibitor, Calphostin C, activates ERK 1/2 signaling but does not block FAK phosphorylation

Earlier studies have documented that the PKC inhibitor Calphostin C results in reduced integrin-dependent FAK phosphorylation [13]. In order to verify the that effect in Calu-1 cells, Calphostin C (100 and 250 nM) was added to suspended Calu-1 cells prior to plating on Coll IV for 30 min. In contrast to the published observations, Calphostin C treatment did not change the Coll IV stimulated FAK phosphorylation in Calu-1 cells (Fig. 6A), but it increased the ERK1 and ERK2 phosphorylation in a dose dependent manner (1.4 to 2 fold from Coll IV stimulation, which is 3.6 fold compared to the phospho ERKs on BSA coated plates, Fig. 6B). In order to determine the effect of Calphostin C alone, we did control on BSA coated plates which shows no change of either FAK or ERK1/2 phosphorylation status (data not shown).

Physical association of β1-integrin with FAK during activation

In our previous studies [14], we have provided evidence that β_1-integrin is essential for Coll IV mediated adhesion of Calu-1 cells which results in FAK phosphorylation. In order to investigate the physical association of FAK with the β_1-integrin network during Coll IV adhesion, we immunoprecipitated FAK from Coll IV coated or BSA coated plates and blotted for β_1-integrin protein. As indicated in Fig. 7, the β_1-integrin is undetectable in FAK immunoprecipitate irrespective of matrix coating, although it is present in the whole cell extract (Fig. 7 lane1). As a positive control of co-immunoprecipitation ability of FAK antibody, we detected paxillin in FAK immunoprecipitate (data not shown).

Discussion

There is accumulating evidence that supports a critical role of FAK in promoting cell migration, proliferation and focal contact remodeling stimulated by different types of cell surface ligand receptors. Activation of FAK following adhesion to Coll IV and integrin engagement has also been reported for many different cell types [15–17]. We have observed phosphorylation and activation of FAK after Calu-1 cell adhesion to Coll IV, which might be important for integrin signaling in this lung cancer squamous cell line.

Richardson *et al*, showed that the Tyr[397] residue of FAK efficiently binds to the SH2 domain of pp60Src and activates its catalytic activity [18]. Binding of FAK to pp60Src allows FAK to have contact with the Ras signaling pathway through the adapter protein Grb2 [19]. In agreement with other reports in different cell lines, we have also found the Tyr[397] phosphorylation of FAK in Calu-1 cells, which can link FAK not only to the Ras signaling pathway, but also to the PI-3 kinase pathway by association with the p85 subunit of PI-3 kinase [20]. A number of signaling molecules known to associate with FAK in focal adhesion such as paxillin [8] and p130Cas [21] represent potential immediate downstream targets for FAK. Although several reports have identified the phosphorylation of p130Cas following Coll IV adhesion in other cell lines, we did not see phosphorylation of this protein. We did see, however, that the phosphorylation of paxillin rose in a time dependent manner after adhesion from 15 to 60 min to Coll IV.

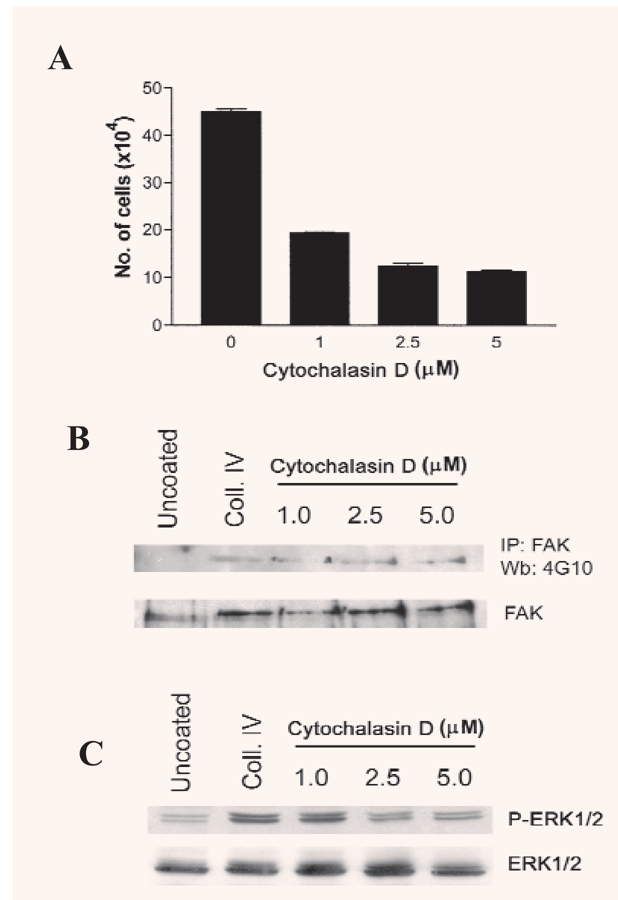

Fig. 5 Effect of Cytochalasin D on cell adhesion, FAK and ERK1/2 phosphorylation. A 0.5×10^6 cells either treated or untreated with Cytochalasin D (1-5 μM), were plated on Coll IV coated plates for 30 min. Plates were washed with cold PBS twice and the attached cells were counted (triplicate). B 600 μg of Cytochalasin D treated or untreated cells from uncoated or Coll IV treated plates were immunoprecipitated with FAK antibody and was blotted with 4G10 and FAK antibody respectively. C Whole cell extracts (50 μg total protein) from Cytochalasin D treated or untreated cells were run on a 10% polyacrylamide gel and was blotted 1st for phospho Erk1/2 antibody followed by ERK1/2 antibody.

The phosphorylation of FAK in Calu-1 cells was undetectable without Coll IV adhesion. In order to

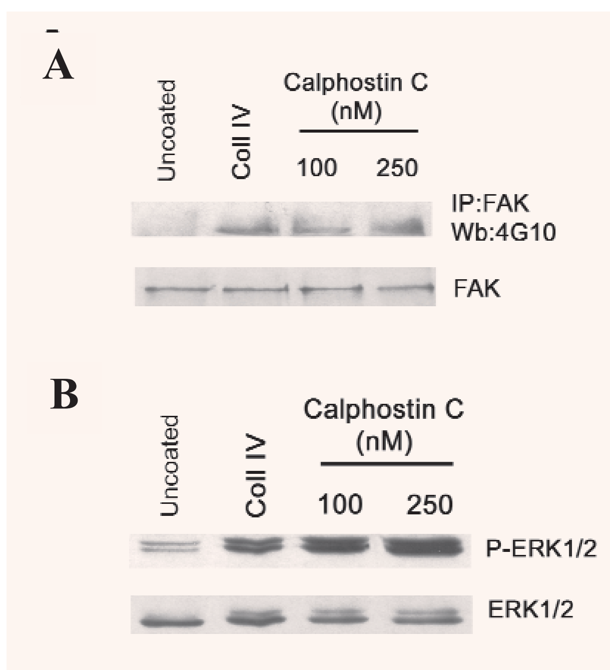

Fig. 6 Effect of Calphostin C on Coll IV induced FAK and ERK1/2 activation. A 600 μg of cell lysates prepared from serum-starved cells from uncoated, Coll IV coated and Calphostin C (100 and 250 nM) treated cells from Coll IV coated plates were immunoprecipitated with FAK antibody and was blotted for 4G10 and FAK antibody respectively. B Whole cell lysates (50 μg total protein) from Calphostin C treated or untreated cells were blotted first with phospho ERK1/2 antibody followed by ERK1/2 antibody after striping.

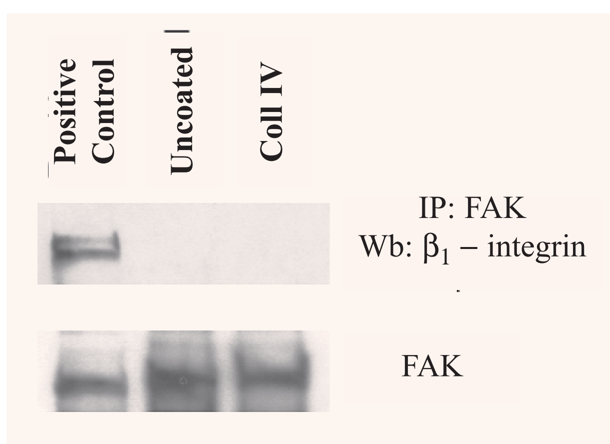

Fig. 7 β1-Integrin is not directly associated with FAK. Whole cell lysates (750 μg protein) from control and Coll IV treated cells were immunoprecipated with FAK antibody and was blotted against β1-integrin and FAK antibody respectively. Positive control indicates the amount of β1-integrin and FAK present in 80 μg of Calu-1 whole cell extracts.

determine whether tyrosine phosphorylation of FAK in Calu-1 cells was a spontaneous event with cell proliferation or causally related to Coll IV binding, we designed two different approaches to uncouple the phosphorylation/dephosphorylation cascade. First, we showed that FAK maintained its phosphorylation status even after 72 h on Coll IV. Probably, adhesion of Calu-1 cells to Coll IV maintained a stable and open structure of FAK which would be accessible to other tyrosine kinases like Src or Src related kinases. However, it was also possible that integrins could stimulate the activity of a protein-tyrosine phosphatase leading to the dephosphorylation of C-terminal Src family tyrosine residues and resulting in the activation of Src family protein tyrosine kinases. Our second approach through peroxivanadate treatment was designed to understand the status of *in vivo* phosphorylation of FAK in Calu-1 cells. As phosphorylation of FAK is undetectable without blocking the dephosphorylation, it is clear that a phosphorylation/dephosphorylation event was going on in Calu-1 cells which possibly through the interaction with Coll IV.

The tyrosine phosphorylation of FAK was suppressed by genistein, a potent inhibitor of several tyrosine kinases including c-Src. It is possible that genistein may inhibit the activities of PTKs, which are active in the upstream of the FAK signal transduction pathway. Considering the effect of peroxivanadate and genistein on FAK phosphorylation, we can speculate that the induction of FAK phosphorylation in Calu-1 cells may proceed primarily through the inhibition of a specific protein tyrosine phosphatase. For instance, a dual-specific phosphatase, PTEN, has been shown to be associated with FAK and capable of FAK dephosphorylation [22]. It would be interesting to determine whether PTEN is associated with FAK in Calu-1 cells after Coll IV induction.

It has been reported in many cell lines including fibroblasts [10, 23] and human salivary gland cells [24] that integrin dependent activation of MAP kinases requires focal adhesion kinase. Collagen type IV dependent ERK1/2 activation in Caco-2 intestinal epithelial cells [4] also requires FAK. MAP kinase has been demonstrated to regulate cell migration through the enhancement of myosin light chain phosphorylation [25]. Thus, the requirement for FAK activity in the efficient

activation of MAP kinase could also be related to the effects of FAK on cell motility, as demonstrated in previous studies [26, 27].

Some controversy exists in the literature over the mechanisms by which ERK1/2 gets activated after integrin dependent adhesion and the role of FAK in this event. It is by no means clear that the same mechanisms would be operational for different cell types or different matrix proteins. Our observation that adhesion of Calu-1 cells to Coll IV activates ERK1/2 is not necessarily predictable from the previous literature. Comparison of the time course over which FAK and ERK1/2 are activated in response to adhesion of Calu-1 cells to Coll IV reveals potentially important similarities and differences between FAK and ERK1/2 activation. Both signals are rapidly stimulated by 15 min after plating. However, the activation profile of ERK1/2 and FAK did not precisely correlate, since ERK1/2 activation began to decline after 30 min while FAK activation remained constant more than 60 min after adhesion.

Integrin mediated signaling to ERK1/2 is dependent on the integrity of the actin cytoskeleton [11, 12]. In fibroblast cells, the disruption of cytoskeleton integrity completely inhibited fibronectin stimulated FAK tyrosine phosphorylation and ERK1/2 signaling. In contrast to that observation, treatment of Calu-1 cells with Cytochalasin D did not prevent the Coll IV dependent FAK phosphorylation in this cell line, although it significantly inhibited the adhesion process and disrupted the Coll IV induced ERK1/2 activation in a dose dependent manner. Therefore, in Calu-1 cells, the integrity of the cytoskeleton structure is required for ECM generated signals to ERK1/2.

Several reports indicate a role of protein kinase C (PKC) in the integrin dependent activation of FAK and other PTKs associated with focal adhesion [13, 28]. Here we have shown that the downregulation of PKC activity by Calphostin C treatment of Coll IV attached Calu-1 cells induced the activation of ERK1/2 without any effect on FAK phosphorylation. Although the use of pharmacological inhibitors is an indirect means for determining protein function, the fact that Calphostin C treatment did not change FAK phosphorylation suggests that the Coll IV induced ERK1/2 activation does not depend totally on FAK phosphorylation in Calu-1 cells. It would be interesting to test whether the induction of ERK1/2 activity through some other molecules in the Ras signaling pathway is independent of FAK and whether this activation is dependent upon the integrity of the actin cytoskeleton.

In our previous report [14], we have demonstrated that the adhesion of Calu-1 cells to Coll IV is dependent on the presence of β_1-integrin. In this work, we explored whether β_1-integrin is physically associated with FAK or the activation of β_1-integrin mediated signaling is sufficient to activate FAK in Calu-1 cells. Our data indicates clearly that the Coll IV induced phosphorylation and activation of FAK does not require physical association of FAK with β_1-integrin in this cell line.

A number of different signaling pathways have been proposed to mediate Coll IV dependent and β_1-integrin mediated FAK and ERKs activation in different cells. Data in our previous report [14] and the kinetic and pharmacologic studies with different inhibitors in Calu-1 cells show that the adhesion of these cells on Coll IV matrix and its subsequent intracellular signaling depends on a delicate balance of protein tyrosine phosphorylation and β_1-integrin engagement (Fig. 8). The outcome of this signaling indicates that phosphorylation of FAK and ERKs probably follow two independent pathways, or at least is not exclusively dependent on FAK phosphorylation. Cytoskeleton integrity is required for adhesion and signaling to ERKs without any influences on FAK, which also indicates that β_1-integrin mediated FAK phosphorylation in Calu-1 cells is an upstream event compared to ERKs phosphorylation. Protein Kinase C is working upstream from ERKs, but its inhibition did not influence FAK activation, indicating that either FAK is upstream of PKC or PKC follows an independent pathway. It is possible that Integrin engagement brings down Src kinase with FAK and then Src can influence the Grb-2 mediated Ras and ERKs activation which probably partly depends on PKC mediated signaling for its full activation. In summary, our data indicates that multiple and potentially parallel Coll IV and β_1-integrin mediated signaling exists in Calu-1 cells. Therefore, any potential intervention to disrupt the binding and migration advantage of Calu-1

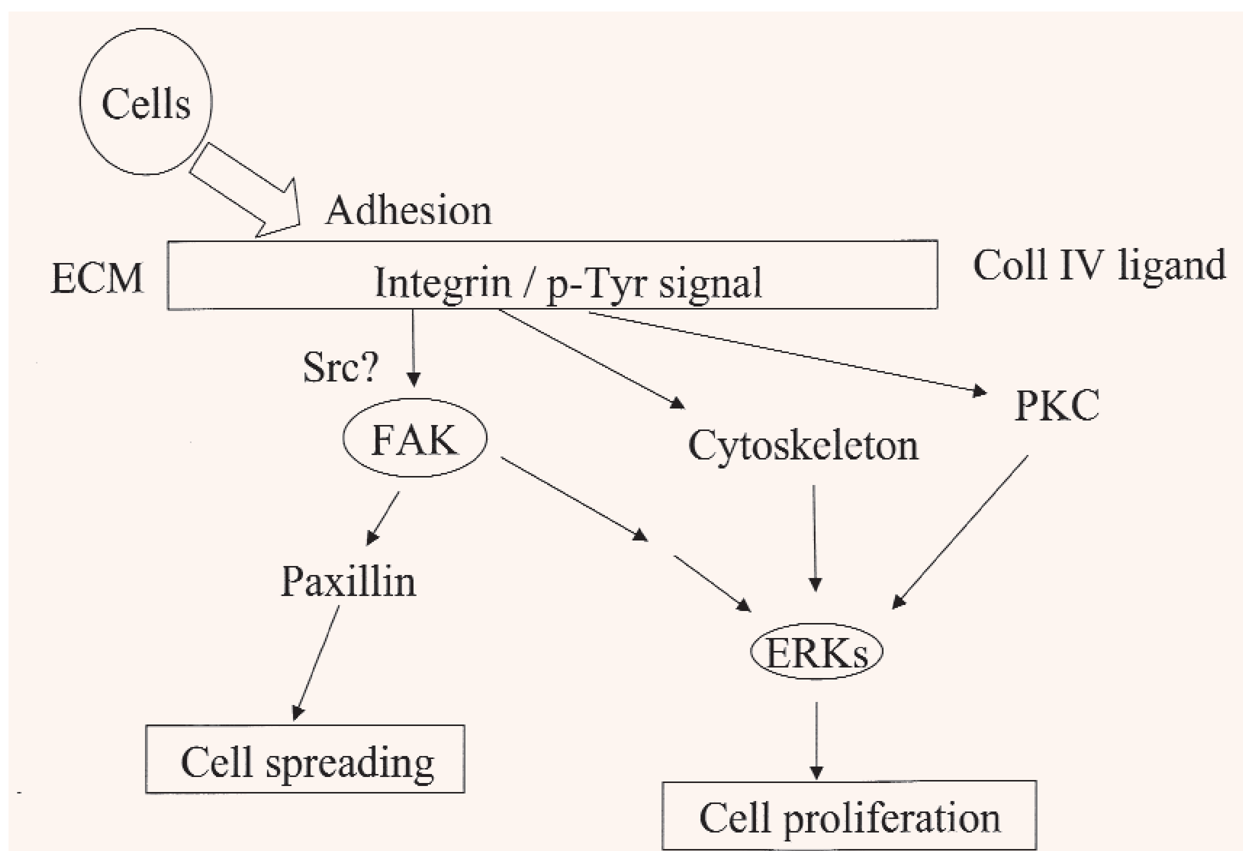

Fig. 8 βA simplified model of β1-integrin signaling to FAK and ERKs in Calu-1 cells. Adhesion of Calu-1 cells to Coll IV results in β1-integrin dependent activation of FAK, ERKs, and PKC. This activation is apparently independent of each other or at least not exclusively dependent on FAK activation.

cells to Coll IV matrix should be aimed at the β_1-integrin, since this is the last identified single pathway before multiple signal pathways develop.

Acknowledgements

The authors would like to thank Dr. Gautam Maulik for critical reading, Debra H Cole and Amy YM Wong for secretarial support, Anastasia N Pappas for statistical support.

References

1. **Liotta LA, Stetler-Stevenson WG**. Tumor invasion and metastasis: an imbalance of positive and negative regulation. *Cancer Res.* 1991; 51: 5054–9.
2. **Furuta SM, Ilic D, Kanazawa S, Takeda N, Yamamto T, Aizawa S.** Mesodermal defect in late phase of gastrulation by a targeted mutation of focal adhesion kinase FAK. *Oncogene.*1995; 11: 1989–95.
3. **Guan J-L, Shalloway D**. Regulation of pp125FAK both by cellular adhesion and by oncogenic transformation. *Nature.* 1992; 358: 690–2.
4. **Sanders MA, Basson MD.** Collagen IV-dependent ERK activation in human Caco-2 intestinal epithelial cells requires focal adhesion kinase. *J Biol Chem.* 2000; 275: 38040–7.
5. **Akasaka T, van Leeuwen RL, Yoshinaga IG, Mihm MC, Byers HH.** Focal adhesion kinase (p125FAK) expression correlates with motility of human melanoma cell lines. *J Invest Darmatol.* 1995; 105: 104–8.
6. **Weiner TM, Liu ET, Craven RJ, Cance WG.** Expression of focal adhesion kinase gene and invasive cancer. *Lancet* 1993; 342: 1024–5.
7. **Owens LV, Xu L, Craven RG, Dent GA, Weiner TM, Kornberg L, Liu E, Cance WG.** Overexpression of the focal adhesion kinase (p125FAK) in invasive human tumors. *Cancer Res.* 1995; 55: 2752–5.
8. **Richardson A, Malik RK, Hildebrand JD, Parsons JT.** Inhibition of cell spreading by expression of the C-terminal domain of focal adhesion kinase (FAK) is rescued by coexpres-

sion of Src or catalytically inactive FAK: a role for paxillin tyrosine phosphorylation. *Mol Cell Biol.* 1997; 17: 6906–14.

9. **Schlaepfer DD, Hunter T.** FAK overexpression enhances Ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. *J Biol Chem.* 1997; 272: 13189–95.
10. **Schalaepfer DD, Jones KC**, **Hunter T.** Multiple Grb2-mediated integrin stimulated signaling pathways to ERK2/mitogen-activated protein kinase: Summation of both c-Src and focal adhesion kinase-initiated tyrosine phosphorylation events. *Mol Cell Biol.* 1998; 18: 1–32.
11. **Miyamoto S, Teramoto H, Coso OA.** Integrin function: molecular hierarchies of cytoskeletal and signaling molecules. *J Cell Biol.* 1995; 131: 791–05.
12. **Clark EA, Hynes RO.** Ras activation is necessary for integrin-mediated activation of extracellular signal regulated kinase 2 and cytosolic phospholipase A2 but not for cytoskeletal organization. *J Biol Chem.* 1996; 271: 14814–8.
13. **Lewis JM, Cheresh DA, Schwartz MA.** Protein kinase C regulates $\alpha_v\beta_5$-dependent cytoskeletal associations and focal adhesion kinase phosphorylation. *J Cell Biol.* 1996; 134: 1323–32.
14. **Mukhopadhay NK, Gillchrist D, Gordon JG, Cheng CJ, Bueno R, Lu ML, Salgia R, Sugarbaker DJ, Jaklitsch MT.** Integrin dependent tyrosine phosphorylation is a key regulatory event in collagen IV mediated adhesion and proliferation of human lung cancer cell line. *Calu-1. Ann Thorac Surg.* 2004; 78: 450–7.
15. **Sakamoto M, Ino Y, Ochiai A, Kanai Y, Akimoto S, Hirohashi S.** Formation of focal adhesion and spreading of polarized human colon cancer cells in association with tyrosine phosphorylation of paxillin in response to phorbol ester. *Lab Invest.* 1996; 74: 199–08.
16. **Miles AJ, Skubitz AP, Furcht LT, Fields GB**. Promotion of cell adhesion by single-stranded and triple-helical peptide models of basement membrane collagen alpha 1(IV) 531-543. Evidence for conformationally dependent and conformationally independent type IV collagen cell adhesion sites. *J Biol Chem.* 1994; 269: 30939–45.
17. **Schaller MD, Hildebrand JD, Shannon JD, Fox JW, Vines RR, Parsons JT.** Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol.* 1994; 14: 1680–8.
18. **Richardson A, Malik RK, Hildebrand JD, Parsons JT.** Inhibition of cell spreading by expression of the C-terminal domain of focal adhesion kinase (FAK) is rescued by coexpression of Src or catalytically inactive FAK: a role for paxillin tyrosine phosphorylation. *Mol Cell Biol.* 1997; 17: 6906-14.
19. **Schlaepfer DD, Hanks SK, Hunter T.** Integrin-mediated signal transduction linked to Ras pathway by Grb2 binding to focal adhesion kinase. *Nature.* 1994; 372: 786–91.
20. **Chen HC, Guan JL.** Association of focal adhesion kinase with its potential substrate phosphatidylinositol 3-kinase. *Proc Natl Acad Sci USA.* 1994; 91: 10148–52.
21. **Polte ZTR, Hanks SK.** Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130cas. *Proc Natl Acad Sci USA.* 1995; 92: 10678–82.
22. **Tamura M, Gu J, Tran H, Yamada KM.** PTEN gene and integrin signaling in cancer. *J Natl Cancer Inst.* 1999; 91: 1820–8.
23. **Renshaw MW, Price LS, Schwartz MA.** Focal adhesion kinase mediates the integrin signaling requirement for growth factor activation of MAP kinase. *J Cell Biol.* 1999; 147: 611–8.
24. **Zhang L, Bewick M, Lafrenie RM**. Role of Raf-1 and FAK in cell density dependent regulation of integrin-dependent activation of MAP kinase. *Carcinogenesis.* 2002; 23: 1251–8.
25. **Klemke RL, Cai S, Giannini AL, Gallagher PJ, de Lanerolle P, Cheresh DA.** Regulation of cell motility by mitogen-activated protein kinase. *J Cell Biol.* 1997; 137: 481–92.
26. **Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsji N, Nomur S, Fujimoto J, Okada M, Yamamoto T.** Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* 1995; 377: 539–44.
27. **Gilmore AP, Romer LH.** Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation. *Mol Biol Cell.* 1996; 7: 1209–24.
28. **Vouri K, Ruoslanti E.** Actvation of protein Kinase C proceeds $\alpha5\beta1$ integrin-mediated cell spreading on fibronectin. *J Biol. Chem.* 1993; 268: 459–62.