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Modulation of Protein Tyrosine Phosphorylation by the Extracellular Matrix[1]

Siobhan A. Corbett, M.D.,*,†,[2] and Jean E. Schwarzbauer, Ph.D.†

*Department of Surgery, UMDNJ–Robert Wood Johnson Medical School, New Brunswick, New Jersey 08903; and †Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544

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Fibronectin (FN) cross-linked to fibrin following injury provides the provisional matrix required for cells to begin tissue repair. Our previous work has demonstrated that fibroblasts adherent to multimeric FN within the context of a fibrin matrix (FN–fibrin) exhibit clear phenotypic differences from those adherent to a dimeric FN-coated surface. We hypothesize that this response to multimeric FN may be mediated by altered protein tyrosine phosphatase activity following integrin activation. Methods: NIH 3T3 cells were plated in the presence or absence of pervanadate (PV), a phosphotyrosine phosphatase inhibitor, on wells coated with FN or FN–fibrin matrix. Spread cell areas were measured after increasing incubation times and are recorded as mean cell area (mm^2) ± SEM. Alternatively, cells were lysed and equal amounts of protein were analyzed by immunoblot using a monoclonal antibody specific for phosphotyrosine. Results: PV significantly inhibited cell spreading on FN–fibrin matrices. In contrast, PV treatment had little effect on cell area on FN alone. Analysis of cell lysates revealed that protein tyrosine phosphorylation events differ in a substrate-dependent manner. Conclusion: Cell attachment to a FN–fibrin matrix induces distinct cell shape and cytoskeletal organization. Inactivation of tyrosine-specific phosphatases enhances this distinction and inhibits the spreading of cells attached to this substrate. The phosphotyrosyl protein content of treated cells on FN–fibrin matrix is also diminished. These results suggest that cell–extracellular matrix interactions affect the tyrosine phosphorylation balance of the cell, thus modifying cytoskeletal organization and related signaling events.

INTRODUCTION

The cellular response to injury is mediated by complex interactions between cells and the extracellular matrix (ECM). Following injury, the extravasation of plasma proteins leads to the formation of a blood clot that is vital for hemostasis and the restoration of vascular integrity [1, 2]. Additionally, the clot forms a provisional ECM which serves as a substrate for cell attachment and migration into the wound, events which are essential to the tissue repair process [3].

The clot's major protein components, fibrin and plasma fibronectin (FN), form a fibrillar matrix that is stabilized by activated Factor XIII [4, 5]. Plasma FN is covalently bound to fibrin in this process, forming high-molecular-weight multimers that markedly enhance cell attachment and spreading to the fibrin matrix [6]. Further, previous work from this laboratory has determined that the FN multimer found in clots is a distinct substrate for cells when compared to a dimeric FN-coated surface [7]. Using a synthetic matrix of cross-linked FN and fibrin as an *in vitro* substrate for cell–matrix interaction, we have demonstrated that cells attached to a FN–fibrin matrix are smaller and display characteristic differences in the organization of their actin cytoskeleton. These results suggest that variation in the molecular form of FN as determined by its mechanism of immobilization can modify receptor-mediated signaling events [7].

Integrins are members of a large family of heterodimeric membrane glycoproteins which mediate cell attachment to the ECM [8, 9]. Integrin–ECM interactions establish a physical link between the cells and the ECM, allowing clustering of the receptors, cytoskeletal reorganization, and the formation of large complexes of signaling and cytoskeletal proteins, termed focal adhesions [10, 11]. Recent evidence suggests that the phosphorylation of focal adhesion proteins plays a critical role in regulating cell shape [12–14]. Thus, a mechanism for regulating the tyrosine phosphorylation of proteins at these sites must exist, involving not only protein tyrosine kinases but also protein tyrosine phosphatases (PTP). While the role of protein tyrosine kinases following integrin engagement has been well described, the role of PTP is less clear. However, their structural features and association with signaling complexes suggest that they are an essential component of signal transduction cascades [15, 16].

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[2] To whom correspondence should be addressed at 301 Schultz Laboratory, Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544. Fax: (609) 258-1035.

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We have used a synthetic FN–fibrin clot to study cell interactions with matrix-immobilized FN [7]. As mentioned above, cells attached to multimeric FN display significant differences in cytoskeletal organization when compared to cells on dimeric FN-coated plastic. To determine whether PTP plays a role in mediating these differences, we treated NIH 3T3 cells attached to both substrates with the potent PTP inhibitor pervanadate. Pervanadate treatment induced distinct morphologic features in these cells in an ECM-dependent fashion. Further, examination of total cell lysates from cells on FN and FN–fibrin matrix demonstrates a difference in the intensity of the phosphotyrosyl protein pattern between the two. These results suggest that the variability in cell shape and protein tyrosine phosphorylation may reflect altered receptor-mediated signaling as determined by the molecular form of FN. Therefore, this provides evidence that PTP activity can be modified by the ECM.

MATERIALS AND METHODS

Preparation of ligand-coated dishes. Rat pFN was purified from fresh rat plasma by gelatin–agarose affinity chromatography [17]. Concentrations of FN were determined by optical density at 280 nm [ϵ = 1.3 (0.1%)]. Protein purity was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and silver staining [18]. pFNs were dialyzed into cyclohexylaminopropanesulfonic acid (Caps)-buffered saline (10 m*M* Caps, 150 m*M* NaCl, pH 11.0) and stored at −80°C. Lyophilized human fibrinogen (98.8% clottable) and bovine thrombin were purchased from American Diagnostica, Inc. (Greenwich, CT) and Sigma Chemical Co. (St. Louis, MO), respectively, and reconstituted according to the manufacturer's directions. To form a pFN–fibrin matrix, clotting components were mixed in volumes of 0.25 to 1.0 ml to give the following concentrations: 2.4 mg/ml fibrinogen, 240 μg/ml pFN, 10 m*M* $CaCl_2$, 10 m*M* Tris–HCl (pH 7.4), 150 m*M* NaCl, thus maintaining a physiologic ratio of fibrinogen to pFN of 10:1. With the addition of thrombin (1 U/ml), the clotting reaction was initiated and the components were quickly transferred to either a 48-well or a 35-mm nontissue culture dish where clot formation was allowed to proceed. Clots were incubated overnight at 4°C to maximize cross-linking. The clots were then carefully removed from the dish with forceps, leaving a pFN–fibrin matrix for use in biologic assays [7]. For pFN alone, a 10 μg/ml solution of rat pFN in Caps buffer was allowed to adsorb to nontissue culture dishes overnight at 4°C. Dishes coated with either pFN or pFN–fibrin matrix were then washed three times with phosphate-buffered saline (PBS) and blocked with 20 mg/ml bovine serum albumin in PBS (heat-denatured at 80°C for 10 min) for 1 hr at 37°C. The dishes were then washed three times with PBS and once with serum-free Dulbecco's modified Eagle's medium (DMEM) prior to use.

Cell spreading assay. Mouse NIH 3T3 fibroblasts were cultured in DMEM supplemented with 10% calf serum (Hyclone Laboratories, Logan, UT). For cell spreading assays and for the preparation of total cell lysates, cells were grown to confluence on 10-cm dishes, washed once with PBS, and released from the dishes by the addition of 2.5 ml TPCK–trypsin (Sigma) at a concentration of 0.1 mg/ml in Versene. The trypsin was stopped by the addition of an equal volume of 0.5 mg/ml soybean trypsin inhibitor in PBS. Cells were then recovered by centrifugation for 5 min and were resuspended in soybean trypsin inhibitor for two additional washes, with the final resuspension in serum-free DMEM.

To determine the cell area, 4×10^4 cells were applied to substrates and allowed to attach for increasing time periods. At designated intervals, attached and spread cells were examined using inverted phase-contrast optics. Where indicated, attached cells were treated after 5 min with 50 μM sodium orthovanadate (Sigma) and 50 μM H_2O_2, yielding vanadyl hydroperoxide (pervanadate) (20). Photographic images were then captured using videoimaging as previously described [7]. Cell area was measured using NIH Image software. Experiments were repeated in triplicate with 50 to 70 measurements per time point for each experiment. Data are reported as the mean area at specific time points ± standard error of the mean (SEM). Statistical analysis of the data at each time point was performed using a Student's unpaired *t* test.

Preparation of cell lysates. Total cell lysates were prepared as previously described [7]. Briefly, equal numbers of cells were plated in 35-mm dishes coated with either pFN or pFN–fibrin matrix. Cells were allowed to attach for specific times as indicated and were either treated or untreated with pervanadate as noted above. At specific intervals the cells were washed once with PBS and lysed on ice with 100 μl of lysis buffer [150 m*M* NaCl, 50 m*M* Tris–HCl, pH 7.5, 1% NP-40, 0.25% deoxycholate plus 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 1 m*M* sodium orthovanadate, 2 m*M* EDTA, 50 μg/ml leupeptin, 0.5% aprotinin]. The cells were scraped with a rubber policeman. The lysate was collected and centrifuged in the cold at 14,000 rpm. The nuclear pellet was discarded, and the supernatant was stored at −80°C. Protein concentrations were determined for each sample by using the Pierce BCA protein assay (Pierce Chemical Co., Rockford, IL). Equal amounts of protein were separated by size using SDS–polyacrylamide minigels and transferred to nitrocellulose (Sartorius Corp., Bohemia, NY) using the transfer apparatus (Mini-Protean II, Bio-Rad, Hercules, CA) as suggested by the manufacturer. Filters were blocked with Buffer A (25 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 0.1% Tween 20) overnight at room temperature. Intracellular protein tyrosine phosphorylation was detected with a monoclonal antibody against phosphotyrosine, PY-20 (Transduction Laboratories, Lexington, KY), at 1:2000 dilution in Buffer A. PY-20 was incubated with the filter for 1 hr. The filter was then washed three times with Buffer A. Biotinylated goat anti-mouse IgG (Life Technologies/Gibco-BRL, Grand Island, NY) was added at 1:10,000 dilution in Buffer A. After a 1-hr incubation and three additional washes with Buffer A, a 1:10,000 dilution of streptavidin–horseradish peroxidase (Life Technologies/Gibco-BRL) in Buffer A was added for 30 min and washed. Immunoblots were then developed with chemiluminescent reagents (Dupont NEN, Boston, MA) according to the manufacturer's instructions and exposed to film (X-Omat, Eastman Kodak Co., Rochester, NY).

RESULTS

Pervanadate Inhibits Cell Spreading on a FN–Fibrin Matrix

Previous work from this laboratory has determined that cells attached to a synthetic FN–fibrin matrix exhibit clear phenotypic differences from those attached to a dimeric FN-coated surface [7]. To determine the role of PTP in the cellular response to this FN–fibrin provisional matrix, NIH 3T3 cells were plated in serum-free DMEM on wells coated with adhesive substrates as described above. Cells were then allowed to attach and spread at 37°C. Where indicated, 50 μM sodium orthovanadate and 50 μM hydrogen peroxide were added to the culture medium after 5 min, yielding vanadyl hydroperoxide (pervanadate), a potent PTP inhibitor. Cells incubated on FN in the presence of pervanadate rapidly assumed a distinct morphology characterized by extreme flattening and a very round shape when compared to the stellate cells seen without pervanadate treatment (Figs. 1A and 1C). The percentage of cells exhibiting this spread morphology on FN de-

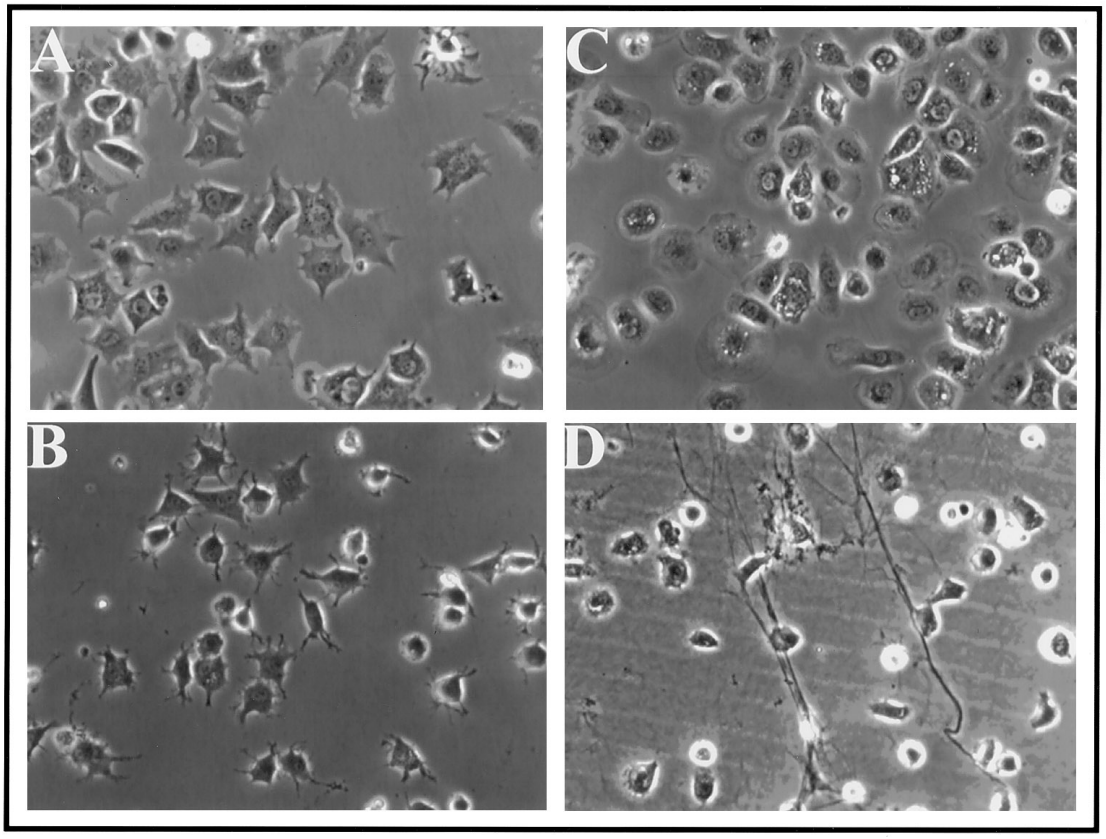

FIG. 1. Pervanadate inhibits spreading on a FN–fibrin matrix. NIH 3T3 cells in serum-free DMEM were allowed to attach and spread on either FN (A and C) or a FN–fibrin matrix (B and D) with either no treatment (A and B) or 50 μM H_2O_2 and 50 μM sodium orthovanadate (C and D). Cells were examined using inverted phase-contrast optics after 1 hr at 37°C.

creased as the dose of pervanadate increased and over 100 μM, the pervanadate quickly became toxic to the cells (data not shown). In contrast, cells attached to a FN–fibrin matrix remained compact (Fig. 1B) and appeared to spread less when pervanadate was added (Fig. 1D).

To determine whether these observed differences corresponded to a measurable change in cell area, NIH 3T3 cells were plated on either FN or FN–fibrin matrix and allowed to attach for increasing time intervals. Spread cell images were then captured at specific time points and cell areas were calculated using NIH Image software. On dimeric FN, pervanadate-treated cells displayed a modest increase in cell area at 15 and 30 min (Fig. 2A). In contrast, pervanadate treatment significantly inhibited cell spreading in cells adherent to FN–fibrin matrix (Fig. 2B). Mean cell areas were 359 and 376 μm^2 at 30 and 60 min, respectively, compared to 498 and 530 μm^2 in untreated cells. This demonstrates that pervanadate treatment enhanced the distinct morphological features observed in NIH 3T3 cells adherent to FN–fibrin matrix and suggests that this phenotype does not result simply from inadequate tyrosine phosphorylation events.

Protein Tyrosine Phosphorylation Is Increased on FN Compared to a FN–Fibrin Matrix

To determine whether pervanadate treatment results in an ECM-dependent difference in phosphotyrosyl proteins, NIH 3T3 cells were plated on FN, FN–fibrin matrix, or poly-L-lysine. At designated time points, cells were lysed and the phosphotyrosine patterns were compared by SDS–PAGE and immunoblotting using a monoclonal antibody against phosphotyrosine. The phosphotyrosine pattern in untreated cells was similar between FN and FN–fibrin (Fig. 3A). The predominant tyrosine phosphorylated protein was detected at 120–130 kDa, probably corresponding to $pp125^{FAK}$ (focal adhesion kinase). As expected, there was little protein tyrosine phosphorylation in untreated cells adherent to poly-L-lysine, a nonspecific substrate for cell attachment. When pervanadate-treated cell lysates from different substrates were compared (Fig. 3B), a change in the intensity of the phosphotyrosyl proteins was detected. The increase in phosphorylation on poly-L-lysine was marked but occurred gradually, with the highest level detected at 60 min. On both FN and FN–fibrin matrix tyrosine phosphorylation appeared rapidly. It was easily detected at 15 min, peaked at 30 min, and then began to diminish at 60 min, suggesting that some dephosphorylation events occurred during this time. Further, there was an increase in intensity noted at all time points on FN when compared to the FN–fibrin matrix. Although the overall patterns of tyrosine phosphorylation between substrates are similar, bands detected in the 30- to 50-kDa range are particularly distinct (Fig. 3C). These

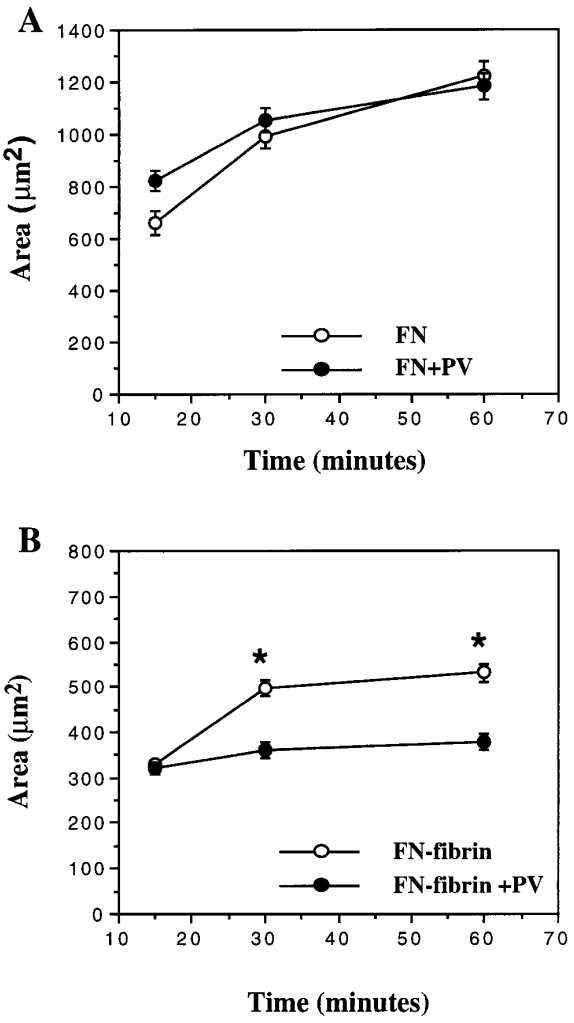

FIG. 2. Effect of phosphatase inhibition is substrate dependent. NIH 3T3 cells in serum-free DMEM were allowed to attach and spread on either FN (A) or a FN–fibrin matrix (B) with either no treatment (open circles) or 50 μM H_2O_2 and 50 μM sodium orthovanadate (solid circles). Cell areas for 50–70 cells from random fields were measured at each time point using NIH Image software and were recorded as the mean cell area (μm^2) + standard error of the mean (SEM). Each point represents the average of three triplicate experiments. Asterisks indicate time points with statistically significant differences as measured by Student's unpaired *t* test.

results suggest that the differences in cell morphology observed on FN and FN–fibrin matrix with pervanadate treatment may correspond to differences in intracellular phosphotyrosyl proteins. Further, it provides evidence that the activity of PTP may be modulated by the ECM.

DISCUSSION

In this study , we have used the PTP inhibitor pervanadate to dissect the signals transduced by cell adhesion to a FN–fibrin matrix. FN within the clot is covalently bound to fibrin by Factor XIIIa as the fibrin polymerizes, forming high-molecular-weight multimers. Our previous work had demonstrated that cells attached to this multimeric form of FN displayed distinct cellular morphology and cytoskeletal organization when compared with cells attached to dimeric FN-coated plastic [7]. These data suggested that the form of FN, i.e., multimer or dimer, could determine the pattern of organization of the cytoskeleton by providing unique sites for integrin-mediated cell attachment. Here, we have demonstrated that pervanadate inhibits the spreading of cells attached to multimeric FN by almost 30%. This difference in cell area corresponds to a distinct difference in cell morphology. Further, intracellular protein tyrosine phosphorylation levels also vary between substrates as detected by immunoblotting with an anti-phosphotyrosine monoclonal antibody. Intensity of phosphotyrosyl protein bands is greater on FN than on FN–fibrin matrix at all time points, suggesting that this increase in phosphoryla-

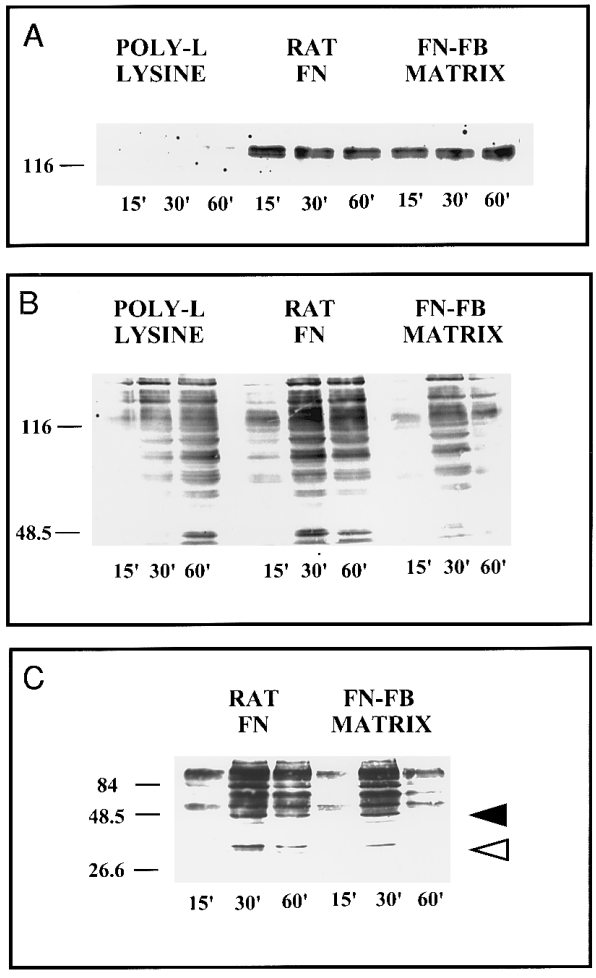

FIG. 3. Protein tyrosine phosphorylation is increased on FN compared to a FN–fibrin matrix. NIH 3T3 cells in serum-free DMEM were allowed to attach and spread at 37°C for 30 min on poly-L-lysine, FN, or FN–fibrin matrix with either no treatment (A) or 50 μM H_2O_2 and 50 μM sodium orthovanadate (B and C). Equal amounts of proteins from cell lysates were separated on either 7% (A and B) or 10% (C) polyacrylamide–SDS gels. Lysates were analyzed for phosphotyrosyl-containing proteins by immunoblot using monoclonal antibody PY-20 developed with chemiluminescence reagents. Arrowheads indicate proteins with molecular weights of approximately 45 kDa (solid) and 30 kDa (open) which appear differentially phosphorylated.

tion correlates with cell shape changes. Together, these data indicate that PTP activity may be differentially modulated by the components of the ECM.

Integrins are the principle family of cell surface receptors that transduce signals from the ECM, allowing cells to better interpret environmental cues [8, 9]. Integrin-mediated signaling depends on a hierarchy of events that include receptor occupancy and clustering, cytoskeletal rearrangement, and tyrosine phosphorylation [10]. Separation of these receptor occupancy and aggregation events results in a different pattern not only of tyrosine phosphorylation, but also of cytoskeletal protein recruitment [19]. This suggests that the spatial organization of the ECM may regulate transmembrane signaling by differential recruitment of integrin receptors. Thus, the multimeric form of FN found within the fibrin clot may present a polyvalent ligand distinct from dimeric FN so that the occupancy and aggregation of the cell surface receptors are modified. As a direct result, signaling complexes and tyrosine phosphorylation events dependent on integrin–cytoskeleton interactions may also be modified.

Several lines of evidence suggest that there is a correlation between the level of phosphorylated proteins and cell adhesion [19–21]. For example, hyperphosphorylation of focal contact proteins in Rous sarcoma virus-transformed cells leads to decreased adhesivity and changes in morphology typical of tumor cells [21]. In contrast, treatment of cells with herbimycin, a protein tyrosine kinase inhibitor, blocks the events required for focal adhesion assembly and inhibits cell spreading [14]. Finally, PTP inhibition in Madin–Darby canine kidney cells results in a change in cell morphology with deterioration of F-actin-containing bundles [20]. Clearly, the phosphotyrosyl levels of focal adhesion proteins are critical to many aspects of cell function. Protein tyrosine kinase regulation of focal adhesion component function has been well described [12, 22]. In particular, $pp125^{FAK}$ is phosphorylated and recruited to focal adhesions as an early step in integrin signaling [23, 24]. Our results identified a protein of a similar molecular weight to FAK which is strongly phosphorylated in adherent untreated cells. The pattern of phosphorylation was similar between substrates and corresponds to previous reports [24]. However, in treated cells this protein is phosphorylated to a greater extent on dimeric FN when compared to FN–fibrin matrix, suggesting an altered PTP activity that is substrate dependent.

The role of PTP in integrin-mediated cell adhesion events has not been well described. PTPs are a structurally diverse family of enzymes composed of both membrane-spanning and intracellular proteins. Previous work has determined a direct role for PTP in both positive and negative regulation of signaling events [15, 16]. For example, Syp (SH-PTP2, PTP1D, PTP2C), an intracellular PTP with wide tissue distribution, appears to be a positive transducer of mitogenic signals via its association with the platelet-derived growth factor and epidermal growth factor receptors [25, 26]. In contrast, the hematopoietic cell PTP HCP (SH-PTP1, PTP1C) plays an essential role in hematopoiesis and immune defense by negatively regulating both B-cell and cytokine receptor-mediated signaling [27, 28]. More recently, the transmembrane PTP LAR and a LAR-interacting protein were found to colocalize at focal adhesions [29, 30]. These proteins were identified in regions of the complex undergoing disassembly, suggesting that they regulate focal adhesion size by acting on available phosphorylated substrates. PTP activity has also been found to be associated with the cytoskeleton in thrombin-stimulated platelets, where it may be regulated by the integrin $\alpha_{IIb}\beta_3$ [31, 32]. These results support the idea that PTP can localize to focal adhesions where they may play a role in modulating cell attachment and spreading. We have demonstrated that pervanadate-treated fibroblasts demonstrate substrate-specific changes in cell morphology which appear to correlate with differences in intracellular protein tyrosine phosphorylation. This suggests that there may be a variability in PTP activity which is linked to cell–ECM interactions [33], presumably via integrin receptor recruitment mediated by the ECM.

In summary, our data demonstrate that the multimeric form of FN typical of the provisional wound ECM induces distinct cell shape and cytoskeletal organization. This distinction is enhanced by inactivation of tyrosine-specific phosphatases which inhibits the spreading of cells attached to a FN–fibrin matrix. These results suggest that cell–ECM interactions can modulate the tyrosine phosphorylation balance of the cell and direct the organization of the cytoskeleton and related downstream signaling events.

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