Protein tyrosine phosphatase 1B negatively regulates integrin signaling

Feng Liu, Mary Ann Sells and Jonathan Chernoff

Protein tyrosine phosphatase (PTP) 1B has long been known to regulate cell proliferation negatively, but the mechanism by which this inhibition occurs is poorly defined. We have shown previously that PTP1B binds to, and dephosphorylates, p130^{Cas} (Crk-associated substrate) [1], a protein that is thought to play a role in integrin signaling [2,3]. In this report, we present evidence that PTP1B interferes specifically with cell-adhesion-stimulated, but not growth-factor-stimulated, signaling pathways. In rat fibroblasts that overexpress PTP1B, the activation of mitogen-activated protein (MAP) kinase by growth factors was not affected, but activation by cell adhesion was markedly impaired. The inhibition of adhesion-dependent MAP kinase activation by PTP1B required an intact proline-rich region in the carboxyl terminus of PTP1B, a region we have shown to mediate binding to the Src-homology 3 (SH3) domain of p130^{Cas} [1]. Overexpression of wild-type PTP1B, but not of a proline-to-alanine mutant form (PA-PTP1B) that is unable to bind or dephosphorylate p130^{Cas}, interfered with cell spreading, cytoskeletal architecture, and the formation of focal adhesion complexes. Cells overexpressing wild-type PTP1B also displayed markedly reduced migration in response to a fibronectin gradient, whereas cells expressing the PA-PTP1B mutant migrated normally. These data indicate that PTP1B exerts its inhibitory effects via proline-dependent interactions with one or more critical components of the adhesion-dependent signaling apparatus, and suggest that one of these components may be p130^{Cas}.

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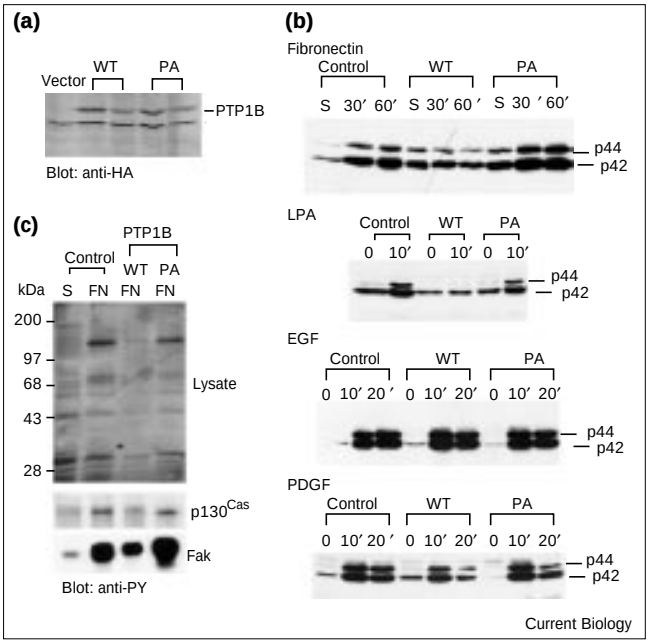
Results and discussion

Having previously shown that PTP1B binds to and dephosphorylates p130^{Cas} [1], we examined the effect of PTP1B on the MAP kinase activity initiated by cell adhesion or by various growth factors. Rat 3Y1 fibroblasts were transfected with expression vectors bearing no insert, or plasmids that drove the stable expression of wild-type PTP1B (WT-PTP1B) or the PA-PTP1B mutant [1]. The expression of these forms of PTP1B in two independent stably expressing 3Y1 clones is shown in Figure 1a. In each case, exogenous PTP1B was expressed at 3–5-fold higher levels than endogenous PTP1B, as assessed by immunoblot with a monoclonal anti-PTP1B antibody (data not shown).

We examined the ability of the PTP1B-expressing cells to signal in response to cell adhesion or soluble growth factors. In cell adhesion experiments, the 3Y1 stable cell lines were kept in suspension or plated on fibronectin-coated dishes and incubated for 30–60 minutes. In growth factor experiments, the cells were serum-starved overnight, then treated with buffer alone, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), or lysophosphatidic acid (LPA) for 10 or 20 minutes. MAP kinase activation was then examined by immunoblot using an antibody that recognizes only the activated (di-phosphorylated) form of the MAP kinases Erk1 and Erk2 (extracellular signal-regulated kinases 1 and 2). When control 3Y1 cells adhered to fibronectin, MAP kinase activity increased substantially within 30 minutes (Figure 1b). This effect was not seen in cells plated on poly-D-lysine or collagen (data not shown). Although the basal activity of MAP kinase in suspension cells was slightly increased, fibronectin-dependent MAP kinase activation was markedly repressed in cells expressing WT-PTP1B, but not in cells expressing the PA-PTP1B mutant. PA-PTP1B is fully active in terms of phosphatase activity, but cannot bind p130^{Cas} or other proteins containing an SH3 domain [1]. Similar results were observed in cells treated with LPA, a compound known to induce focal adhesion formation, p130^{Cas} tyrosine phosphorylation and MAP kinase activation [4–6]. In contrast to these results, neither wild-type nor the mutant form of PTP1B affected MAP kinase activation by EGF. In the case of PDGF, MAP kinase activation was slightly inhibited by WT-PTP1B, but these effects were independent of the proline-rich domain. Similar results were obtained in COS1 cells transiently transfected with WT-PTP1B or PA-PTP1B (see Figure S1 in Supplementary material), indicating that the effects of this phosphatase on MAP kinase activation in the stable 3Y1 cells are not due to chronic PTP1B expression inducing changes in integrin or growth-factor receptor levels. Furthermore, the direct measurement of surface integrin expression showed that three major fibronectin-binding integrins, α3, α5, and αv, were expressed at equal levels on the surface of control cells or 3Y1 cells stably expressing WT-PTP1B or PA-PTP1B (see Figure S2 in Supplementary material). In summary, these results show that PTP1B specifically represses integrin-mediated MAP kinase activation, and that this repression requires the proline-rich domain in PTP1B.

Figure 1



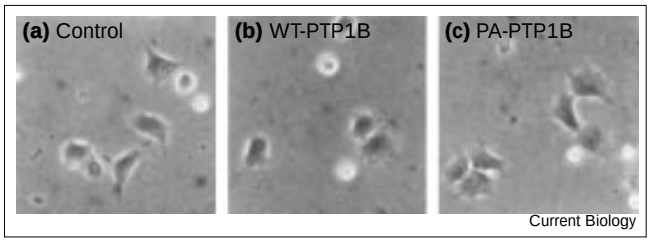
Effect of PTP1B on signaling by cell adhesion and by growth factors. (a) Expression of tagged PTP1B proteins. Lysates were prepared from 3Y1 fibroblasts stably transfected with a control plasmid or expression vectors encoding hemagglutinin (HA) epitope-tagged wild-type (WT) PTP1B, or proline-to-alanine (PA) mutant PTP1B [1]. Two independent clones for each construct were analyzed. An anti-HA (HA.11, BabCo), chemiluminescent (Pierce) immunoblot is shown. (b) Effect of PTP1B on growth-factor-stimulated MAP kinase activity. Control cells and cells expressing WT-PTP1B or PA-PTP1B were serum-starved overnight, then stimulated by plating on fibronectin or collagen (data not shown), or by treatment with LPA (5 μg/ml), EGF (500 ng/ml) or PDGF (10 ng/ml). Lysates from these cells were analyzed by immunoblot with anti-phospho-MAP kinase antibodies (Promega). The phosphorylated forms of the MAP kinases Erk1 and Erk2 are indicated by p42 and p44. S indicates cells in suspension. (c) Effect of PTP1B on adhesion-stimulated tyrosine phosphorylation. Lysates were prepared from cells in suspension (S) or cells plated on fibronectin (FN), then $p130^{Cas}$ and Fak were immunoprecipitated from these lysates using polyclonal anti-$p130^{Cas}$ and anti-Fak (Santa Cruz) antibodies, respectively. Cell lysates and immunoprecipitates were separated by 7% SDS–PAGE, transferred to Immobilon membranes, and blotted with anti-phosphotyrosine (PY20, Transduction Laboratories) antibodies. The $p130^{Cas}$ and Fak immunoprecipitates were also blotted with a monoclonal anti-$p130^{Cas}$ antibody (Transduction Laboratories) or anti-Fak antibody (a gift from Tom Parsons), to demonstrate that protein levels were similar (data not shown).

We next examined the effects of PTP1B on the tyrosine-phosphorylation levels of cellular proteins following integrin engagement. When control cells were plated on fibronectin, a number of proteins became tyrosine phosphorylated (Figure 1c). The most prominent of these proteins migrated at approximately 125–130 kDa, which may represent Fak (focal adhesion kinase) and $p130^{Cas}$, and at approximately 68 kDa, which may represent paxillin. In cells expressing WT-PTP1B, the overall level of tyrosine phosphorylation decreased dramatically, whereas expression of PA-PTP1B had little effect on overall tyrosine phosphorylation levels. Analysis of $p130^{Cas}$ and Fak immunoprecipitates revealed that levels of tyrosine phosphorylation of these proteins increased upon integrin engagement, and that the levels were substantially reduced in cells expressing WT-PTP1B, but not in cells expressing PA-PTP1B. These data indicate that the negative effect of PTP1B on integrin-stimulated MAP kinase activity correlates with the tyrosine-phosphorylation state of a number of phosphotyrosyl proteins including $p130^{Cas}$ and Fak, and that these effects are likely to involve SH3-dependent interactions.

We examined the effect of PTP1B on other adhesion-related phenomena, including the formation of stress fibers and focal adhesions, and cell motility. When plated on fibronectin for 1 hour, WT-PTP1B-expressing 3Y1 cells were more rounded and poorly spread compared to control cells or cells expressing PA-PTP1B (Figure 2). These differences were less apparent in cells plated on uncoated tissue culture dishes (data not shown). Despite these differences, the growth rates of control and WT-PTP1B-expressing cells are similar [7]. Cells expressing WT-PTP1B did eventually spread, but this took an average of 3–4 hours. In addition to these morphologic changes, WT-PTP1B cells also displayed a motility defect. Chemotaxis in response to soluble fibronectin was almost completely abolished in cells expressing WT-PTP1B. In contrast, cells expressing PA-PTP1B were only slightly less motile than control cells (Figure 3). Chemotaxis in response to PDGF (10 ng/ml), soluble collagen (12 μg/ml), or poly-D-lysine (12 μg/ml) was unaffected by WT-PTP1B or PA-PTP1B (data not shown).

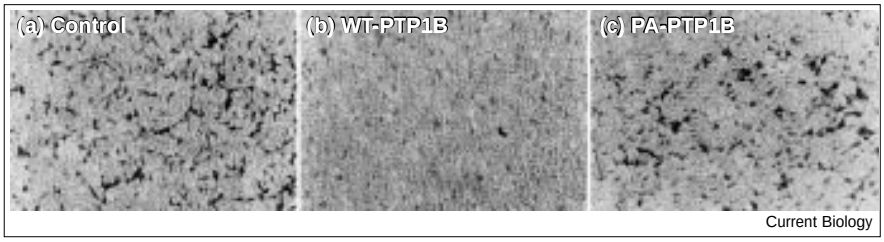
To investigate the mechanism leading to the impaired migratory activity of cells overexpressing WT-PTP1B, we examined the organization of their actin cytoskeleton. Fluorescein isothiocyanate-conjugated phalloidin staining of the cells plated onto fibronectin-coated dishes indicated the presence of abnormal stress fibers in WT-PTP1B-expressing cells: F-actin accumulated in dense bundles of stress fibers that accumulated around the cell

Figure 2



Effect of PTP1B on cell spreading. (a) Control 3Y1 cells and 3Y1 cells expressing (b) WT-PTP1B or (c) PA-PTP1B were trypsinized, then replated onto tissue culture dishes as described in Materials and methods. The cells were examined by phase-contrast microscopy 30 min after being plated. A representative field for each cell line is shown.

Figure 3



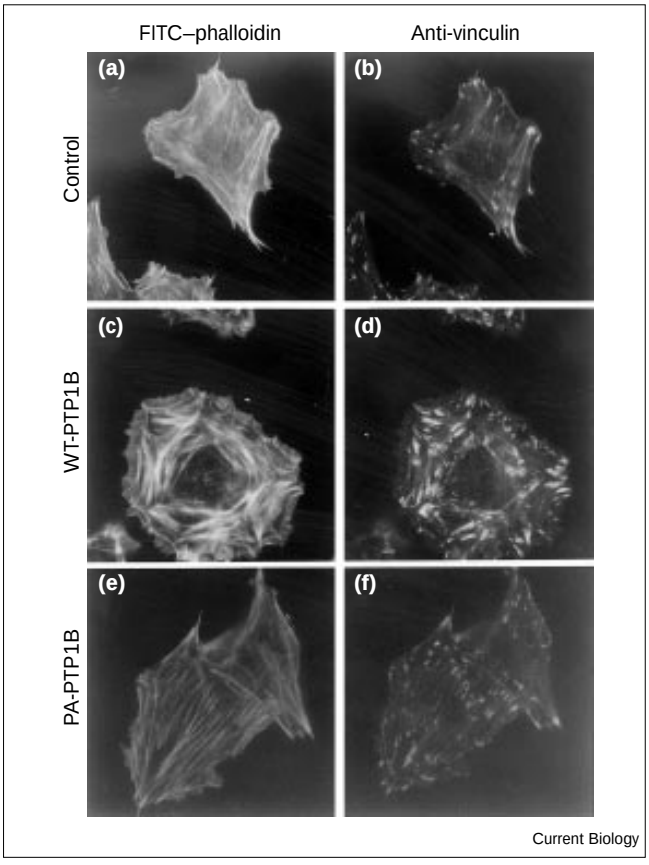
Effect of PTP1B on chemotaxis. Exponentially growing (a) control 3Y1 cells and 3Y1 cells expressing (b) WT-PTP1B or (c) PA-PTP1B were placed in a chemotaxis chamber and assayed for movement in response to a fibronectin gradient (12 μg/ml) for 4 h as described by Cary *et al*. [14] and in the Supplementary material. Cells that successfully migrated through the membrane were identified by a modified hematoxylin stain.

periphery (Figure 4c). In addition, such cells displayed circumferential lamellipodia. This phenotype occurred in virtually every cell upon spreading on the plate. Control cells and cells expressing the PA-PTP1B mutant displayed normal F-actin organization, consisting mostly of thin stress fibers without accompanying lamellipodia (Figure 4a,e). The abnormal spreading and F-actin distribution in WT-PTP1B-expressing cells could be related to a disordered formation of focal adhesions. This was examined by staining the cells with an antibody against vinculin, which is a major component of focal adhesions. In cells expressing WT-PTP1B, vinculin staining revealed the presence of numerous, very large, elongated focal complexes scattered over the ventral surfaces (Figure 4d). Under the same conditions, control cells or cells expressing PA-PTP1B showed vinculin-positive patches at the cell periphery in the form of typical focal adhesions (Figure 4b,f).

We have shown here that PTP1B overexpression inhibits several integrin-regulated events: adhesion-dependent MAP kinase activation, tyrosine phosphorylation of a variety of proteins including $p130^{Cas}$, cell spreading, motility, focal adhesion assembly and actin reorganization. All these effects require the presence of the proline-rich domain of PTP1B, which we have previously shown to be required for PTP1B to interact with SH3-domain-containing proteins. PTP1B also inhibits signaling by LPA, a compound whose effects on cells resemble those generated upon integrin engagement, perhaps through activation of the GTPase Rho [8]. In contrast, PTP1B does not affect MAP kinase activation by mitogens such as EGF and PDGF that act through receptor protein tyrosine kinases. Although substrate-trapping experiments have shown that PTP1B may form complexes with activated receptor tyrosine kinases such as the EGF [9,10], PDGF [10], and insulin [11] receptors, the physiological importance of these associations is uncertain. If PTP1B downregulates signaling from these receptors, then overexpression of this phosphatase would be expected to inhibit signaling by the relevant growth factors. In 3Y1 cells, this is clearly not the case (Figure 1b). Instead, our results indicate that the effects of PTP1B on cell proliferation are likely to be due to interactions with substrates that are unique to integrin signaling pathways, rather than growth-factor pathways.

A dominant-negative form of the Ras GTPase blocks integrin-mediated activation of MAP kinase without inhibiting cell adhesion, spreading, focal-complex formation, or

Figure 4



Effect of PTP1B on F-actin distribution and focal complex formation. Exponentially growing (a,b) control 3Y1 cells and 3Y1 cells expressing (c,d) WT-PTP1B or (e,f) PA-PTP1B were stained with FITC-conjugated phalloidin to indicate F-actin organization or anti-vinculin (Sigma) to indicate focal-adhesion formation, followed by a fluorescently labeled secondary antibody (LRSC-conjugated goat anti-mouse serum). See Supplementary material for details. The cells were examined by indirect immunofluorescence.

stress-fiber formation [12]. These data imply that cell adhesion generates a signal that diverges before Ras is activated, with one or more branches devoted to actin reorganization, and others to Ras (and subsequent MAP kinase) activation. The fact that PTP1B overexpression interferes with both adhesion-dependent MAP kinase activation and cytoskeletal organization suggests that the target(s) of PTP1B is/are located upstream of Ras in the integrin signaling pathway. Such upstream targets might include adhesion-stimulated protein tyrosine kinases such as Fak, Pyk2, Src, Fyn, and Csk, and tyrosine-phosphorylated focal-complex proteins such as $p130^{Cas}$, Hef1, paxillin, cortactin, and tensin [13]. Interestingly, most of these proteins contain SH3 domains and could potentially bind to PTP1B.

PTP1B-induced tyrosine dephosphorylation of $p130^{Cas}$ reduces the association of $p130^{Cas}$ with signaling molecules such as Crk, which may link $p130^{Cas}$ to Ras and the MAP kinase pathway [7]. Failure to form such signaling complexes following integrin engagement might be expected to interfere with adhesion-dependent signaling events, such as MAP kinase activation and reorganization of cortical actin, with consequent abnormalities in gene expression, cell spreading, focal-adhesion complex formation, and motility. Indeed, whereas overexpression of Fak (which normally binds to $p130^{Cas}$ and may mediate the tyrosine phosphorylation of $p130^{Cas}$ by Src) in Chinese hamster ovary cells increases cell motility [14], overexpression of a Fak mutant that cannot bind $p130^{Cas}$ fails to elicit this effect [15]. Although on the basis of our results we cannot be certain whether the effects of PTP1B overexpression can be explained solely as resulting from its interactions with $p130^{Cas}$ or Fak, it is clear that the proline-dependent interactions mediate the ability of this phosphatase to regulate adhesion-dependent signaling negatively. The identification of molecules that bind PTP1B is a major aim of our future work.

Materials and methods

Construction of 3Y1 cell lines expressing PTP1B

Rat 3Y1 cells were grown to 40% confluence and transfected by a calcium phosphate method with either pJ3H, pJ3H-WT-PTP1B, or pJ3H-PA-PTP1B [1] together with a plasmid encoding a puromycin-resistance marker. The cells were selected by growth in 2 μg/ml puromycin, and colonies were isolated with cloning cylinders.

Adhesion assays

Cells derived from 3Y1 fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum (FBS) and 2 μg/ml puromycin. Cells were starved overnight in serum-free DMEM and dissociated from culture flasks with 0.04% trypsin, 0.53 mM EDTA. The suspended cells were washed once in serum-free DMEM containing 0.1 mg/ml soybean trypsin inhibitor (Sigma), twice with serum-free DMEM alone, and held in suspension in DMEM plus 2% bovine serum albumin (BSA) at 37°C for 30 min with rotation. An equivalent number of cells were then plated on tissue-culture dishes or glass cover slips coated with 25 μg/ml fibronectin (prepared by adsorbing a 25 μg/ml solution of fibronectin to 60 mm tissue-culture dishes at room temperature for 1 h) or retained in suspension, and incubated at 37°C for the times indicated in the figure legends. Following the incubations, the cells were washed twice with ice-cold phosphate-buffered saline and then lysed in 200 μl of 1% NP-40 lysis buffer (20 mM Tris·HCl pH 8.0, 1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 50 mM NaF, 10 mM β-glycerolphosphate) plus 1 mM *p*-nitrophenyl phosphate, 10 μg/ml aprotinin and 1 mM sodium vanadate. Protein concentration was determined using bicinchoninic acid (Pierce).

Supplementary material

Additional methodological detail and two figures showing the effect of transient PTP1B expression on signaling by cell adhesion and by growth factors, and showing the expression of cell-surface integrins, are published with this paper on the internet.

Acknowledgements

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Supplementary material

Protein tyrosine phosphatase 1B negatively regulates integrin signaling

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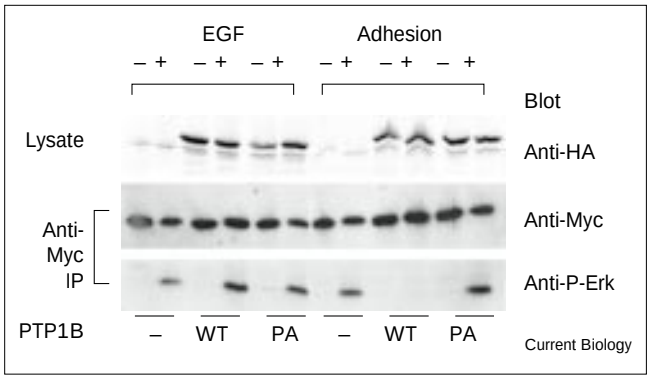
Figure S1



Effect of transient PTP1B expression on signaling by cell adhesion and by growth factors. COS1 cells were co-transfected with plasmids encoding Erk1 tagged with the Myc epitope and either an empty expression vector, or plasmids encoding HA-tagged WT-PTP1B or HA-tagged PA-PTP1B. The cells were serum-starved, then stimulated with EGF (left-hand side) or by plating to fibronectin-coated dishes (right-hand side) as described in Figure 1b. Lysates were examined for PTP1B expression, and Myc immunoprecipitates were examined for Myc–Erk1 expression (with an anti-Myc antibody) and for activated Erk1 (with an anti-phospho-Erk antibody) by immunoblot.

Materials and methods

Chemotaxis

Migration studies were performed using a 48-well chemotaxis chamber (NeuroProbe). Buffer alone, various growth factors, or extracellular matrix proteins in DMEM containing 0.5% BSA were added to the lower chamber. Cells were harvested as for adhesion experiments, resuspended in DMEM plus 0.5% BSA, and added to the upper chamber at 1.5×10^4 cells per well. The lower and upper chambers were separated by a collagen (rat tail, type I, Collaborative Biomedical Products)-coated, PVP-free polycarbonate membrane (8 μm pore size, Poretics). Cells were allowed to migrate for 6 h at 37°C in a humidified atmosphere containing 5% CO_2. Cells on the upper side of the membrane were removed by gentle scraping. The membrane was then fixed in ethanol for 10 min and stained with Gill No. 2 hematoxylin stain (Sigma) for 1 h. Cells on the lower side of the membrane were counted and photographed with an inverted microscope. For each mutant, a minimum of two clones were assayed in triplicate in three independent experiments, and migrating cells from two microscopic field were examined (at least 54 microscopic fields were examined per class of mutant).

Transient transfections

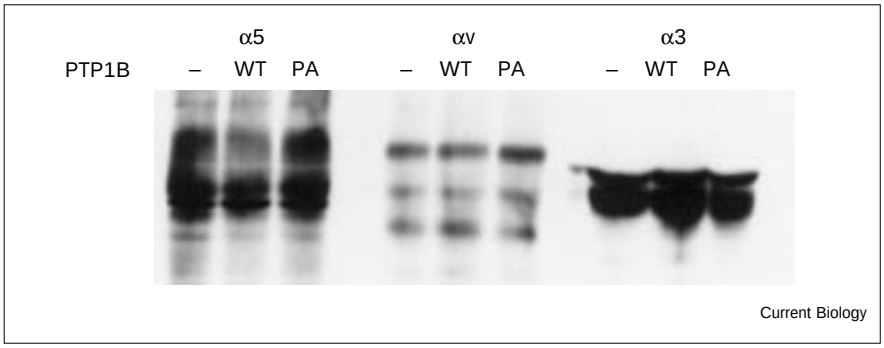
COS1 cells (5×10^5) grown on 60 mm plates were co-transfected with 0.5 μg pJ3M-Erk1 [1] plus 1.5 μg pJ3H, pJ3H-WT-PTP1B, or pJ3H-PA-PTP1B, using 10 μl LipofectAmine (BRL/GibCo), according to the manufacturer's recommendations. After 24 h, the cells were serum-starved overnight, then stimulated with EGF or fibronectin as described in Figure 1b. Myc-tagged Erk1 was immunoprecipitated from lysed cells using the monoclonal antibody 9E10 (Santa Cruz Biotechnology). Lysates and immunoprecipitates were fractionated by 10% SDS–PAGE, then immunoblotted with monoclonal anti-HA (HA.11, BabCo), polyclonal anti-Myc (Santa Cruz Biotechnology), or polyclonal anti-phospho-MAP kinase (Promega) antisera.

Determination of surface integrin levels

Dishes (150 mm) of control or PTP1B-transfected 3Y1 cells were detached non-enzymatically using Cell Dissociation Solution (Sigma). The cells were washed in DMEM, then in PBS containing 1 mM Ca^{2+} and 1 mM Mg^{2+}. Surface proteins were then biotinylated by incubating the cells with 1 ml EZ-link Sulfo-NHS-LC-Biotin (Sigma) in a solution containing 10 mM triethanolamine pH 8.5, 2 mM $CaCl_2$, 150 mM NaCl for 20 min, twice. The cells were then washed with PBS containing 1 mM Ca^{2+}, 1 mM Mg^{2+}, and 100 mM glycine, to quench reactive biotin, then with PBS containing 1 mM Ca^{2+}, 1 mM Mg^{2+}, to remove free biotin. The cells were lysed in 50 mM Tris·HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, on ice for 30 min. Cellular debris was removed by centrifugation at $14{,}000 \times g$ for 10 min at 4°C. The supernatant was precleared with rabbit IgG agarose, then immunoprecipitated with antisera against α3, α5, or αv integrins. The immunoprecipitates were separated by 7% SDS–PAGE, then blotted with streptavidin-HRPO, and developed using a chemiluminescent procedure (Pierce).

Figure S2

Expression of cell-surface integrins. Surface proteins on rat 3Y1 clones were biotinylated as described in the Supplementary Materials and methods. Following cell lysis, α3, α5 and αv integrins were immunoprecipitated, and biotinylated integrins were identified by immunoblot using streptavidin–HRPO (horseradish peroxidase) and chemiluminescent detection.



Fluorescence microscopy

Cells derived from 3Y1 fibroblasts were plated on glass coverslips coated with fibronectin and allowed to adhere and spread for 60 min at 37°C. The cells were then fixed for 5 min in 3.5% formaldehyde, permeabilized in 0.2% Triton X-100 KB buffer (0.1 M Tris pH 7.5, 1.5 M NaCl, 1.0% BSA) for 5 min at room temperature, rinsed with KB buffer without Triton X-100 for 5 min and incubated for 1 h at 37°C with monoclonal anti-vinculin antibody. Following the incubation with primary antibody, the cells were rinsed in KB buffer with 0.2% Triton X-100 once, and KB buffer without Triton once, then incubated with fluorescently labeled secondary antibody (lissamine–rhodamine B sulfonyl chloride (LRSC)-goat anti-mouse IgG) and fluorescein isothiocyanate (FITC)-conjugated phalloidin (Molecular Probes) for 1 h at 37°C. The cover slips were washed in KB buffer and mounted with Vectashield (Vector). Coverslips were viewed on an epifluorescence microscope equipped with a 35 mm camera and fluorescence micrographs taken on T-max ASA 400 film (Kodak).

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