PTP1B triggers integrin-mediated repression of myosin activity and modulates cell contractility

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ABSTRACT

Cell contractility and migration by integrins depends on precise regulation of protein tyrosine kinase and Rho-family GTPase activities in specific spatiotemporal patterns. Here we show that protein tyrosine phosphatase PTP1B cooperates with β3 integrin to activate the Src/FAK signalling pathway which represses RhoA-myosin-dependent contractility. Using PTP1B null (KO) cells and PTP1B reconstituted (WT) cells, we determined that some early steps following cell adhesion to fibronectin and vitronectin occurred robustly in WT cells, including aggregation of β3 integrins and adaptor proteins, and activation of Src/FAK-dependent signalling at small puncta in a lamellipodium. However, these events were significantly impared in KO cells. We established that cytoskeletal strain and cell contractility was highly enhanced at the periphery of KO cells compared to WT cells. Inhibition of the Src/FAK signalling pathway or expression of constitutive active RhoA in WT cells induced a KO cell phenotype. Conversely, expression of constitutive active Src or myosin inhibition in KO cells restored the WT phenotype. We propose that this novel function of PTP1B stimulates permissive conditions for adhesion and lamellipodium assembly at the protruding edge during cell spreading and migration.

KEY WORDS: PTP1B, Src, FAK, Integrin, Myosin, Contractility

INTRODUCTION

Protein tyrosine phosphatases, including PTP1B, have been established as important regulators of integrin-mediated signal transduction implied in cytoskeletal rearrangements and cell migration (Larsen et al., 2003; Burridge et al., 2006; Arregui et al., 2013).

PTP1B is an endoplasmic reticulum (ER)-anchored enzyme whose access to substrates is partly dependent on the ER distribution and dynamics (Frangioni et al., 1992; Hernández et al., 2006; Anderie et al., 2007; Fuentes and Arregui, 2009; Nievergall et al., 2010; Haj et al., 2012; Monteleone et al., 2012; Burdisso et al., 2013). PTP1B dephosphorylates the autoinhibitory tyrosine of Src (Tyr 529 in mouse), contributing to its activation (Arregui et al., 2010; Haj et al., 2012; Monteleone et al., 2012; Burdisso et al., 2013). PTP1B dephosphorylates the autoinhibitory tyrosine of Src (Tyr 529 in mouse), contributing to its activation (Arregui et al., 2010; Haj et al., 2012; Monteleone et al., 2012; Burdisso et al., 2013). BiFC (bimolecular fluorescence complementation) analysis demonstrated that ER-bound PTP1B targets Src associated with the plasma membrane in contact with the substrate (Monteleone et al., 2012).

RESULTS

PTP1B is required for early and transient integrin-dependent Src activation

Integrin stimulation induces Src and FAK activity (Burridge et al., 1992; Hanks et al., 1992; Lipfert et al., 1992; Kaplan et al., 1995). PTP1B is essential for δIbβ3 activation of Src in platelets (Arias-Salgado et al., 2005). However, the spatiotemporal coordinates of these events are unknown. To address this issue we monitored active Src by immunodetection of Src phosphoTyr-418 in cell lines derived from PTP1B null mice, KO cells, and reconstituted with wild type PTP1B, WT cells (Haj et al., 2002). Serum-starved cells were plated for 5, 10, 20, 30 and 60 min on polylysine, as an unspecific substrate, and fibronectin, as a substrate to stimulate integrins. At all time points after plating on polylysine (only the 10 min time point is shown),...
Src-pY418 distributed throughout the cell, with no accumulation at the periphery in both, WT and KO cells (Fig. 1A,B). In contrast, at 5 and 10 min post-plating on fibronectin, Src-pY418 was strongly accumulated at a peripheral ring of puncta in WT cells (Fig. 1D,G). This signal declined with time, being undetectable by 20 min post-plating (Fig. 1J). Src-pY418 did not accumulate at the periphery of KO cells plated on fibronectin at any time point (Fig. 1E,H,K). We quantified the peripheral signal by two methods, one measuring the fluorescence intensity along line scans orthogonal to the cell margin (Fig. 1C,F,I,L) (>20 cells per condition, cell border=0 µm). The magnitude of Src-pY418 fluorescent signal at the peripheral ring of puncta in WT cells did not correlate with cell area (not shown), and, on average, was 6-fold higher than the signal in the inner lamella (Fig. 1C,F,I,L) (>20 cells per condition, cell border=0 µm). The pan-Src antibody did not reveal Src accumulation at the periphery of WT and KO cells (Fig. S1A-C). The specificity of Src-pY418 labeling was confirmed in SYF cells, which do not express Src, Fyn and Yes members of the Src family (Klinghoffer et al., 1999). SYF cells plated for 10 min on fibronectin show a faint perinuclear Src-pY418 signal (Fig. S1D,F). As expected, SYF cells expressing HA-tagged Src display a peripheral ring of Src-pY418 puncta, similar to that observed in WT cells. The total exogenous Src (revealed by HA labeling), did not accumulate at the cell periphery, as the endogenous pool (Fig. S1E,F). These results demonstrate that PTP1B and integrin stimulation are both required for transient Src activation at the periphery of spreading cells.

High resolution fluorescence microscopy reveals the localization of ER-bound PTP1B in the cell periphery, reaching the lamellipodium and the ring of active Src puncta (Fig. S1H-N). To examine whether peripheral ER positioning was required for Src activation, we transfected WT cells with a soluble GFP-kinectin construct (GFP-knt) which uncouples the ER from microtubules, leading to the collapse of the peripheral ER.
required for Src-pY418 activation on fibronectin, in which α5β1 heterodimer is functional. WT cells were incubated with the cyclic GpenGRGDSPCA (GPen) peptide. At the concentration used (1 mM), GPen efficiently inhibits the vitronectin receptor but does not block cell attachment to fibronectin (Fig. 2F, Pierschbacher and Ruoslahti, 1987; Arregui et al., 1998). Cell spreading was reduced by GPen; however, in the fraction of spreading cells Src-pY418 accumulation at the cell periphery did not occur (Fig. 2G,H). Similar results were obtained in WT cells pre-incubated with a function-blocking anti-β3 integrin (not shown). Although we cannot rule out the contribution of additional vitronectin receptors, like αvβ1 and αvβ5 integrins, our results implicate that PTP1B and β3 integrin cooperate to activate Src at peripheral puncta.

To determine whether active Src puncta colocalize with adhesions, WT cells were transfected with β3 integrin-GFP and immunolabeled for Src-pY418. Confocal sections at the cell-substratum interface reveal that peripheral Src-pY418 puncta colocalize with the distal tips of β3 integrin aggregates (Fig. 2I-M). The distal localization of Src-pY418 in adhesive puncta was confirmed by TIRF microscopy (Fig. 2N-R). We determined whether the peripheral ring of adhesive puncta in WT cells localize within the lamellipodium, a thin layer of peripheral cytoplasm rich in actin filaments and accessory proteins like filamin, α-actinin and cortactin, among others (Small et al., 2002). Double immunofluorescence analysis revealed that Src-pY418 puncta overlapped with the peripheral labeling of F-actin, cortactin and α-actinin (Fig. 2S-V, only F-actin is shown). Surface reflectance interference contrast (SRIC) analysis reveals the membrane proximity to the substrate within the nanometer range, with low and high reflectance intensities representing membrane...
PTP1B promotes integrin-dependent FAK activation and paxillin phosphorylation

Integrin aggregation induces autophosphorylation of FAK at tyrosine 397, creating a binding site for the Src-homology 2 domain of Src. Src phosphorylates and promotes the full activation of FAK (Schaller et al., 1994; Xing et al., 1994; Calalb et al., 1995; Mitra and Schlaepfer, 2006). The active Src/FAK complex phosphorylates the adaptor protein paxillin at Tyr-31 and Tyr-118 residues after cell-matrix adhesion (Schaller and Parsons, 1995; Bellis et al., 1997; Schaller et al., 1999). We examined the spatiotemporal activation of FAK in starved WT and KO cells plated on fibronectin, double labeled for FAK and FAK-pY397. FAK and FAK-pY397 accumulated in a peripheral ring of puncta at 5 and 10 min in WT cells (Fig. 3A-D,G-I). These puncta localized within the lamellipodium. FAK-pY397 signal persisted at high intensity in elongated peripheral adhesions at 30 and 60 min post-plating (Fig. 3E,F; only the 30 min time point is shown). KO cells did not show detectable FAK and FAK-pY397 accumulation at the cell periphery at 5 min post-plating (Fig. 3J,K), but discrete FAK-pY397 puncta were detected at the cell periphery by 10 min (Fig. 3M,Q,R) and the signal increased in elongated peripheral adhesions at 30 and 60 min post-plating (Fig. 3O, only the 30 min time point is shown). Quantification of the fluorescence intensity along line scans perpendicular to the cell border at 10 min post-plating revealed that the FAK-pY397 signal in WT cells was 3-fold higher than in KO cells (Fig. 3S). Likely, this result could be explained by deficient FAK aggregation at the periphery of KO cells (Fig. 3T).

We next analyzed the phosphorylation of paxillin. In WT cells, paxillin and paxillin-pY118 accumulated at a peripheral ring of puncta at 5 and 10 min post-plating on fibronectin (Fig. 4A-D,G-I). In KO cells paxillin and paxillin-pY118 puncta were less developed than in WT cells (Fig. 4I-M,P-R). Quantification of paxillin-pY118 fluorescent signal at 10 min revealed that the peak intensity at the cell border was 40% lower in KO cells compared with WT cells (Fig. 4S). At later time points (30 and 60 min), WT and KO cells displayed similar elongated peripheral paxillin adhesions and strong pY118 signal (Fig. 4E,F,N,O; only the 30 min time point is shown). The lower phosphorylation of paxillin was confirmed by western blot analysis (Fig. 4U).

We compared WT cells, KO cells also displayed reduced peripheral aggregation of β3 integrin and vinculin, lower phosphorytrosine content, and lower accumulation of lamellipodium markers like cortactin and F-actin (Fig. S2).

PTP1B-dependent Src/FAK signaling represses myosin and promotes lamellipodium and adhesion assembly

RhoA activity is transiently downregulated immediately after integrin stimulation, event that requires functional Src/FAK (Arthur et al., 2000; Ren et al., 2000; Pirone et al., 2006; Schober et al., 2007; Bass et al., 2008; Tomar and Schlaepfer, 2009), and PTP1B (Burdisso et al., 2013). RhoA activity stimulates myosin-driven contractile forces (Geiger and Bershadsky, 2001; Zaidel-Bar et al., 2003). We hypothesized that inefficient activation of the Src/FAK signaling pathway in KO cells impairs lamellipodium and adhesion formation due to enhanced myosin activity. To test this we examined whether myosin inhibition by blebbistatin restores integrin-dependent signaling, lamellipodium and adhesion assembly at the periphery of KO cells plated for 10 min on fibronectin. Blebbistatin did not affect these processes in WT cells (Fig. S3). Remarkably, in KO cells blebbistatin induced the appearance of a well defined F-actin rich lamellipodium, which is not present in control cells (Fig. 5A-C, Fig. S2M-O). Blebbistatin also strongly induced the assembly of a peripheral ring of puncta containing FAK-pY397, Src-pY418 and paxillin-pY118 (Fig. 5D-L) as well as paxillin and β3 integrin (Fig. S4). Interestingly, blebbistatin did not have effect at longer times after plating (30 and
PTP1B decreases peripheral cell contractility during spreading

PTP1B regulates cell-matrix adhesion and motility through dephosphorylation of adaptors and scaffolds associated to integrin receptors, including p130Cas (Liu et al., 1996), paxillin (Takino et al., 2003), and α-actinin (Zhang et al., 2006; Burdisso et al., 2013). Our current data suggest an additional role of PTP1B in promoting the transient suppression of myosin-dependent contractility at the cell cortex during spreading. We evaluated the contractility at the cell cortex of WT and KO cells using a recently developed mechano-transduction sensor based on filamin A (Nakamura et al., 2014). Filamin A is an extended homodimeric protein that binds F-actin at the cell periphery (Nakamura et al., 2011; Razinia et al., 2012). The sensor, named FLNA-CS (Filamin A conformational sensor), consists of a FRET pair designed to quench the fluorescence of monomeric EGFP when the conformation of filamin is closed, as it occurs in the absence of cytoskeletal forces, and to unquench it (with fluorescence emission) when its conformation is open, as expected when contractile forces develop. The sensor has mCherry added at its C-terminal as an internal control to normalize for probe concentration. Determination of mEGFP/mCherry ratios in a pixel by pixel basis of cell images allows the reconstruction of

60 min, Fig. S3). Thus, attenuation of myosin activity in KO cells compensates for the lack of PTP1B function without altering the normal dynamics of peripheral puncta, which are undetectable by 30 min post-plating in both WT and KO cells. Unrestricted myosin-dependent mechanical strain at the cell periphery may promote Rac1 inhibition by FilGAP, a filamin A-associated Rac GAP that can suppress the formation of Rac-dependent lamellipodia (Shifrin et al., 2009; Ehrlicher et al., 2011; Nakamura, 2013). In agreement with this, expression of constitutive active Rac1 L61 in KO cells restored the lamellipodium formation (Fig. S4F-J), but it was unable to induce the assembly of peripheral adhesion puncta (not shown).

Our results suggest that early after cell contact with the matrix, PTP1B cooperates with β3 integrins to activate a Src/FAK signaling pathway leading to the transient repression of RhoA and myosin-dependent contractility, allowing adhesion and lamellipodium assembly. To further substantiate this idea, we tested a number of predictions. First, artificially increasing Src/FAK signaling in WT cells should promote the formation of new adhesions at the cell periphery. To test this, we expressed a constitutive active Src mutant (Src Y529F) in KO cells. Transfected cells showed enhanced accumulation of paxillin-pY118 at a ring of peripheral puncta compared to non transfected KO cells (Fig. 6A-D). Second, impairing Src/FAK signaling in WT cells should reproduce the effects caused by PTP1B deficiency in KO cells. WT cells transfected with a dominant negative mutant of Src, SrcKD/Y529F (Mukhopadhyay et al., 1995; Burdisso et al., 2013) or with FRNK (FAK-related non kinase), a dominant negative mutant of FAK (Richardson and Parsons, 1996), displayed a significant reduction of Src-pY418 (not shown) and paxillin-pY118 at the cell periphery in comparison with non transfected WT cells (Fig. 6E-L). Third, inhibition of myosin should reverse the effect of Src/FAK impairment in WT cells. In fact, incubation of WT cells expressing SrcKD/Y529F or FRNK with blebbistatin completely reversed the negative effect of the constructs and restored Src-pY418 (not shown) and paxillin-pY118 puncta to levels similar to non transfected cells (Fig. 6M-T). Fourth, we predicted that expression of constitutively active RhoA in WT cells should impair adhesion formation and spreading. In fact, cells transfected with RhoA L63 have reduced spreading capacity (not shown), lack of paxillin-pY118 (not shown) and Src-pY418 accumulation at the cell periphery compared to non transfected cells (Fig. 6U-X).

Fig. 4. PTP1B is required for integrin-dependent paxillin phosphorylation. WT (A-I) and KO cells (J-R) plated on fibronectin for 5, 10 and 30 min after plating. Cells were double immunolabeled for paxillin and paxillin-pY118. (A-F) In WT cells, paxillin-pY118 strongly accumulated in a peripheral ring of puncta at 5 and 10 min and in elongated peripheral adhesions at 30 min (yellow arrowheads). (G-I) Enlarged views (4×) of the boxed regions in C and D. (J-O) In KO cells, paxillin-pY118 accumulation at the cell periphery was barely detectable at 5 and 10 min post-plating (K,M), but the signal increases at elongated peripheral adhesions by 30 min (O) (yellow arrowheads). (P-R) Enlarged views of the boxed regions shown in L and M. (S,T) Quantification of peripheral paxillin-pY118 (S, n=47 cells) and paxillin (T, n=29 cells) fluorescent signals as described in Fig. 1. Differences between mean values at the peak were statistically significant P<0.0001, two-tailed Student’s t-test. (U) Western blot analysis of paxillin-pY118 and paxillin in cells kept in suspension or plated on fibronectin for 10 min. The plot represents averaged values of paxillin-pY118 normalized to total paxillin (n=3 experiments). Scale bar, 20 µm. Values (in arbitrary units) in S,T represent mean±s.e.m. from >20 cells per condition.

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two-dimensional maps of the sensor signal response. WT and KO cells expressing the FLNA-CS sensor were plated on fibronectin for 10, 60 and 90 min and analyzed in absence or presence of serum, with similar results. At 10 min after plating, WT cells showed low FLNA-CS signal at the cell periphery (Fig. 7A,D). By contrast, KO cells showed a strong sensor signal response at the cell edge, which decreases to reach a lower plateau ~2 µm away of the cell border (Fig. 7B,D). Similar results were observed in cells plated for 60 and 90 min (Fig. 7E-G, only the 90 min time point is shown). As expected, incubation of KO cells with blebbistatin eliminated the strong sensor response at the cell periphery (Fig. 7C,D), and cells plated on polylysine showed a relatively uniform response of the sensor throughout the cell, with a slightly higher basal level in KO cells compared to WT cells (not shown).

As in Fig. 1 we used two methods to quantify the variations of the peripheral signal from the cell border (=0 distance in the plots) to the cell center (Fig. 7D,G). To examine further the spatiotemporal FLNA-CS sensor response in migrating WT and KO cells we performed time lapse and kymograph analysis. WT cells consistently displayed a low sensor response at the protruding leading edge (Fig. 7H and Movie 1). In contrast, the protruding leading edge in KO cells showed a more variable sensor response, alternating bursts of moderate and high signal.

Fibroblasts are able to contract collagen gels in a myosin-dependent manner (Meshel et al., 2005). To determine the impact of PTP1B function in the capacity of cells to remodel 3D collagen gels, we measured the contraction of floating collagen gels containing WT and KO cells in complete culture medium. Determinations over time reveal that gels containing KO cells are consistent and significantly (P<0.05, two-tailed Student’s t-test) more contracted than those containing WT cells (Fig. 7I,J). This result could not be attributed to differences in cell number (data not shown). Blebbistatin reverses the contractility capacity of KO cells to the level of WT cells. However, blebbistatin does not have effect on the contractile capacity of WT cells. To determine the role of Src activity, which is impaired in KO cells, we expressed a constitutive form of the kinase in WT and KO cells.
active Src mutant (Src Y529F) in KO cells. Under this condition, the contraction of gels was reduced at all time points (Fig. 7I,K). These results indicate that PTP1B contributes to reduce myosin-dependent contractility at the cell periphery, with detectable consequences to the attached extracellular matrix.

DISCUSSION

Early integrin-dependent signal transduction events include the activation of the non receptor protein tyrosine kinases Src and FAK, which are responsible for most tyrosine phosphorylation activity occurring in adhesion complexes (Guan, 1997; Mitra and Schlaepfer, 2006; Huveneers and Danen, 2009). An important downstream consequence of these early molecular events is the modulation of Rho family GTPases, which control fundamental aspects of cell behavior, including cell spreading and migration (Ridley et al., 2003; Scales and Parsons, 2011; Spiering and Hodgson, 2011). The fibronectin receptors expressed in fibroblasts, α5β1 and αvβ3, display overlapping and distinctive effects on modulating RhoA activity (Morgan et al., 2009; Schiller and Fässler, 2013). Both heterodimers induce transient downregulation of RhoA activity immediately after cell adhesion; however, only α5β1 promotes the subsequent increment of RhoA activity required for focal adhesion maturation (Danen et al., 2002). They also drive divergent migratory behaviors; α5β1 promoting thin cell protrusions and random cell migration, and αvβ3 promoting the extension of broad lamellipodia and persistent migration (Danen et al., 2005). Our previous work suggests that the migratory behavior of KO cells was compatible with a α5β1-mediated pattern of migration, likely reflecting an impaired αvβ3 function (Hernández et al., 2006; Burdisso et al., 2013). Here we demonstrate that PTP1B promotes lamellipodium and adhesion formation at the protruding cell edge by ensuring the efficient β3 integrin-dependent activation of Src/FAK signaling. WT cells attached to fibronectin, which stimulates β1 and β3 integrins, or vitronectin, which selectively stimulates β3 integrin, develop lamellipodium and adhesive puncta eliciting Src/FAK activation...
and paxillin phosphorylation. This induction is inhibited by blocking β3 integrin function and occurs inefficiently in KO cells. NIH3T3 fibroblasts expressing a dominant-negative mutant of PTP1B (C215S) (Arregui et al., 1998) recreate some of the phenotypes described in KO cells (data not shown), suggesting that results are not biased in the PTP1B WT and KO cell lines. Our results agree with previous findings in fibrinogen-stimulated platelets, showing that PTP1B is recruited to a αIIbβ3/Src complex and is essential for Src activation (Arias-Salgado et al., 2005). Between 5-15 min after plating most WT cells exhibit a circumferential lamellipodium and a rounded shape, compatible with isotropic spreading (Dubin-Thaler et al., 2004). As time progresses, cells become increasingly asymmetric in shape and peripheral Src activation become spatially and temporally restricted to random and transient protrusions (Gulyani et al., 2011). This may explain the limitation of our quantification methods to detect significant Src-pY418 signal at longer times post-plating.

How impaired Src/FAK signaling in KO cells relates to the inefficient adhesion and lamellipodium assembly at the cell periphery? It is well established that after fibroblast attachment to fibronectin RhoA activity is modulated in a biphasic manner (Lawson and Burridge, 2014). A first phase of RhoA downregulation occurs during the first 30 min after integrin stimulation, which is then followed by a stimulatory phase (Ren et al., 1999; Arthur et al., 2000). RhoA downregulation correlates temporally with strong induction of Src and FAK activities.
and Bershadsky, 2001; Ridley et al., 2003; Huveneers and Danen, 2013). Rac is required for lamellipodium and focal complex assembly, and is induced by integrin stimulation (Geiger, 2013). Rac is required for lamellipodium and focal complexes (Zaidel-Bar et al., 2007). Expression of Rac1Y31F/Y118F in fibroblasts, increases lamellipodial protrusions and focal complexes (Zaidel-Bar et al., 2007). Expression of rac1 is tyrosine phosphorylated by Src/FAK in response to fibronectin (Burridge et al., 1992; Deakin and Turner, 2008; Robertson et al., 2015). Our results show reduced levels of paxillin phosphorylation at peripheral puncta in KO cells. It has been shown that expression of the phosphomimetic mutant of paxillin, Y31E/Y118E in fibroblasts, increases lamellipodial protrusions and focal complexes (Zaidel-Bar et al., 2007).

The acute and robust effect of blebbistatin in KO cells argues against major structural alterations that could prevent the assembly of adhesions and the lamellipodium in these cells. Instead, our results suggest a defect in Src/FAK signaling, which ultimately results in the inefficient assembly of the lamellipodium and adhesions. In agreement with this view, migrating KO cells display lamellar extensions and adhesion complexes of short duration compared to WT cells (Burdisso et al., 2013). The enhanced contractility of KO cells reported in the present work may explain, at least partly, these previous results. Enhanced cell contractility alters the conformation of filamin, with concomitant modulation of its interaction with partners (Ehrlicher et al., 2011; Razinia et al., 2012; Nakamura et al., 2011). Filamin is a large homodimeric protein, which interacts with F-actin to form orthogonal branches and provide network flexibility (Nakamura et al., 2007, 2011; Razinia et al., 2012). Using a filamin-based, force sensor in live cells, allowed to visualize specific spatiotemporal force patterns in WT cells, with lower sensor responses associated with the cell cortex in active protrusions. These patterns are altered in KO cells, which show high sensor responses at the cell cortex, blocked by blebbistatin incubation. Mechanical strain applied to F-actin/filamin networks reconstituted in vitro induced opposed effects on filament binding to partners, with an increase of β-integrin binding and a decrease of FilGAP association (Ehrlicher et al., 2011). Enhanced filamin interaction with β-integrin inhibits integrin activation (Kiema et al., 2006; Das et al., 2011), effect that could explain, at least in part, the absence of adhesion puncta at the periphery of KO cells. FilGAP promotes GTP hydrolysis in Rac1, inhibiting its activity (Nakamura, 2013). Rac is required for lamellipodium and focal complex assembly, and is induced by integrin stimulation (Geiger and Bershadsky, 2001; Ridley et al., 2003; Huveneers and Danen, 2009; Lawson and Burridge, 2014). We recently showed that integrin-dependent Rac induction is impaired in KO cells (Burdisso et al., 2013). Our new findings revealing an enhanced contractility in KO cells suggest a negative regulation of Rac1 through the dissociation of FilGAP from filamin, and/or reducing the availability of Rac1 GEFs, such as β-Pix (Kuo et al., 2011; Kutys and Yamada, 2014). In fact, expression of constitutively active Rac1L61 in KO cells restored the formation of an F-actin-rich lamellipodium (Fig. S4). However, Rac1L61 was insufficient to induce the assembly of a rim of peripheral vinculin and paxillin pY118 puncta (results not shown), suggesting that additional signaling branches depending on Src/FAK activation by PTP1B are required for adhesion assembly and growth (Zaidel-Bar and Geiger, 2010; Robertson et al., 2015). One important hub of the phospho-adhesome network is the adaptor protein paxillin, which is tyrosine phosphorylated by Src/FAK in response to fibronectin adhesion (Burridge et al., 1992; Deakin and Turner, 2008; Robertson et al., 2015). Our results show reduced levels of paxillin phosphorylation at peripheral puncta in KO cells. It has been shown that expression of the phosphomimetic mutant of paxillin, Y31E/Y118E in fibroblasts, increases lamellipodial protrusions and peripheral puncta (results not shown), arguing that the main constraint in KO cells is likely an enhanced myosin-dependent contractility at the cell periphery. The higher FLNA-CS response and collagen contraction capacity observed in KO cells, compared to WT cells, demonstrate the medium- and long-range effects of PTP1B deficiency.

Our results support a model in which PTP1B cooperates with β3 integrin to set in motion a feed-forward mechanism at the cell periphery during initial stages of contact with the substratum. This mechanism involves activation of the Src/FAK signaling pathway and inhibition of RhoA-myosin activity. The biological consequence is a reduction of contractile forces at the periphery, generating permissive conditions for adhesion, lamellipodium assembly, and spreading (Fig. 8). Myosin deregulation in KO cells may have a wide range of physiological implications. Remarkably, we demonstrated a significant effect on collagen contraction. Higher contractile capacity of PTP1B-deficient cells may explain defects in clot retraction in platelets (Arias-Salgado et al., 2005), cell migration in fibroblasts (Hernández et al., 2006; Burdisso et al., 2013), axon elongation (Fuentes and Arrequi, 2009), and dendritic spine maturation (Fuentes et al., 2012).

MATERIALS AND METHODS

Cell culture and treatments

PTP1B null (KO) cells and PTP1B reconstituted (WT) cells (Haj et al., 2002) and SYF cells (ATCC) were cultured in high glucose DMEM containing L-glutamine, supplemented with 10% fetal bovine serum, penicillin and streptomycin (Invitrogen). Unless indicated, cells were serum-starved for 4 h, and then resuspended with 0.05% trypsin in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) containing 1 mM EDTA. Trypsin was neutralized with soybean trypsin inhibitor (Sigma-Aldrich). Cells (1×104) were plated in plain DMEM on coverslips coated with poly-L-lysine, (150 μg/ml), fibronectin (20 μg/ml), both from Sigma-Aldrich, or with vitronectin (5 μg/ml) obtained from BD Biosciences. After 5, 10, 20, 30 and 60 min cells were fixed for subsequent analysis. When indicated, cells were pre-incubated with blebbistatin (20 μM, Sigma-Aldrich) for 60 min at 37°C, with G-Pen peptide (1 mM, GRGDSPCA, American Peptide Company), with anti-β3 antibody, or isotype control IgG (20 μg/ml), for 30 min at 4°C, before plating in the presence of the reagents. The effect on attachment and spreading was evaluated by microscopy using Metamorph (Molecular Devices).
Antibodies and other labeling reagents
Phalloidin-TRITC, and monoclonal antibodies against vinculin (hVIN-1), HA (HA-7), c-myc (9E10) and α-tubulin (DM 1A) were from Sigma-Aldrich. Hamster anti-mouse CD29 (HMβ1-1), hamster anti-mouse CD61 (2C9.G2), hamster IgG isotype control and FITC goat anti-hamster IgG were from Biolegend, and HRP-conjugated rabbit anti-hamster IgG was from Abcam. Polyclonal antibody against FAK-pY397 was from Biosource International. Polyclonal antibodies against Src-pan, Src-pY418 and paxillin-pY118 were from Invitrogen. Monoclonal anti-paxillin (349), anti-phosphotyrosine (PY20), anti-PTP1B (15/PTP1B) and anti-FAK (77) were from BD Transduction Laboratories. Alexa Fluor 488- and Alexa Fluor 568-conjugated secondary antibodies were from Invitrogen. HRP-conjugated antibodies were from Jackson Immunoresearch.

DNA constructs and transfections
Cells were transfected using Lipofectamine 2000 (Invitrogen) and processed 24 h post-transfection. The pCMV-myc-FRNK was provided by J. T. Parsons (University of Virginia); EGFP-knt by H. Yu (National University of Singapore); RhoA L63 and Rac1 L61 in pRK5-myc by A. Hall (Memorial Sloan-Kettering Cancer Center); and pSP73-β3-integrin by A. Teitelbaum (University of Washington). To obtain β3 integrin-GFP, β3 integrin cDNA was amplified by PCR and inserted into Bgl II/Age I sites of pEGFP-N1. Both proteins are joined by a GPVAT spacer. Transfected β3-GFP localizes in cell-matrix adhesions only when cells are seeded on fibronectin and vitronectin but not on laminin (not shown). GFP-PTP1B, mRFP-PTP1B D181A, Src-HA, SrcY529F-HA and Src KD/Y529F-HA were previously described (Hernández et al., 2006; Burdisso et al., 2013).

Flow cytometry
Surface expression of β1 and β3 integrins in WT and KO cells was analyzed by flow cytometry. Cells (1×10⁶) were harvested with trypsin/EDTA, diluted with complete medium and centrifuged. Cells were resuspended in PBS/BSA 0.1%, and incubated on ice for 60 min with hamster anti-CD61, hamster anti-CD61, or hamster IgG isotype control (20 µg/ml). After washing with PBS, cells were incubated with FITC-conjugated goat anti-hamster (1:100) on ice for 60 min. Cells were washed, fixed in 1% parafomaldehyde, and analyzed on a FlowMax cytometer PASHI (Partec). Data were plotted using WinMdi 2.9 software (Bio-Soft Net).
Microscope analysis

Cells were fixed with 4% paraformaldehyde in PBS (20 min), permeabilized with 0.5% Triton X-100 (5 min) and blocked with 3% BSA (1 h). Primary and secondary antibodies were incubated in a humid chamber for 1 h. Samples were mounted in Vectashield (Vector Laboratories) and observed through a 60×/1.4 NA objective in an Olympus FX1000 confocal microscope, or by wide-field in a Nikon TE2000-U microscope coupled to an ORCA-ER CCD camera (Hamamatsu). For SRIC, a cube with a green excitation filter, a UV diroic mirror and without barrier filter was set in place in the epi-filter rotating turret. For TIRFM, cells were observed with a 60×1.4 NA objective in a Nikon TE2000-E inverted microscope coupled to an ORCA II ER CCD camera controlled by Metamorph. A 100 W mercury lamp was used for SRIC and wide-field observation, and a 488 nm argon laser and a 543 nm helium/neon laser for confocal and TIRFM. Penetration depth of the evanescent field (∼210 nm) was calculated as described (Monteleone et al., 2012).

Mechanical strain was determined using the FLNA-CS force sensor (Nakamura et al., 2014). Transfected WT and KO cells were fixed and analyzed by wide-field fluorescence microscopy. Incident light from a 100 W mercury lamp was attenuated to 25% using neutral density filters. Monomeric EGFP was imaged using a B-2E/C filter cube [excitation filter, 480/30, dicroic mirror 505 (LP), emission filter 535/40], and mCherry was imaged using filters in wheels [excitation filter 565/25, emission filter 620/60] in combination with a 86007bs dichroic mirror (Chroma). Images were acquired using binning 2 and exposure times ranging 500-1500 ms. For time lapse imaging, serum-starved cells were plated on MatTek’s coverglass-bottom 35 mm culture dishes coated with fibronectin in 2.5 ml phenol red-free DMEM, supplemented with 25 mM NaVO₃, 10 mM NaF and protease inhibitors (Sigma-Aldrich). Cell lysates were centrifuged at 13,600 × g for 15 min at 4°C and ~30 µg of the supernatants were fractionated by SDS-PAGE and transferred to polyvinyl difluoride membranes. After blocking with 3% BSA, membranes were probed with primary antibodies (2 µg/ml) followed by peroxidase-conjugated secondary antibodies and revealed by ECL (SuperSignal West Femto Maximum Sensitivity Substrate, Thermo Scientific).

Image analysis and quantitative procedures

All quantitative procedures were performed using ImageJ. Microscopy 12-bit images were corrected for shading and background-subtracted. The cell border was defined by thresholding. Variations of signal intensity at the cell periphery were quantified along four equidistant line scans (5 µm width) orthogonal to the cell border, which in total covered 15-25% of the entire cell perimeter. Pixel values from the four line scans were averaged per cell. Means and s.e.m. values of more than 15 cells per condition were used for representation using Kaleidagraph (Synergy Software). As an alternative quantification method, we used the ‘ADAPT’ software (Barré et al., 2015) to calculate the mean fluorescence intensity of segmented image boundaries taken successively from the cell border to the cell center. The FLNA-CS sensor response was quantified according to published procedures (Nakamura et al., 2014), mEGFP and mCherry images were aligned (MultistackReg plugin), and the time lapse stacks corrected for bleaching (corr_blech050405, EMBL) before ratio calculation. For visualization, ratio images were pseudo-colored using the ratio lookup table. Kymographs of a representative time lapse experiment (Movie 1) were generated using the MultipleKymograph plug-in for ImageJ. The ratio values were represented along line scans (1-pixel wide) orthogonal to the cell border in images recorded every min during 25 min.

Collagen gel contraction assays

Type I collagen was prepared from rat tail tendons (Price, 1975). Gel contraction assays were performed as previously described (Jean et al., 2013). Briefly, collagen diluted in DMEM was adjusted to a pH 7.4 with 1 M NaOH and mixed with a cell suspension, so that 500 µl containing 0.6 mg collagen and 5×10⁶ cells was added to each well of 24-well cell culture plates (Costar), previously blocked with 3% BSA. Collagen was allowed to polymerize for 1 h at 37°C. Then, complete DMEM was added to the wells and collagen gels were gently detached using a pipette tip. Floating gels were incubated for 72 h at 37°C, and photographed every 24 h. Gel area was measured using ImageJ. When indicated, collagen gels were incubated with blebbistatin (20 µM) from the beginning of the experiment.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.E.G.W. and A.G. performed the experiments; A.E.G.W. and C.O.A. designed the experiments and analyzed the data; F.N. contributed with reagents and analysis tools; A.E.G.W. and C.O.A. wrote the paper.

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Supplementary information

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References

References


