ER-bound PTP1B is targeted to newly forming cell-matrix adhesions

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Summary

Here, we define the mechanism through which protein tyrosine phosphatase 1B (PTP1B) is targeted to cell-matrix adhesion sites. Green fluorescent protein (GFP)-labeled PTP1B bearing the substrate-trapping mutation D181A was found in punctate structures in lamellae. The puncta co-localized with focal adhesion kinase (FAK) and Src, and defined the distal tips of cell-matrix adhesion sites identified with paxillin and vinculin. PTP1B is largely associated with the external face of the endoplasmic reticulum (ER) and the puncta develop from ER projections over cell-matrix adhesion sites, a process dependent on microtubules. Deletion of the ER-targeting sequence resulted in cytosolic localization and altered the distribution of PTP1B at cell-matrix foci, whereas mutations disrupting interactions with Src homology 3 (SH3) domains, and the insulin and cadherin receptors had no effect. PTP1B recognizes substrates within forming adhesion foci as revealed by its preferential association with paxillin as opposed to zyxin-containing foci. Our results suggest that PTP1B targets to immature cell-matrix foci in newly forming lamellae by dynamic extensions of the ER and contributes to the maturation of these sites.

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Introduction

Protein tyrosine phosphatase 1B (PTP1B) is targeted to the cytosolic face of the membranes of the endoplasmic reticulum (ER) via a hydrophobic sequence at its C terminus (Frangioni et al., 1992). As the catalytic domain faces the cytosol, ER bound enzyme has the potential for substrate dephosphorylation throughout the extensive branching network occupied by the ER. Indeed, recent biochemical, as well as FRET (fluorescence resonance energy transfer) and BRET (bioluminescence resonance energy transfer) analyses show that PTP1B at the ER interacts directly with, and dephosphorylates, several cell surface receptors, either during their biosynthetic route to the cell surface, or after being endocytosed (Haj et al., 2002; Boute et al., 2003; Romsicki et al., 2004; Cohen et al., 2004). PTP1B can be cleaved by calpains at a site upstream from the ER targeting sequence, producing a cytosolic ~42 kDa form (Frangioni et al., 1993). Such a cleaved form of PTP1B has been shown to associate with N-cadherin (Balsamo et al., 1998) and to depend on phosphorylation of PTP1B Y152 (Rhee et al., 2001) catalyzed by the tyrosine kinase Fer (Xu et al., 2004). In contrast to the association of this cleaved and presumably diffusible form with cadherin, full length PTP1B is associated with the β1-integrin complex of cytosolic proteins (Arregui et al., 1998). PTP1B has also been shown to interact directly with the adaptor protein p130^{Cas} (Liu et al., 1996), which is localized at focal adhesions (Harte et al., 1996). The interaction between PTP1B and p130^{Cas} is mediated by a proline-rich motif in PTP1B that interacts with the Src homology 3 (SH3) domain of p130^{Cas} (Liu et al., 1996). Mutation of the proline-rich motif impairs

many of the integrin-dependent effects regulated by PTP1B (Liu et al., 1998; Dadke and Chernoff, 2002). Thus, the proline-rich motif of PTP1B may be involved in the association of PTP1B with cell-matrix adhesions.

Cell-matrix adhesion sites are heterogeneous in composition, shape and behavior (Zamir et al., 2000; Zamir and Geiger, 2001; Yamada et al., 2003; Webb et al., 2003). Small dot-like adhesions near the edges of lamellar extensions are known as focal complexes. Larger, elongated adhesions associated with actin stress fibers that can be induced by rho-dependent, acto-myosin contractility, are named focal adhesions. Quantitative studies show that most focal complexes are rich in paxillin and poor in zyxin, α-actinin and tensin; these latter proteins incorporate into focal complexes later during their maturation into focal adhesions (Laukaitis et al., 2001; Zaidel-Bar et al., 2003). The molecular mechanisms involved in the assembly and maturation of cell-matrix sites are largely unknown, but depend to some degree on the combined activity of protein tyrosine kinases and phosphatases (Miyamoto et al., 1995; Volberg et al., 2001; Larsen et al., 2003). The tyrosine phosphatase SHP2 was recently shown to be necessary for the incorporation of α-actinin into focal complexes and for their maturation to focal adhesions (von Wichert et al., 2003).

Turnover of cell-matrix sites and cell motility are also regulated by phosphorylation/dephosphorylation events. Cells derived from mice null for the tyrosine kinases FAK and Src display reduced cell-matrix turnover and cell motility (Ilic et al., 1995; Klinghoffer et al., 1999; Webb et al., 2004). Cells derived from mice that are null for PTP1B also display defects

in cell spreading (Cheng et al., 2001). Furthermore, L-cells expressing a dominant negative form of PTP1B have low Src activity and small cell-matrix sites, and are similarly impaired in cell spreading (Arregui et al., 1998). These observations are consistent with the hypothesis that PTP1B is instrumental in activation of Src through dephosphorylation of the C-terminal tyrosine (Arregui et al., 1998; Bjorge et al., 2000; Cheng et al., 2001; Dadke and Chernoff, 2003). PTP1B also activates integrin-signaling pathways and motility by controlling the level of phosphorylation of additional substrates such as CrkII (Takino et al., 2003).

In the present study we examined the role of the proline-rich motif, the insulin and cadherin-binding motif, and the ER targeting sequence in localization of PTP1B to cell-matrix adhesion sites. For these studies we used the substrate-trapping mutant of PTP1B in which the invariant aspartate residue at position 181 is replaced by alanine. This substrate trapping mutant binds to its substrates with similar affinity as the wild-type enzyme, but catalytic activity is significantly reduced (Flint et al., 1997). The resulting enzyme-substrate complexes have been shown to be stable enough to be visualized by optical techniques (Haj et al., 2002; Boute et al., 2003; Romsicki et al., 2004). We find that PTP1B is positioned at the distal tips of cell-matrix adhesion sites through dynamic extensions of the ER. We also show that PTP1B is preferentially localized to paxillin-rich foci, and that maturation of these foci and lamellar extension is impaired in PTP1B null cells, supporting a role of PTP1B in the maturation and stabilization of focal adhesions.

Results
PTP1B localizes to the distal tip of cell-matrix adhesion sites
We previously found that PTP1B is associated with the $\beta1$-integrin complex of proteins and that it affects cell-matrix adhesion and signaling (Arregui et al., 1998; Pathre et al., 2001). Thus, PTP1B probably acts on substrates located in cell matrix sites, modifying their activity (Arregui et al., 1998; Bjorge et al., 2000; Cheng et al., 2001; Takino et al., 2003). To

identify potential PTP1B substrates at cell-matrix adhesion sites we used the substrate-trapping mutant PTP1B D181A (PTP1B DA) which forms long-lived complexes with substrates. This substrate-trapping mutant has proved to be crucial for detection, by FRET and BRET, of complexes formed between PTP1B and different growth factor receptors at the surface of the ER (Haj et al., 2002; Boute et al., 2003; Romsicki et al., 2004). Mouse fibroblasts derived from mice null for PTP1B (Klaman et al., 2000) were transiently transfected with GFP fusions of the wild-type PTP1B and PTP1B DA. Whereas GFP-wild-type PTP1B has the typical ER distribution (Fig. 1A) (Frangioni et al., 1992; Arregui et al., 1998), equivalent levels of GFP-PTP1B DA also localized to small punctate structures near cell margins (Fig. 1B, also see inset). These puncta are also apparent using anti-PTP1B antibody staining of PTP1B null cells stably reconstituted with PTP1B DA not fused to GFP (Haj et al., 2002) (Fig. 1C) indicating that GFP has no effect on the distribution of PTP1B. The fact that punctate structures were apparent only with PTP1B DA, at equivalent expression levels as the wild-type form (Fig. 1A,B,D) indicates that stabilization of enzyme-substrate complexes is necessary for their visualization. In concert with this, prior treatment of the cells with pervanadate, which inhibits PTPs by binding to and modifying the essential catalytic cysteinyl residues at their active sites (Huyer et al., 1997), leads to the disappearance of the punctate structures at the cell-matrix adhesion foci (not shown).

We next examined the co-localization of the PTP1B DA with several structural proteins commonly used as markers of cell-matrix adhesion complexes. Confocal sections taken at the cell-matrix interface revealed that PTP1B DA localizes at the distal tips of foci stained with vinculin, paxillin (Fig. 2A,B), and zyxin (not shown). The PTP1B DA puncta also localize at the tips of actin stress fibers (Fig. 2C) and microtubules (Fig. 2D) consistent with the fact that cell-matrix adhesion complexes are sites of anchorage to the actin cytoskeleton and the termination of microtubules (Calderwood et al., 2000; DeMali and Burridge, 2003; Small and Kaverina, 2003; Ezratty et al.,
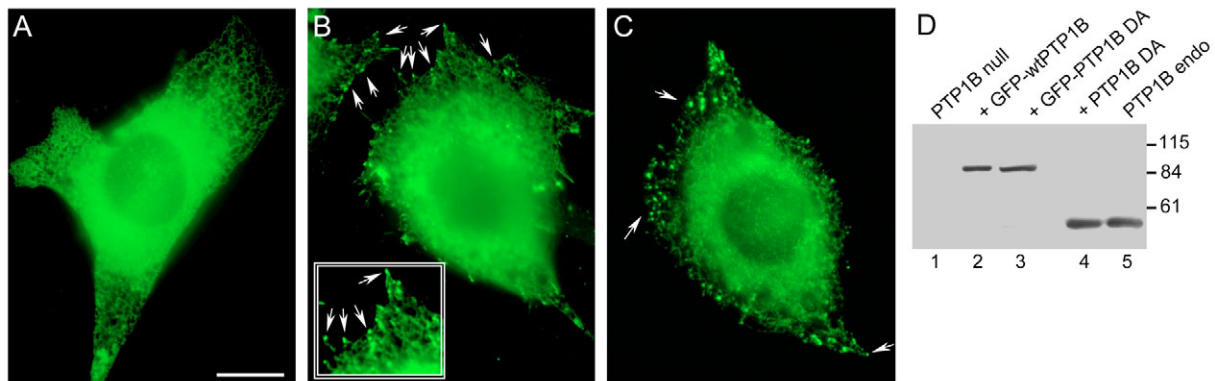
Fig. 1. The substrate-trapping PTP1B DA localizes to punctate structures at cell margins. Cells were plated on fibronectin-coated coverslips, fixed and examined by wide-field fluorescence microscopy. (A) PTP1B null cells were transfected with GFP-wild-type PTP1B or (B) GFP-PTP1B DA and visualized 20 hours later. The arrows indicate punctate structures (B, inset). Note the absence of punctate structures in A. (C) PTP1B null cells stably reconstituted with PTP1B DA were stained with monoclonal anti-PTP1B followed by Alexa Fluor 488-conjugated secondary antibody. Note that the punctate structures look larger because of the signal amplification by the antibodies. Bar, 20 µm. (D) Representative western blot of equivalent amounts of soluble protein prepared from PTP1B null cells (lane 1); PTP1B null cells transiently transfected with GFP-wild type PTP1B (lane 2); GFP-PTP1B DA (lane 3); or stably reconstituted with PTP1B DA (lane 4). The endogenous levels of PTP1B in a non related fibroblast cell line is also shown (lane 5). The blot was probed with anti-PTP1B.

Fig. 2. PTP1B DA puncta co-localize with proteins associated with cell-matrix adhesion sites and the cytoskeleton. PTP1B null cells stably expressing PTP1B DA (A,B) or PTP1B null cells transiently transfected with GFP-PTP1B DA (C,D), or GFP-PTP1B DA/QA (E) were plated on fibronectin-coated coverslips and examined 24 hours later. (A) Cells double-stained with a polyclonal anti-PTP1B and a monoclonal (hVIN-1) anti-vinculin followed by secondary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 568, respectively. Shown is one confocal section (0.5 µm) at the cell-matrix interface. (B) Cells stained with anti-PTP1B and anti-paxillin followed by secondary antibodies conjugated with Alexa Fluor 568 and Alexa Fluor 488, respectively. (C) Cells stained with rhodamine-phalloidin. Dotted line indicates the boundary of the cell. (D) Cells stained with anti-tubulin (DM1 A), followed by a secondary antibody conjugated with Alexa Fluor 568. (E) Cells stained with anti-PTP1B followed by a secondary antibody conjugated with Alexa Fluor 568. In all cases, arrows indicate punctate structures. Note that punctate structures localize at the distal tips of vinculin and paxillin (A,B,E), as well as at the tips of actin (C) and microtubules (D). Bars, (A,B,E) 20 µm; (C,D) 10 µm.
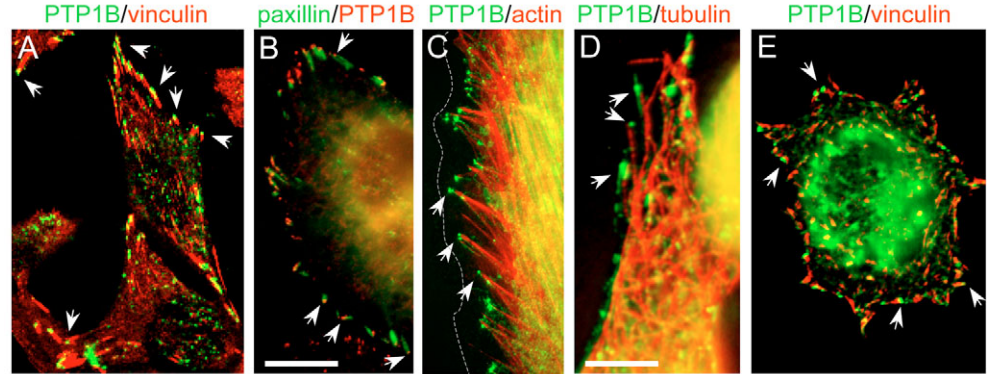
2005). We also assessed the localization of two additional substrate-trapping mutants of PTP1B: PTP1B C215S (PTP1B CS), in which the essential cysteine of the active site is replaced by a serine, localized to the ER and no clear punctate structures were seen over cell-matrix foci (not shown); the double mutant PTP1B DA plus Q262A (PTP1B DA/QA), in which the invariant Gln262 involved in the hydrolysis of the cysteinyl phosphate intermediate has been replaced by alanine, displayed a punctate distribution at the distal tips of cell-matrix foci similar to PTP1B DA (Fig. 2E). These results are consistent with previous reports showing that PTP1B CS is a weak substrate-trapping mutant compared to PTP1B DA and PTP1B DA/QA (Flint et al., 1997; Xie et al., 2002). Again, these data indicate that the stability of the enzyme-substrate complexes is a key feature allowing for visualization of PTP1B at cell-matrix sites.

Cell-matrix adhesion complexes are sites of intense tyrosine phosphorylation/dephosphorylation and sources of signaling complexes that regulate cell behavior, including the turnover of cell-matrix adhesion sites (Giancotti and Ruoslahti, 1999; Webb et al., 2002). Two major tyrosine kinases, FAK and Src, localize to cell-matrix adhesion sites and are responsible for the bulk of tyrosine phosphorylation at these sites (Gilmore and Romer, 1996; Klinghoffer et al., 1999). Several laboratories including ours have demonstrated that Src is activated by PTP1B (Arregui et al., 1998; Bjorge et al., 2000; Cheng et al., 2001; Dadke and Chernoff, 2003). We found that the PTP1B DA puncta do co-localize tightly with Src (Fig. 3A-F) and a subpopulation of the PTP1B DA puncta co-localize with the phospho-active form of Src in lamellae (Fig. 3G). Furthermore, Src and PTP1B co-immunoprecipitate (Fig. 3H) (Liang et al., 2005). These data suggest that the co-localization of PTP1B with Src is probably due to the binding of PTPB DA to the C-terminal regulatory tyrosine of Src (Bjorge et al., 2000; Cheng et al., 2001).

PTP1B puncta also co-localize tightly with FAK (Fig. 4A-C). Dual confocal time-lapse analysis revealed that puncta containing FAK/PTP1B DA are dynamic, giving the appearance of fusing with each other or splitting, and some disappear from the plane of the cell-substrate interface (Fig.
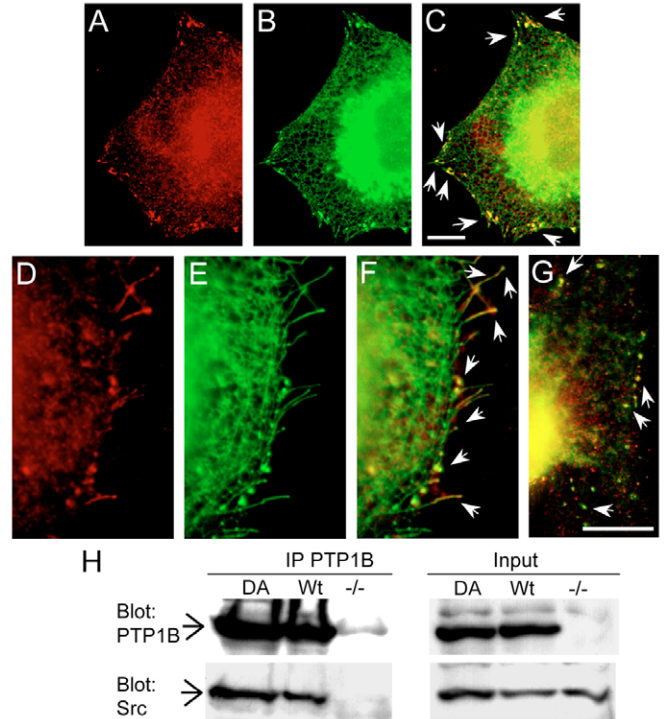
Fig. 3. PTP1B puncta co-localize with Src and Src-pY418 at cell margins. PTP1B null cells were transfected with GFP-PTP1B DA and examined 20 hours later by fluorescence microscopy (A-G). Cells were labeled with anti-Src (A-F) or anti-Src-pY418 (G) followed by a secondary antibody conjugated with Alexa Fluor 568. Note the co-localization (yellow) at large punctate structures formed at the peripheral ER network (C), or in small punctate structures at individual ER tubules at the cell margins (F) Arrows indicate puncta. Note that a subpopulation of PTP1B DA puncta is coincident with the phospho-active form of Src (G). Bars, (A-C) 10 µm; (D-G) 15 µm. (H) The formation of Src-PTP1B complexes was analyzed by immunoprecipitation. PTP1B null cells (–/–), and cells stably expressing PTP1B wild type (Wt) or PTP1B DA (DA) were lysed and immunoprecipitated with anti-PTP1B. The amount of PTP1B and Src in the lysate (input) and in the immunoprecipitates was detected with the respective antibodies (Blot).
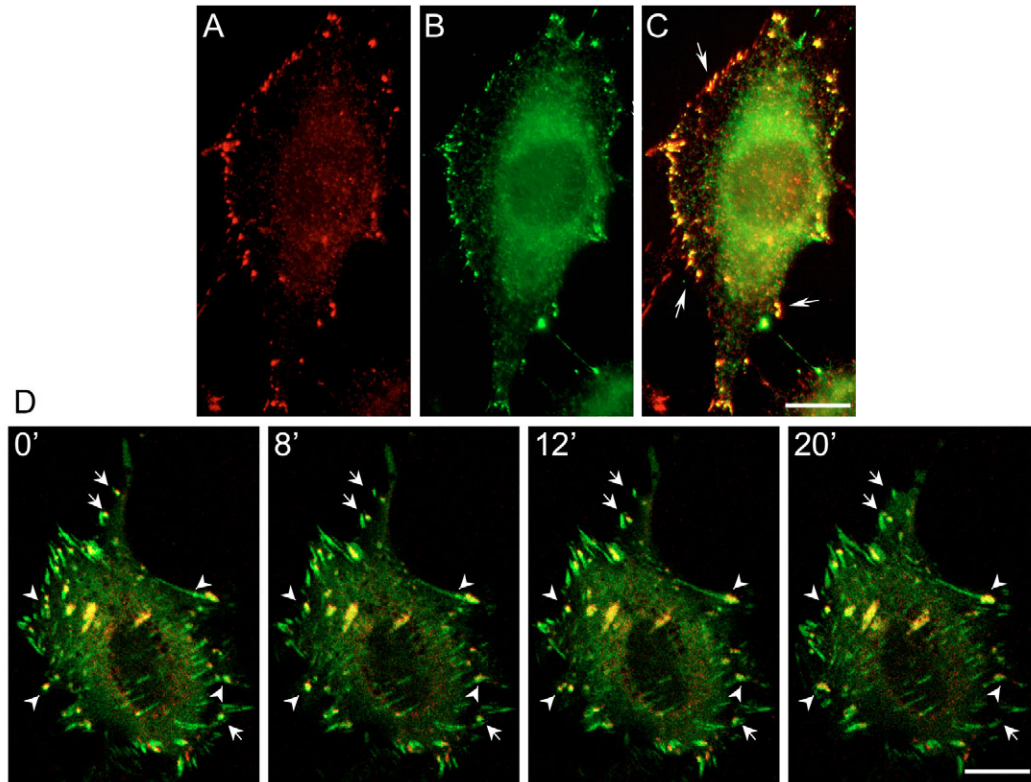
Fig. 4. PTP1B puncta co-localize with FAK in fixed and living cells. (A-C) PTP1B null cells stably expressing PTP1B DA double labeled with anti- FAK and anti-PTP1B, followed by secondary antibodies conjugated with Alexa Fluor 568 (A) and Alexa Fluor 488 (B), respectively. Note the high overlap of both proteins at the cell margins (arrows). (D) PTP1B null cells co-transfected with mRFP-PTP1B DA and GFP-FAK analyzed by time-lapse confocal microscopy after 24 hours. Note that most PTP1B DA puncta also contain FAK (in yellow), however, in instances of non co-localization, FAK has an elongated distribution. Arrows indicate puncta containing both PTP1B DA and FAK that move or disappear from the plane of focus without changing their relative fluorescence. Arrowheads indicate puncta that fuse or split. Bars, 20 μm.

4D). These observations indicate that FAK and PTP1B DA may also be present in the same complex of proteins.

Dynamic projections of ER tubules at the cell margins position full length PTP1B DA near the distal tip of cell-matrix adhesion sites

We compared the co-localization of PTP1B DA with markers of the endosomal compartment and the ER. Markers for distinct endocytic pathways, including Rab5, Rab7, dynamin K44A and caveolin 1 (Pfeffer, 2001; Pelkmans and Helenius, 2002) did not co-localize with PTP1B DA (Fig. 5A-D). However, PTP1B DA puncta co-localized with the ER marker calnexin (Fig. 5E-G). These data suggest that the positioning of PTP1B may be dependent on extension of the ER to the cell margins, a process dependent on microtubules, as demonstrated by retraction of the peripheral ER network on treatment of cells with the microtubule depolymerizing drug nocodazole (Terasaki et al., 1986; Waterman-Storer and Salmon, 1998). Indeed, we find that the puncta do not develop if nocodazole (0.5 μM) is added prior to the expression of transfected GFP-PTP1B DA in PTP1B null cells (Fig. 5I, compare with 5H). Furthermore, puncta disappeared when nocodazole (5 μM) is added after 24 hours of GFP-PTP1B DA transfection, with a halftime of ~1 hour (not shown).

In an attempt to visualize the formation and dynamics of the PTP1B DA puncta we analyzed, using time-lapse videos, the co-distribution of GFP-PTP1B DA and mRFP-paxillin after transfection into PTP1B null cells. Time-lapse series were started 30 minutes post-plating on fibronectin to facilitate the visualization of cells during active spreading. Most paxillin foci were at the extending cell margins and appeared to be accompanied by fast projections of ER tubules containing GFP-PTP1B DA (Fig. 6; Movie 1 in supplementary material). These tubules elongated and retracted along the long axis of the paxillin foci and formed transient puncta at their tips as already noted (Fig. 6A-C, arrows; see also Movie 1 in supplementary material). With time the puncta increased in size as new adjacent ER tubules fuse with the pioneer ones (Fig. 6A, 120-154 minutes; C, 114 minutes; see also Movie 1 in supplementary material). These observations suggest that GFP-PTP1B DA puncta form with the assembly of paxillin-containing foci in association with tubular extensions of the ER; however, the puncta are dynamic and can disappear independently of the turnover of the foci (Fig. 6B, 14-46 minutes, yellow arrows).

ER targeting sequences are required for localization of PTP1B at the distal tip of cell-matrix adhesion complexes

PTP1B interacts with its partners through distinct targeting sequences. A conserved proline-rich motif downstream from the catalytic domain mediates interactions with several SH3 domain-containing proteins. Substitution of two critical proline
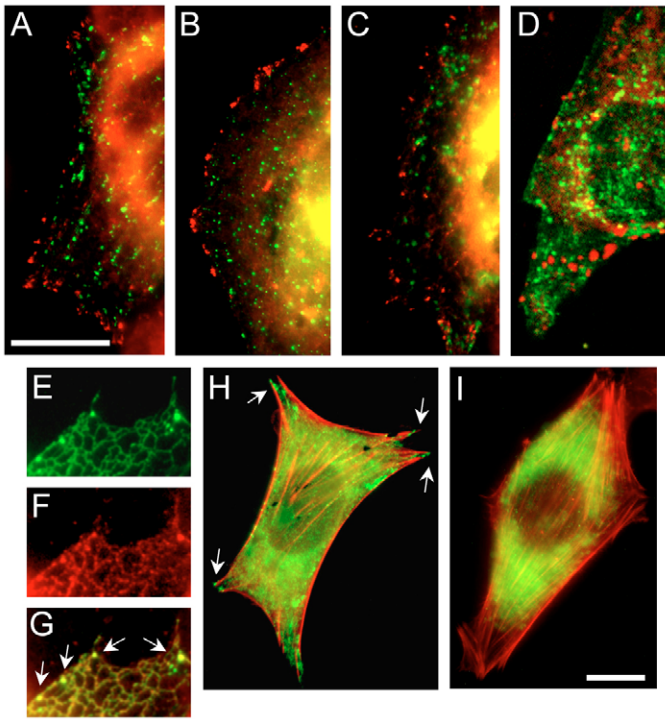
Fig. 5. PTP1B DA puncta do not co-localize with endocytic markers, but do co-localize with ER markers and require intact microtubules. PTP1B null cells stably expressing PTP1B DA were transfected with different endocytic markers: (A) the dominant-negative mutant GFP-dynamin K44A; (B) GFP-Rab 5; (C) GFP-Rab 7; (D) GFP-caveolin 1. Cells were fixed and stained with anti-PTP1B followed by a secondary antibody conjugated with Alexa Fluor 568. (E-G) PTP1B null cells were transfected with GFP-PTP1B DA (E) and stained with anti-calnexin antibody (F) followed by a secondary antibody conjugated with Alexa Fluor 568. The merged image is shown in G. PTP1B null cells were transfected with GFP-PTP1B DA in the absence (H) or presence (I) of Nocodazole (0.5 μM). After 20 hours, cells were fixed and stained with rhodamine-phalloidin. Note that the punctate structures (arrows in H) are not longer visible in the presence of nocodazole (I). Bars, (A-G) 20 μm; (H,I) 25 μm.

GFP. PTP1B DA/PA retained its localization to the ER and formed peripheral punctate structures identical to those seen with GFP-PTP1B DA. Importantly, the co-localization of PTP1B-DA/PA with cell-matrix markers (and actin, not shown) was not affected by disruption of the SH3 binding motif (Fig. 7A,B). These observations indicate that the proline-rich SH3-binding motif is not essential for the targeting of PTP1B to cell-matrix sites.

It was previously shown that PTP1B tyrosine residues 152 and 153 are required for the direct interaction with the insulin receptor (Dadke et al., 2000) and that phosphorylation of one of these tyrosine residues (152) is essential for interaction with N-cadherin (Rhee et al., 2001). We replaced these two tyrosine residues with phenylalanine (PTP1B DA/YF) and tested the resultant construct for its localization to cell-matrix adhesion

residues (Pro309/310) with alanine abrogates the in vivo interaction of PTP1B with the SH3 domain of p130^{Cas} (Liu et al., 1996). We replaced these two proline residues with alanine in PTP1B DA (PTP1B-DA/PA) and fused this construct to

Time lapse (min)
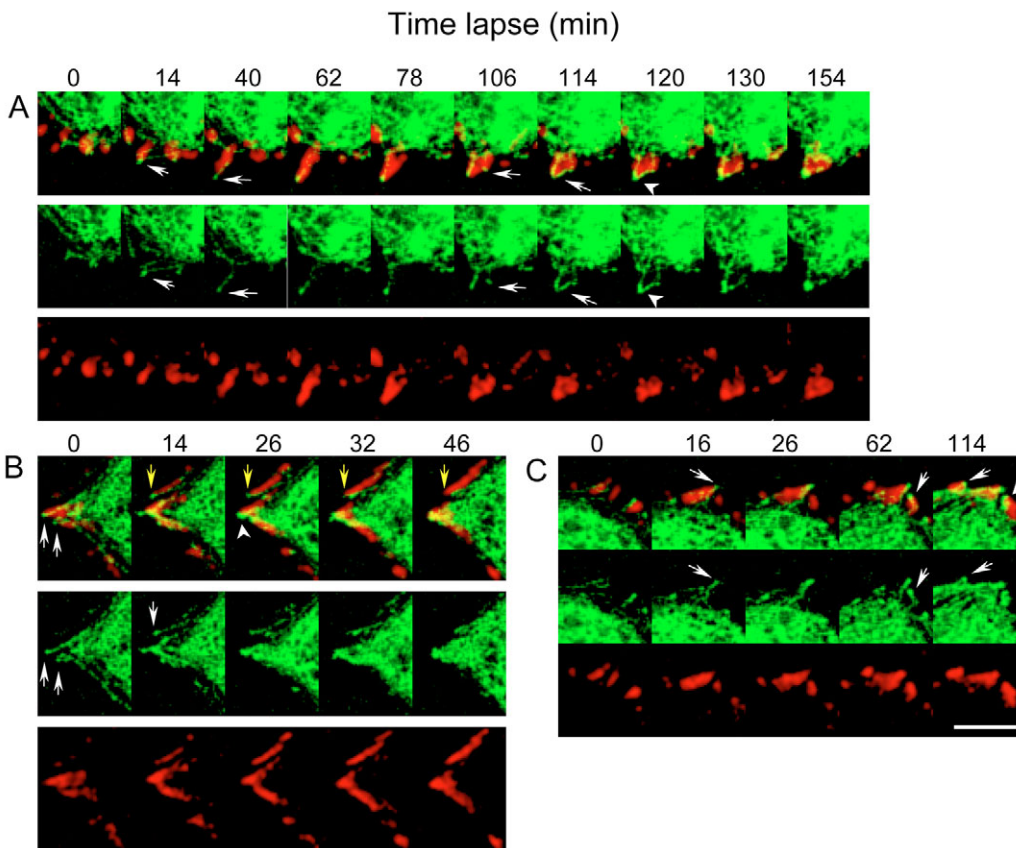
Fig. 6. PTP1B DA puncta develop from ER tubules extending over cell-matrix adhesion foci. PTP1B null cells were co-transfected with GFP-PTP1B DA and mRFP-paxillin, and 24 hours later were replated for 30 minutes on fibronectin-coated coverslips. Cells were immediately analyzed by time-lapse confocal microscopy. Different regions of a cell in selected frames of a time-lapse sequence are shown (A-C). Numbers over the time-lapse series indicate minutes elapsed. Arrows indicate punctate structures that develop from ER tubules at the tips of new paxillin foci. Yellow arrows in B indicate a punctate structure that forms and disappears, whereas the paxillin foci remain. Note that the punctate structures become larger after the arrival of additional ER tubules (arrowheads, 120 minutes in A; 26 minutes in B; 114 minutes in C). Bar, 10 μm. See also Movie 1 in supplementary material.

sites. PTP1B DA/YF targeted correctly to the ER and also formed puncta that were indistinguishable from PTP1B DA (Fig. 7C,D).

The bulk of PTP1B is associated with the cytosolic face of the ER through a hydrophobic C-terminal sequence (Frangioni et al., 1992) which may be cleaved by calpain activation (Frangioni et al., 1993; Tonks, 2003). To examine the contribution of this carboxyl-terminal ER targeting sequence in the localization of PTP1B to cell-matrix adhesion sites, we prepared two deletion constructs; one that terminated at glutamic acid 381, removing just the ER targeting sequence (PTP1B DA/t381), and a larger deletion that terminated at phenylalanine 280, which also removes the SH3 binding motif (PTP1B DA/t280). When expressed in cells derived from PTP1B null mice, both constructs show diffuse fluorescence, consistent with cytosolic localization. Examination of lamellar extensions, however, revealed a clear localization of both constructs at cell-matrix adhesion sites (Fig. 7E,F; only PTP1B DA/t280 shown). However, in contrast to the ER-anchored enzyme, both truncated forms distributed throughout the entire focal adhesion complex (Fig. 7E,F). Similar truncations of non

Fig. 7. PTP1B structural requirements for formation of cell-matrix adhesion site puncta. PTP1B null cells were transfected with GFP-PTP1B DA/PA (A,B), GFP-PTP1B DA/YF (C,D) and GFP-PTP1B DA/t280 (E,F) and fixed 20 hours later. Cells stained with anti-FAK (A,C) or anti-vinculin (B,D,F), followed by secondary antibodies conjugated with Alexa Fluor 568. Note that PTP1B DA/PA and PTP1B DA/YF form typical punctate structures (A-D, arrows) whereas PTP1B DA/t280 has lost the punctate distribution and appears throughout the entire area of the cell-matrix adhesion foci (arrows in E and F). Bar, 20 μm.
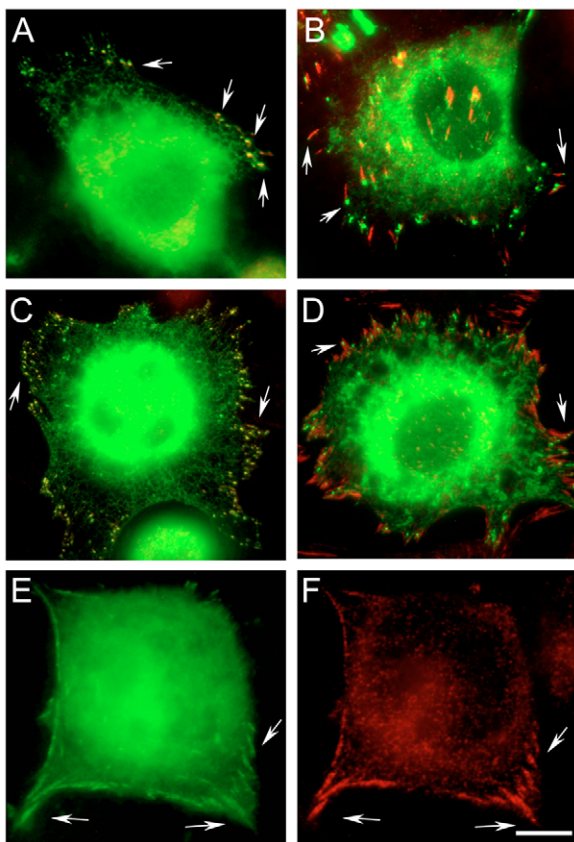
substrate-trapping PTP1B showed the diffuse distribution but lacked the accumulation at cell-matrix sites (not shown), consistent with the prior result indicating that the substrate trapping form of the enzyme is essential for visualization at focal adhesions. These results indicate that the ER-targeting sequence is not essential for gross localization at cell-matrix adhesion sites, but that it is required to achieve the restricted distribution at the distal tips of the focal adhesions.

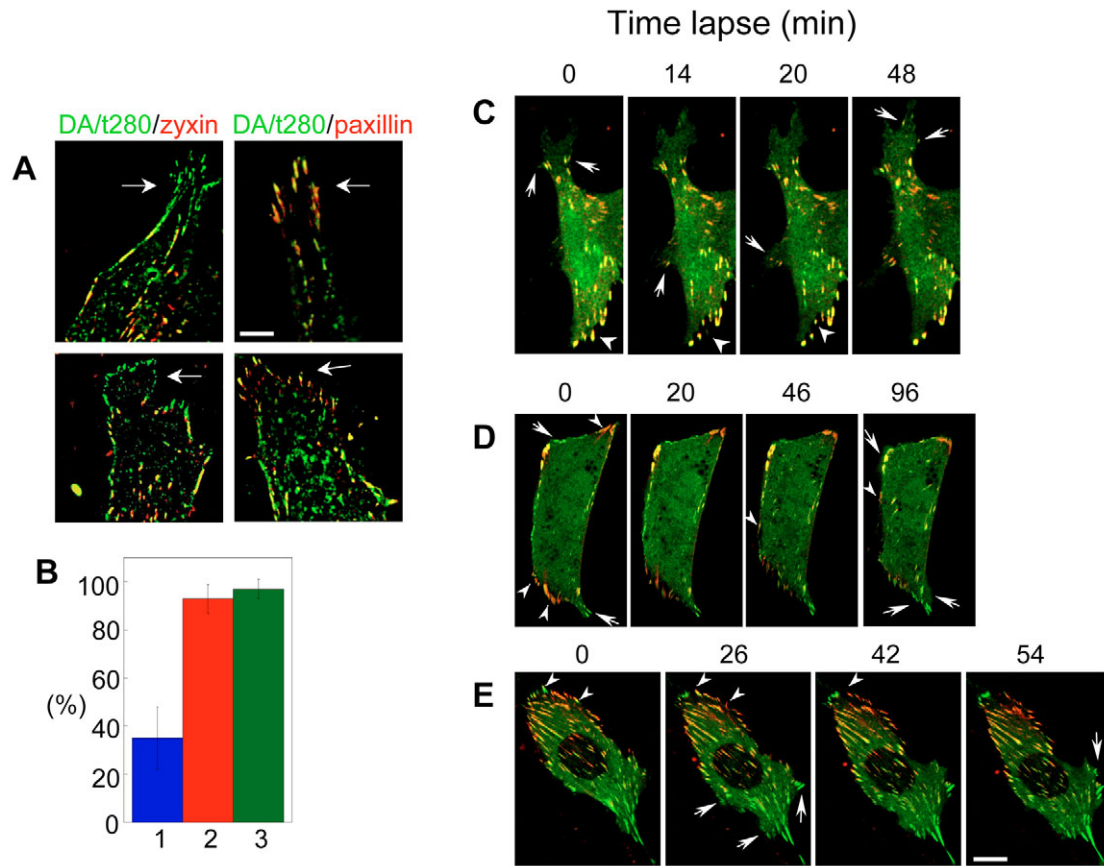## PTP1B associates with newly forming, paxillin-rich focal complexes

Cell-matrix adhesion sites differ in their molecular assemblages (Geiger and Bershadsky, 2001; Webb et al., 2003). Focal complexes are newly forming small adhesions near the cell margins and are rich in paxillin and poor in zyxin, whereas mature focal adhesions are larger and ovoid adhesions that contain a more complex set of proteins including zyxin (Laukaitis et al., 2001; Zaidel-Bar et al., 2003). To determine whether PTP1B DA/t280 associates preferentially with one or another stage of adhesion complex formation, we performed quantitative and dual time-lapse analysis of the GFP-PTP1B DA/t280 with either mRFP-paxillin or DsRed-zyxin. We used the cytosolic, non-ER-targeted form of PTP1B for this analysis because its distribution in adhesion complexes is less restricted and thus more easily visualized, and because it remains associated with cell-matrix sites for longer periods of time; both factors facilitating the dual time-lapse imaging. In lamellar extensions, approximately 93% of PTP1B DA/t280 foci were found to contain paxillin (Fig. 8A, and histogram in B, bar 2) and only 35% also contained zyxin (Fig. 8A, and histogram in B, bar 1). Most paxillin-containing foci at lamellae also contained PTP1B DA/t280 (Fig. 8B, bar 3). We that the distribution and fluorescence intensity of PTP1B DA/t280 (and PTP1B DA/t381, not shown) match tightly with that of paxillin over time, as seen by the relatively invariant yellow color in small growing foci at spreading lamella (Fig. 8C, arrows), as well as in elongated foci in more stationary regions of the cell. Furthermore, cell-matrix sites in the process of disassembly in retracting regions of the cell preserved the relative intensities of PTP1B-DA/t280 and paxillin fluorescence, suggesting a coordinated dynamic behavior for both proteins (Fig. 8C, arrowheads). By contrast, the relative fluorescence intensities of PTP1B-DA/t280 and zyxin, or paxillin and zyxin, vary according to the region of the cell. Stationary or extending regions of the cell contained foci with a predominance of PTP1B DA/t280 and paxillin fluorescence (more green, Fig. 8D,E, arrows), whereas foci in retracting regions incorporated zyxin (turning from green or yellow to red) before disassembly (Fig. 8D,E, arrowheads).

PTP1B expression is required for stabilizing cell-matrix adhesion sites at spreading lamellae

Our time-lapse experiments show that PTP1B DA puncta appear and grow at the tips of forming cell-matrix adhesion sites. The preferential association of PTP1B DA and PTP1B DA/t280 with paxillin foci as compared to zyxin foci suggests that PTP1B activity may contribute to formation and/or stabilization of cell-matrix adhesion sites and thus promote spreading. Consistent with this view, cells derived from the PTP1B null mice spread poorly (Cheng et al., 2001) (our unpublished observations) and L-cells transfected with a

Fig. 8. PTP1B DA/t280 co-localizes preferentially with paxillin-containing foci in lamellae. PTP1B null cells were co-transfected with GFP-PTP1B DA/t281 and mRFP-paxillin, GFP-PTP1B DA/t280 and DsRed-zyxin, or GFP-paxillin and DsRed-zyxin. 20 hours post-transfection cells were fixed and analyzed by fluorescence microscopy (A,B), or replated on fibronectin-coated coverslips and analyzed by time-lapse fluorescence confocal microscopy 24 hours later (C-E). Numbers over the frames indicate the time elapsed. Note that most PTP1B DA/t280 foci in lamellar extensions contain paxillin but not zyxin (A, arrows). Approximately 93% of PTP1B DA/t280 foci contain paxillin (B, bar 2) and about 35% also contain zyxin (B, bar 1). Most paxillin foci also contain PTP1B DA/t280 (B, bar 3). Results shown are the mean ± s.d. of three independent experiments (*n*=30). Time-lapse analysis reveals that the ratio of fluorescence intensities for GFP-PTP1B DA/t280 and mRFP-paxillin (indicated by the yellow color) does not change significantly over time. New foci at extending lamellae (C, arrows), and foci that disassemble at retracting regions of the cell (C, arrowheads) maintain constant relative fluorescence intensities of PTP1B and paxillin. In contrast to paxillin, zyxin and PTP1B DA/t280 do not consistently overlap (D, arrows). New PTP1B DA/t280-rich foci are usually devoid of zyxin (D, arrows). Foci at retracting sites frequently incorporate zyxin before disassembling (revealed by a change of color from green or yellow to red, arrowheads). Co-localization of paxillin and zyxin is partial (E). Foci at extending parts of the cell are rich in paxillin foci (arrows) and foci in retracting lamellae change from paxillin-rich (green or yellow) to zyxin-rich (red). Bars, (A) 20 μm; (C-E) 30 μm.

dominant negative form of PTP1B also show less spreading than control cells (Arregui et al., 1998). To directly visualize this phenomena in living cells, and to determine if the spreading defect is due to the inability to produce lamellar extensions or to their stabilization, we compared the formation and stabilization of cell-matrix adhesion sites and lamellar extensions among PTP1B null cells and cells reconstituted with wild-type PTP1B after transfection with GFP-labeled vinculin and GFP-paxillin. Both cell lines were able to extend lamellae; however, cells reconstituted with wild-type PTP1B quickly stabilized adhesions by forming small cell-matrix sites (Fig. 9, wt). By contrast, lamellar extensions formed by the PTP1B null cells sometimes formed adhesion sites, but these quickly disappeared along with the retraction of the lamella (Fig. 9, null). To examine if the small cell-matrix sites in the PTP1B null cells are defective in incorporating zyxin, a marker of more mature focal adhesions (Laukaitis et al., 2001;

Zaidel-Bar et al., 2003), we determined the percentage of paxillin-containing foci that also contained zyxin in the PTP1B null cells and the cells reconstituted with the wild-type PTP1B. Interestingly, we found that PTP1B null cells exhibited a 30% reduction in paxillin-containing foci that also contained zyxin (Fig. 9, histogram). These observations indicate that PTP1B null cells are defective in lamellar stabilization.

Discussion

PTP1B is possibly the most ubiquitous tyrosine phosphatase and as such has been implicated in many distinct cellular processes that require precise targeting to potential cellular substrates. Consistent with this, several distinct targeting motifs have been identified in addition to the substrate recognition motif. Here, we provide new insight into the targeting and role of PTP1B at cell-matrix adhesion sites.
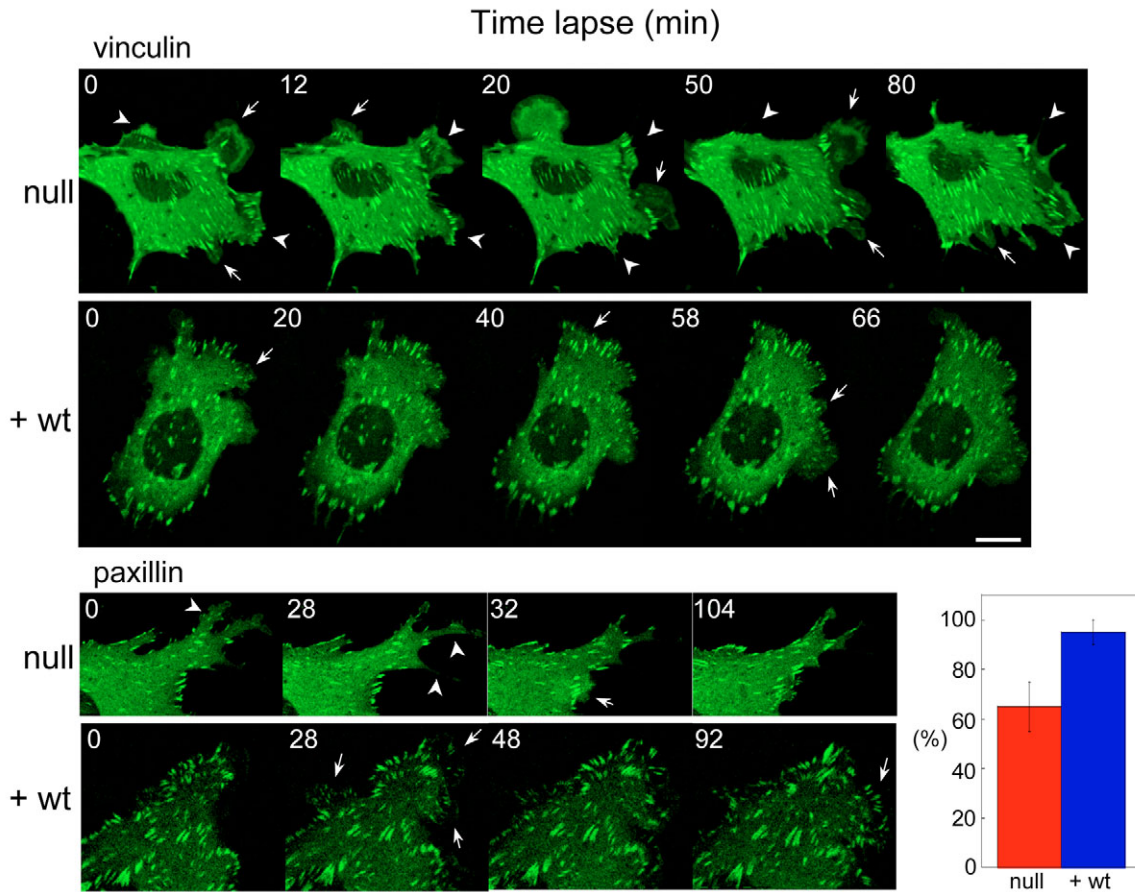
Fig. 9. Lamellar extensions in cells lacking PTP1B are unstable and contain immature cell-matrix sites. PTP1B null cells (null) and cells reconstituted with wild-type PTP1B (+wt) were transfected with GFP-vinculin or GFP-paxillin and analyzed by time-lapse confocal microscopy. Note that PTP1B null cells produce lamellar extensions and assemble vinculin and paxillin foci (arrows); however, the lamellae quickly retract (arrowheads). By contrast, cells expressing wild-type PTP1B extend lamellae and assemble vinculin and paxillin foci that are persistent in time (arrows). Bar: 30 μm. The percentage of paxillin foci that also contain zyxin in PTP1B null cells and cells stably expressing wild-type PTP1B is shown in the histogram in the bottom, right panel. Results shown are the mean ± s.d. of three independent experiments (*n*=44 PTP1B null cells; *n*=39 cells expressing wild-type PTP1B). The number of cell-matrix sites per cell ranged from 50-100.

The subcellular targeting of PTP1B

PTP1B associates with the ER through a C-terminal hydrophobic sequence with the catalytic domain facing the cytosol (Frangioni et al., 1992). A cytosolic species of PTP1B may also result from removal of the hydrophobic sequence by calcium-activated proteases of the calpain family (Frangioni et al., 1993). PTP1B has been shown to dephosphorylate several cell surface receptor tyrosine kinases, including the insulin receptor (IR), epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR) (Tonks, 2003). Recent work also indicates that a cleaved form of PTP1B associates with N-cadherin (Balsamo et al., 1998) and that the full-length form interacts with integrin complexes (Arregui et al., 1998). In both of these latter cases PTP1B dephosphorylates components of these adhesion complexes with significant consequences for their adhesive, as well as signaling, function (Arregui et al., 2000; Lilien et al., 2002; Larsen et al., 2003; Lilien and Balsamo, 2005).

What is the cellular compartment in which PTP1B interacts with and dephosphorylates its substrates has been a recurrent question. Recent BRET and FRET studies showed that the substrate-trapping mutant PTP1B D181A (PTP1B DA) interacts with endocytosed IR, EGFR and PDGFR (Haj et al., 2002; Boute et al., 2003; Romsicki et al., 2004), although at least for the IR and the erythropoietin receptor, the evidence also points to an association with the PTP1B DA during their biosynthetic pathway (Boute et al., 2003; Cohen et al., 2004).

In the current study we showed that the substrate trapping PTP1B DA labeled with GFP (GFP-PTP1B DA) is easily visualized in the ER and at cell-matrix adhesion sites using fluorescence microscopy. Wild-type GFP-PTP1B appears largely associated with the ER, however, the substrate-trapping mutant PTP1B DA appears in punctate structures at the distal tips of cell-matrix sites, presumably reflecting the trapping of substrates at these sites. These punctate structures do not contain Rab5, Rab7, dynamin K44A or caveolin 1, indicating that they are not part of an endocytic compartment; they do co-localize with calnexin, an integral ER membrane chaperone (Schrag et al., 2003). Dual-color time-lapse microscopy also revealed that the formation of these punctate structures is associated with ER tubules that project over cell-matrix sites. Consistent with these observations we found that two deletion

constructs that lack the ER targeting sequence, PTP1B DA/t281 and PTP1B DA/t381, distribute diffusely in the cytosol and in the entire area of adhesion sites, a distribution easily distinguished from the specific localization of PTP1B DA at the distal tip of cell-matrix adhesion sites. Our results suggest that the intrinsic dynamics of the ER positions PTP1B at the distal tips of adhesion sites. This idea is further supported by two additional observations: (1) treatment with nocodazole abolishes the formation of the punctate structures at the peripheral cell-matrix sites, consistent with the role of microtubules in extension of the ER (Terasaki et al., 1986; Waterman-Storer and Salmon, 1998); and (2) the direct visualization of the genesis of the punctate structures over cell-matrix sites as they form during cell spreading. Since the substrate trapping version of PTP1B is required for visualization at cell-matrix adhesion sites, we presume that the enzyme is positioned via interaction with its substrates, also localized specifically at the distal tips of adhesion sites (see below).

Analysis of the subcellular distribution of PTP1B DA with deletions of other known targeting motifs further substantiates the conclusion that it is the interaction with specific substrates that is responsible for localization at the distal tips of adhesion sites. One candidate targeting sequence is the proline-rich motif in PTP1B which acts as a ligand for the SH3 domain of p130[cas] (Liu et al., 1996). It was previously shown that neutralizing mutations of this motif impair many of the integrin-dependent effects regulated by PTP1B (Liu et al., 1998; Dadke and Chernoff, 2002). We find that neutralization of the proline-rich motif in PTP1B DA, or its deletion (PTP1B DA/t280), does not prevent localization at cell-matrix sites. Thus, interaction with SH3 domains is not required for the incorporation of PTP1B DA at cell-matrix sites. However, we cannot rule out the possibility that SH3 domain-mediated interactions contribute to stabilization of PTP1B at cell-matrix sites.

Potential substrates of PTP1B in cell-matrix sites

The formation of enzyme-substrate complexes at cell-matrix sites appears to be largely responsible for the localization of PTP1B at the distal tips of cell-matrix adhesion sites; our data imply that Src is a primary substrate. We and others have reported that PTP1B dephosphorylates and activates Src (Arregui et al., 1998; Liu et al., 1998; Cheng et al., 2001). Furthermore, it has been shown that in fibroblasts, briefly plated on fibronectin, Src redistributes from the perinuclear region to the margins of spreading lamellae and becomes transiently activated (Kaplan et al., 1995). We found that Src co-localizes with the PTP1B DA puncta at the margins of lamellar extensions, and can be isolated in a complex with PTP1B by immunoprecipitation (Fig. 3), consistent with a recent report (Liang et al., 2005). A reasonable assumption is that these puncta and those observed at the distal poles of cell-matrix sites form as a consequence of the stable interactions of the substrate-trapping version of PTP1B with Src. Novel methods for visualizing interactions at the nanometer range, such as fluorescence resonance energy transfer (Sekar and Periasamy, 2003) and bimolecular fluorescence complementation (Hu and Kerppola, 2002) would be valuable for testing the direct interaction between PTP1B and Src in cell-matrix sites. We are in fact pursuing these studies at this time.

Further reinforcing the idea that Src is a/the prominent substrate of PTP1B at cell-matrix adhesion sites is the finding that cells derived from mice null for each of these enzymes have overlapping defects. Cells derived from mice null for Src and FAK are defective in spreading (Ilic et al., 1995; Klinghoffer et al., 1999) suggesting that these proteins play a key role in the maturation or stabilization of cell-matrix adhesion sites. This is consistent with our previous data showing that expression of a dominant-negative PTP1B in L-cells results in decreased Src activity, and defects in integrin-mediated adhesion and spreading (Arregui et al., 1998). Delayed spreading is also seen in cells derived from mice null for PTP1B (Cheng et al., 2001). Our time-lapse observations also indicate that, whereas cells derived from mice null for PTP1B have the capacity to assemble cell-matrix sites, these sites are less stable and lamellar extensions tend to retract quickly. Furthermore, the percentage of paxillin foci that also contain zyxin (as a marker of more complex and mature cell-matrix adhesion sites) in the PTP1B null cells is consistently lower than that in the cells reconstituted with the wild-type PTP1B.

The role of PTP1B at cell-matrix adhesion sites

What would be the consequence of activation of Src by PTP1B in cell-matrix sites? After integrin stimulation, Src phosphorylates and forms signaling complexes with FAK (Hanks et al., 2003; Parsons, 2003; Mitra et al., 2005). By time-lapse microscopy we found that mRFP-PTP1B DA and GFP-FAK are constituents of a common dynamic compartment (Fig. 4). One of the roles of PTP1B in cell-matrix sites may be the activation of the Src-FAK signaling complex (see also Arregui et al., 1998; Liang et al., 2005), an event that may contribute to the maturation of cell-matrix sites from paxillin-rich to zyxin-rich foci. Time-lapse analyses showed that PTP1B DAt/281 co-localizes preferentially with paxillin-rich foci than with zyxin-rich foci. Furthermore, the percentage of paxillin foci that also contain zyxin (as a marker of more complex and mature cell-matrix adhesion sites) in the PTP1B null cells is consistently lower than that in the cells reconstituted with the wild-type PTP1B.

In conclusion, the observations presented here indicate that PTP1B is localized to the distal tip of newly forming cell-matrix adhesion sites through the dynamic activity of the ER and associated microtubules, and that it plays a positive role in the maturation of new cell-matrix adhesion sites.

Materials and Methods

monoclonal anti-PTP1B antibody (3 hours at 4°C), and agarose-conjugated anti-mouse antibody (1.5 hours). After washing with lysis buffer, the immunocomplexes were boiled in SDS-PAGE sample buffer. Supernatants were fractionated by SDS-PAGE and transferred to polyvinyl difluoride membranes. Blots were initially probed with a polyclonal anti-PTP1B and revealed by enhanced chemiluminescence (ECL). Then, blots were stripped (30 minutes at 55°C) with TBS containing 5% 2-mercaptoethanol and 2% SDS, and re-probed with a polyclonal anti-Src antibody.

For the western blot shown in Fig. 1 whole cell extracts were prepared as above and equivalent amounts of soluble protein (~30 µg) were loaded per lane.

Immunofluorescence

Cells grown on fibronectin-coated coverslips (20 µg/ml) were fixed with 4% paraformaldehyde in PBS for 20 minutes, permeabilized with 0.5% Triton X-100 in PBS for 10 minutes, and blocked with 3% BSA in PBS for 60 minutes. After incubation with primary (overnight at 4°C) and fluorescent-conjugated secondary antibodies (45 minutes), the cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and analyzed using a Nikon E600 microscope (Melville, NY, USA) with a 100×/1.3 NA objective coupled to a Spot RT Slider CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA), or with a Bio-Rad MRC 1024 laser scanning confocal microscope (Hercules, CA, USA) with a 60×/1.4 NA objective. For quantitative analysis in Figs 8 and 9, paired, two-color images were acquired, adjusting the exposure times to obtain the maximal dynamic range without saturating the detector. These images were analyzed with the ImageJ software (Wayne Rasband, NIH, Bethesda, MD, USA) to determine co-localization. The intensity of the fluorescence signal in the positive scores was at least twice that of the surrounding area.

DNA constructs

The substrate trapping PTP1B D181A (PTP1B DA) was obtained by PCR using chick PTP1B (Balsamo et al., 1998) as a template. The PCR product was cloned at the C terminus of enhanced GFP (pEGFP-C3; Clontech BD Biosciences, Mountain View, CA, USA) between *Xho*I and *Xma*I sites. Three additional point mutants were performed using the GFP-PTP1B DA as a template: PTP1B DA/PA, in which prolines 309 and 310 were replaced by alanines; PTP1B DA/YF, in which tyrosines 152 and 153 were replaced by phenylalanines, and PTP1B DA/QA, in which glutamine 262 was replaced by alanine. We also prepared, by PCR, two C-terminal deletions of the GFP-PTP1B DA: PTP1B DA/t381 and PTP1B DA/t280, in which PTP1B was truncated after glutamic acid 381 and after phenylalanine 280, respectively. These truncations were cloned between *Xho*I and *Kpn*I sites. All mutations were confirmed by DNA sequencing. To prepare the PTP1B DA fused to the C terminus of monomeric red fluorescent protein (mRFP) (Campbell et al., 2002), the mRFP cDNA (provided by R. Tsien, Howard Hughes Medical Institute, University of California, San Diego, CA, USA) was amplified by PCR and cloned into the *Nhe*I/*Xho*I sites of the pEGFP-C3-PTP1B DA plasmid replacing the EGFP with the mRFP. GFP-paxillin was provided by K. Yamada (National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD, USA) and GFP-FAK was provided by D. Webb (Department of Cell Biology, University of Virginia, Charlottesville, VA, USA). To prepare mRFP-paxillin, the paxillin cDNA was amplified by PCR and cloned in the *Xho*I/*Kpn*I sites of the plasmid pmRFP-C1 (provided by Q. Wang and S. Green, Department of Biological Sciences, University of Iowa, Iowa City, IA, USA), replacing the EGFP with mRFP. GFP-vinculin was provided by T. Volberg and B. Geiger (Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel). DsRed-zyxin was provided by A. Huttenlocher (Department of Pediatrics and Pharmacology, University of Wisconsin, Madison, WI, USA). Plasmids encoding GFP-dynamin K44A, GFP-Rab5 and GFP-Rab7 were provided by Dr J. Martina (National Institute of Child Health and Human Development, NIH, Bethesda, MD, USA). Caveolin-1-GFP was provided by M. Parat (Department of Cell Biology, Cleveland Clinic Foundation, The Lerner Research Institute, Cleveland, OH, USA).

Cell culture and DNA transfection

The generation of the PTP1B null cell line, and those reconstituted with the wild-type PTP1B or the substrate trapping PTP1B DA have been previously described (Klaman et al., 2000; Haj et al., 2002) and were provided by Benjamin Neel (Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA). The control fibroblast cell line used in Fig. 1 was provided by P. Soriano (Fred Hutchinson Research Center, Seattle, WA, USA) and prepared as described previously (Klinghoffer et al., 1999). All cells were cultured in high glucose DMEM containing L-glutamine, supplemented with 10% fetal bovine serum, penicillin and streptomycin (Invitrogen Corp, Carlsbad, CA, USA). Transient transfections of PTP1B null cells were performed in 24-well tissue-culture plates using lipofectamine 2000 (Invitrogen Corp, Carlsbad, CA, USA) and 1.5 µg (single transfections) or 3 µg (equimolar, for double transfections) DNA/well.

Time-lapse imaging

Twenty hours after transfection, cells were resuspended with trypsin and seeded at 30% confluency on fibronectin-coated coverslips attached to the bottom of 60 mm dishes. Single-color time-lapse imaging was performed in cells transfected with GFP-fusion proteins. Dual-color time-lapse imaging was performed in cells co-transfected with GFP and mRFP- or DsRed-fusion proteins. Imaging medium was phenol red-free, high-glucose Dulbecco's modified Eagle's medium containing 4 mM L-glutamine and 25 mM Hepes buffer (Invitrogen Corp, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum and antibiotics. The medium was maintained at 36°C by warm, forced air. Cells were observed using a DMIRE2 Leica inverted microscope with a 63×/1.4 NA oil objective. Confocal images were acquired every 2 minutes on a Leica SP2 AOBS confocal microscope, at a scanning rate of 400 Hz and in a 512×512 pixel array format. Pinhole size was set at 2 Airy disks to improve light collection and PMT gain and offset was set so that at the starting point the highest signal was just below the detector saturation and the background was slightly above the detection floor. For single-color image acquisition the 488-nm line of the Argon laser was used, and the 500-600 nm emission was collected. For dual-color time-lapse analysis, the images were acquired using the line-interlaced sequential excitation mode, with the 488-nm line of the Argon laser for GFP (500-535 nm emission collected) and the 543-nm line of the helium/neon laser for DsRed and mRFP (555-700 nm emission collected). The lasers were used at minimum power and at 25% transmission. Under our experimental conditions we did not detect significant photo-bleaching. Image sequences were built using the ImageJ software (Wayne Rasband, NIH, Bethesda, MD, USA).

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