

Tyrosine phosphorylation of the CrkII adaptor protein modulates cell migration

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Summary

CrkII belongs to a family of adaptor proteins that become tyrosine phosphorylated after various stimuli. We examined the role of CrkII tyrosine phosphorylation in fibronectin-induced cell migration. Overexpression of CrkII inhibited dephosphorylation of focal adhesion components such as p130 Crk-associated substrate (p130^{cas}) and paxillin by protein tyrosine phosphatase 1B (PTP1B). Tyrosine-phosphorylated CrkII was dephosphorylated by PTP1B both in vitro and in vivo, showing for the first time that PTP1B directly dephosphorylates CrkII. A CrkII mutant in which tyrosine residue 221 was substituted by phenylalanine (CrkII-Y221F) could not be tyrosine phosphorylated, and it showed significantly increased binding to p130^{cas} and paxillin. Enhanced binding of CrkII to p130^{cas} has been

reported to promote cell migration. Nonphosphorylated CrkII-Y221F promoted HT1080 cell migration on fibronectin, whereas wild-type CrkII did not at moderate expression levels. Moreover, co-expression of CrkII and PTP1B promoted HT1080 cell migration on fibronectin and retained tyrosine phosphorylation and binding of p130^{cas} to CrkII, whereas paxillin tyrosine phosphorylation was reduced. These findings support the concepts that CrkII binding activity is regulated by tyrosine kinases and phosphatases, and that tyrosine phosphorylation of CrkII can downmodulate cell migration mediated by the focal adhesion kinase/p130^{cas} pathway.

Key words: CrkII, PTP1B, Cell migration, Tyrosine phosphorylation, Phosphatase

Introduction

Crk was originally isolated as an oncogene product of the CT10 chicken retrovirus. It belongs to a group of adaptor proteins that are comprised of Src homology 2 (SH2) and Src homology 3 (SH3) domains, which interact with phosphotyrosine and proline-rich regions, respectively (Mayer et al., 1988). The SH2 domain of Crk can bind to phosphotyrosine-containing proteins such as the focal adhesion components paxillin, p130 Crk-associate substrate (p130^{cas}) and growth factor receptors. The N-terminal SH3 domain of Crk has been shown to bind to C3G (a guanine nucleotide exchange factor/GEF for Rap1), Sos (GEF for Ras), DOCK180 (which activates Rac1 after binding to CrkII-p130^{cas} complexes), the protein tyrosine kinase c-Abl, protein tyrosine phosphatase 1B (PTP1B), the phosphatidylinositol 3-kinase p85 regulatory subunit and also c-Jun N-terminal kinase (JNK) (Feller et al., 1998; Girardin and Yaniv, 2001). The C-terminal SH3 domain of CrkII has been recently reported to interact with the nuclear export receptor Crm1 (Smith et al., 2002). CrkII has been implicated in integrin-mediated cell migration by forming a complex with tyrosine-phosphorylated p130^{cas} (Klemke et al., 1998; Kain and Klemke, 2001).

CrkII is known to be tyrosine phosphorylated after many types of stimulation (Feller et al., 1998). Most of the total population of CrkII molecules is tyrosine phosphorylated in

HeLa cells, but less than 40% of total CrkII is phosphorylated on tyrosine 221 in various human cell lines (Hashimoto et al., 1998). The tyrosine-phosphorylation site of CrkII is known to serve as a high-affinity binding site for the SH2 domain of CrkII, resulting in intramolecular binding of the SH2 domain to phosphotyrosine 221 (Rosen et al., 1995). This intramolecular binding results not only in a blockade of CrkII SH2-mediated binding to phosphotyrosine residues in other molecules, but also in reduced affinity for C3G due to masking or modification of the CrkII central SH3 domain (Okada et al., 1998).

Tyrosine phosphorylation of CrkII may induce negative regulation. Substitution of tyrosine 221 to phenylalanine greatly reduces the tyrosine phosphorylation of CrkII, yet it increases the binding activity of CrkII to other phosphorylated proteins (Escalante et al., 2000). Abl family tyrosine kinases phosphorylate tyrosine 221 on CrkII, resulting in decreased CrkII-to-p130^{cas} association; Abl kinase also inhibits cell migration (Kain and Klemke, 2001). CrkI, which is an alternative splicing variant and lacks tyrosine 221 and the C-terminal SH3 domain of CrkII, is upregulated and promotes cell migration and invasion in glioblastoma (Takino et al., 2003). However, it is not yet clear whether tyrosine-phosphorylated CrkII is dephosphorylated by cellular phosphatases, nor how crucial phosphorylation of CrkII at this specific site is to the process of cell migration.

In the present study, we examined whether PTP1B could dephosphorylate CrkII. PTP1B was able to directly dephosphorylate CrkII, as well as its SH2 binding proteins p130^{cas} and paxillin. A CrkII mutant with tyrosine residue 221 substituted by phenylalanine (CrkII-Y221F) could not be tyrosine phosphorylated, and it showed significantly increased binding to p130^{cas} and paxillin. The CrkII-Y221F mutant, but not wild-type CrkII, promoted cell migration on fibronectin concomitant with increased binding to p130^{cas} and its SH3-binding proteins. This CrkII-Y221F-induced cell migration was suppressed by co-transfecting with dominant-negative (DN) Rac1 but not wild-type Rac1. Moreover, co-expression of CrkII and PTP1B promoted HT1080 cell migration on fibronectin, whereas expression of either regulatory protein alone did not. These findings provide evidence that the regulation of CrkII activity by tyrosine phosphatase/kinase targeting of tyrosine 221 is an important factor in cell migration mediated by the focal adhesion kinase (FAK)/p130^{cas}/CrkII pathway.

Materials and Methods

Expression plasmids

Green fluorescent protein (GFP) expression plasmids based on pGZ21δxZ that contained no insert, full-length wild-type chicken PTP1B or dominant-negative p130^{cas} (psSRα-DN-p130^{cas}) were constructed as described previously (Tamura et al., 1999a; Tamura et al., 1999b). The pRK-VSV-CrkII (where VSV is vesicular stomatitis virus epitope) and pcDNA-CrkII expression plasmids were generated by transferring the CrkII cDNA insert from pCEFL-GST-CrkII to pRK-VSV and pcDNA3 after *Bam*HI and *Not*I digestion. The CrkII SH2 mutant (CrkII-R38L), the CrkII N-terminal SH3 mutant (CrkII-W169L) and CrkII-Y221F were generated by substitutions of arginine 38 to leucine, tryptophan 169 to leucine, and tyrosine 221 to phenylalanine using the QuikChangeTM site-directed mutagenesis kit (Stratagene), respectively, and then they were transferred to pcDNA3 (Invitrogen) expression vectors. Catalytically inactive PTP1B (PTP1B-C215S) was constructed by substitution of cysteine 215 within the consensus PTP catalytic domain to serine. The pHA262pur puromycin-resistance plasmid was kindly provided by Hein te Riele (Netherlands Cancer Institute, Amsterdam, The Netherlands).

Antibodies and reagents

Mouse monoclonal anti-phosphotyrosine (PY20), anti-p130^{cas}, anti-Crk, anti-FAK (focal adhesion kinase), and anti-paxillin antibodies were purchased from Transduction Laboratories. Rabbit polyclonal anti-p130^{cas} (C-20), anti-CrkII and anti-GFP antibodies were from Santa Cruz Biotechnology. Mouse monoclonal anti-α-tubulin, anti-VSV glycoprotein and anti-GST antibodies, and Dulbecco's modified Eagle's medium (DMEM) were from Sigma-Aldrich. Mouse monoclonal anti-PTP1B antibody was from Calbiochem. Cy3-conjugated mouse immunoglobulin G from Jackson ImmunoResearch Laboratories and rhodamine-labeled phalloidin from Molecular Probes were used at 1:1000 dilution. Recombinant human PTP1B was from Upstate Biotechnology.

Cell culture and transfection

The human embryo kidney 293-EBNA cell line was purchased from Invitrogen. HT1080 cells were obtained from ATCC. Cells were maintained in DMEM supplemented with 5% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin and cultured in 5% CO₂ at 37°C. HT1080 cells were seeded at 5×10⁴ cells/ml and 293-EBNA cells at 1×10⁵ cells/ml at 24 hours before transfection.

Transient transfections were performed by standard calcium phosphate methods.

Silencing of endogenous PTP1B with small interfering RNA (siRNA)

Purified, duplexed siRNA for PTP1B and β-actin were purchased from Dharmacon. The siRNA sequence targeting human PTP1B (GenBank accession number M31724) was from position 799-819. Twenty microlitres of siRNA (20 µM) plus 1 µg of pcDNA3 and 1 µg of pRK-GFP plasmids were transfected into 293-EBNA cells cultured in 60 mm diameter dishes by calcium phosphate co-precipitation. At 36 hours after transfection, the cells were trypsinized, suspended in DMEM containing 1 mg/ml bovine serum albumin (BSA) for 20 minutes, and replated on fibronectin-coated dishes for the indicated periods.

Immunoprecipitation and immunoblotting

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and homogenized in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF, 1% NP-40, 0.25% sodium deoxycholate and protease inhibitor cocktail (Boehringer Mannheim, Germany). Cell lysates were centrifuged at 15,000 *g* for 15 minutes at 4°C to remove insoluble material. Protein concentrations of lysates were determined using a bicinchoninic acid protein assay kit (Pierce) and samples were adjusted to equal protein concentration and volume. The samples were used for immunoprecipitation with the indicated antibodies for 2 hours at 4°C followed by sedimentation with GammaBind Plus SepharoseTM (Amersham Pharmacia Biotech). The immunoprecipitates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with 3% BSA in TBS-T (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) for 1 hour at room temperature (RT), then probed with the indicated antibodies for 2 hours at RT. After washing the membranes in three 10 minute washes with TBS-T, the membranes were incubated with horseradish peroxidase-linked secondary antibody (Amersham Pharmacia Biotech) for 1 hour at RT followed by enhanced chemiluminescence detection using SuperSignalTM (Pierce). The membranes were then stripped with 2% SDS, 100 mM β-mercaptoethanol in 62.5 mM Tris-HCl, pH 6.8, for 20 minutes at 70°C. Stripped membranes were washed extensively in TBS and placed in 3% BSA blocking buffer overnight, and then were re-probed with another antibody as indicated.

Protein phosphatase assay

PTP1B dephosphorylation of CrkII was examined using an in-blot phosphatase assay. In brief, phosphorylated CrkII and FAK were obtained from immunoprecipitates with anti-VSV antibody from lysates of 293-EBNA cells co-transfected with VSV-FAK and VSV-CrkII. Immunoprecipitated FAK and CrkII were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membrane was incubated with 20 U/ml recombinant PTP1B in assay buffer containing 25 mM HEPES (pH 7.2), 50 mM NaCl, 2.5 mM EDTA, and 5 mM dithiothreitol (DTT) at 37°C for 30 minutes. The phosphorylation state of FAK and CrkII was determined with anti-phosphotyrosine antibody (PY20). The membrane was then re-probed with anti-VSV antibody.

Cell motility

HT1080 cells were co-transfected with 2 µg pcDNA-CrkII-WT, pcDNA-CrkII-W169L or pSSRα-DN-p130^{cas}, or either 0.5 µg pcDNA-CrkII-WT or pcDNA-CrkII-Y221F together with 0.5 µg GFP

(pGZ218xZ) and pHA262pur puromycin-resistance plasmids. The cells were subcultured at a 1:3 dilution 12 hours after transfection and maintained for 36 hours in 1.5 $\mu\text{g/ml}$ puromycin-containing medium. This selection for transient transfectants routinely resulted in 90% positive cells expressing GFP as determined by fluorescence microscopy. After puromycin selection, cells expressing various constructs were washed twice with DMEM containing 1 mg/ml BSA and replated on 35 mm glass-bottom microwell dishes (MatTek) coated with 10 $\mu\text{g/ml}$ fibronectin; the cells were cultured overnight in the same serum-free medium. Cell movements were monitored using Zeiss inverted microscopes. Video images were collected with Newvicon cameras (model 2400; Hamamatsu Photonics) at 20 minute intervals, digitized and stored as image stacks using MetaMorph 3.5 software (Universal Imaging). Image stacks were converted to QuickTime movies, and the positions of nuclei were tracked to quantify cell motility using Move-tr/2D software (Library, Tokyo, Japan).

Alternatively, migration assays were performed in 48-well chemotaxis chambers (Neuro Probe). Cells selected by puromycin were trypsinized, kept in suspension for 20 minutes to recover from the trypsinization, then added to the upper wells of chambers separated from the lower wells by a polycarbonate membrane coated with 10 $\mu\text{g/ml}$ fibronectin. Cells were allowed to migrate for 10 hours at 37°C. The membrane was fixed, stained and scanned before and after wiping the upper side to remove non-migrating cells.

Immunofluorescence staining

Glass coverslips (12 mm diameter) were coated with 10 $\mu\text{g/ml}$ fibronectin in PBS overnight at 4°C, and then blocked with 5 mg/ml BSA for an additional 1 hour at 37°C. After puromycin selection, cells expressing GFP-PTP1B were washed twice with DMEM containing 1 mg/ml BSA and replated on the coverslips, and were then cultured for 2 hours in DMEM containing 1 mg/ml BSA. The cells were fixed with 4% paraformaldehyde in PBS for 20 minutes, then permeabilized with 0.5% Triton X-100 and 4% paraformaldehyde in PBS for 5 minutes. Focal adhesions were visualized by incubating first with mouse anti-paxillin monoclonal antibody for 1 hour at RT, then with Cy3-conjugated goat antibody to mouse immunoglobulin G. Actin filaments were stained with rhodamine-labeled phalloidin. Localization was evaluated by confocal laser microscopy (Carl Zeiss, LSM510).

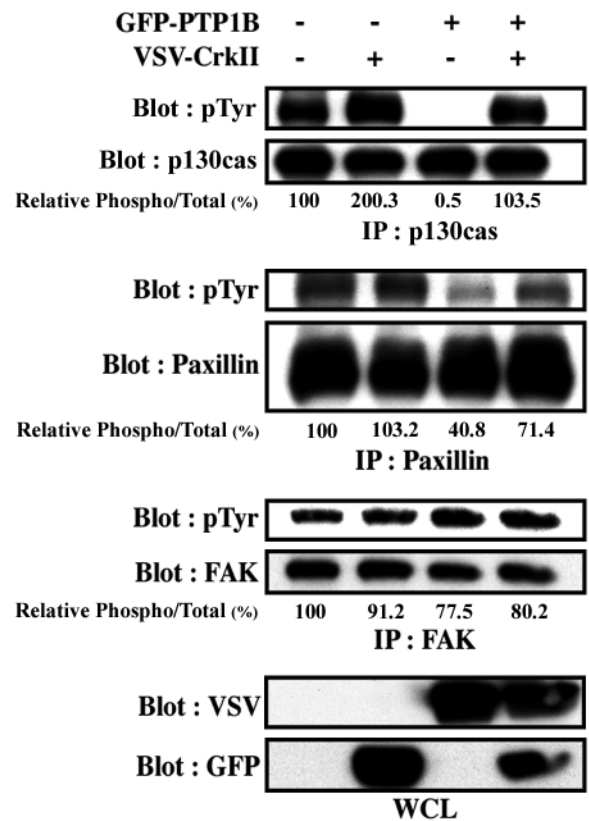
Fig. 1. CrkII protects p130^{cas} from dephosphorylation. (A) 293-EBNA cells were transfected with either pRK-VSV-CrkII (2 μg) or pRK-GFP-PTP1B (2 μg) expression plasmids, or co-transfected with pRK-VSV-CrkII and pRK-GFP-PTP1B. Cell lysates were immunoprecipitated with anti-p130^{cas} (IP: p130cas), anti-paxillin (IP: paxillin) or anti-FAK (IP: FAK) antibodies, and then immunoblotted with anti-phosphotyrosine (Blot: pTyr), anti-p130^{cas} (Blot: p130cas), anti-paxillin (Blot: paxillin) or anti-FAK (Blot: FAK) antibodies. The whole-cell lysates (WCL) were immunoblotted with anti-GFP (Blot: GFP) or anti-VSV (Blot: VSV) antibodies. The numerical values indicate relative ratios as a percentage of the control for each band of phosphoprotein/total protein after scanning and analysis using NIH Image software. (B) pRK-GFP-PTP1B (2 μg) and various quantities of pcDNA-CrkII expression plasmids as indicated were co-transfected in 293-EBNA cells. Cell lysates were immunoprecipitated with anti-p130^{cas} (IP: p130cas) and then immunoblotted with anti-phosphotyrosine (Blot: pTyr), anti-p130^{cas} (Blot: p130cas) or anti-Crk (Blot: Crk) antibodies. The whole-cell lysates (WCL) were immunoblotted with anti-GFP (Blot: GFP) or anti-Crk (Blot: Crk) antibodies. The numbers indicate relative ratios as a percentage of controls for phosphoprotein/total protein or for CrkII bound to p130^{cas} compared with the control.

Results

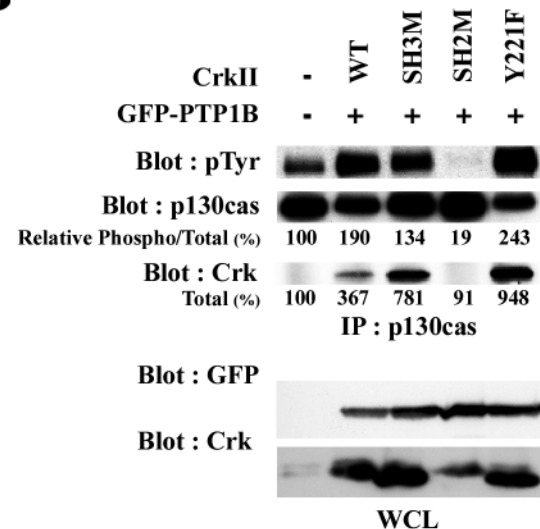
CrkII protects p130^{cas} from dephosphorylation

We first analyzed the effects of CrkII expression on the levels of tyrosine phosphorylation of the focal adhesion components FAK, paxillin and p130^{cas}. As shown in Fig. 1A, overexpression of CrkII (2 μg) by transfection moderately enhanced the tyrosine phosphorylation of p130^{cas}, and it

A



B



slightly elevated the phosphorylation of paxillin without affecting that of FAK. CrkII was previously shown to bind preferentially to phosphotyrosine on the epidermal growth factor receptor and p130^{cas}, and CrkII binding protects these phosphotyrosine sites from cellular phosphatase activities (Birge et al., 1992). From other studies, it was known that PTP1B can dephosphorylate p130^{cas} and paxillin (Liu et al., 1996). Consequently, we investigated whether CrkII could protect p130^{cas} and paxillin from dephosphorylation by PTP1B. Expression by transfection of GFP-PTP1B (2 µg) markedly reduced the tyrosine phosphorylation of p130^{cas} and paxillin, while phosphorylation levels of FAK were not altered (Fig. 1A). Total protein levels of all three proteins remained unchanged. Co-expression of CrkII with GFP-PTP1B using equal plasmid concentrations completely restored the phosphorylation levels of p130^{cas} and slightly enhanced those of paxillin, without affecting FAK phosphorylation (Fig. 1A).

To determine which domain of CrkII is responsible for protecting p130^{cas} from dephosphorylation by PTP1B, 293-EBNA cells were co-transfected with GFP-PTP1B and mutants of CrkII. As shown in Fig. 1B, only the CrkII SH2

mutant (CrkII-R38L) failed to protect and bind to tyrosine-phosphorylated p130^{cas}, whereas two other mutants including Y221F (mutation of tyrosine 221 to phenylalanine) bound similarly or better than wild-type. Thus, although GFP-PTP1B can effectively induce p130^{cas} dephosphorylation, co-expression of CrkII restores tyrosine phosphorylation of p130^{cas} by binding to p130^{cas} through its SH2 domain. These results indicate that CrkII binding to p130^{cas} can protect its

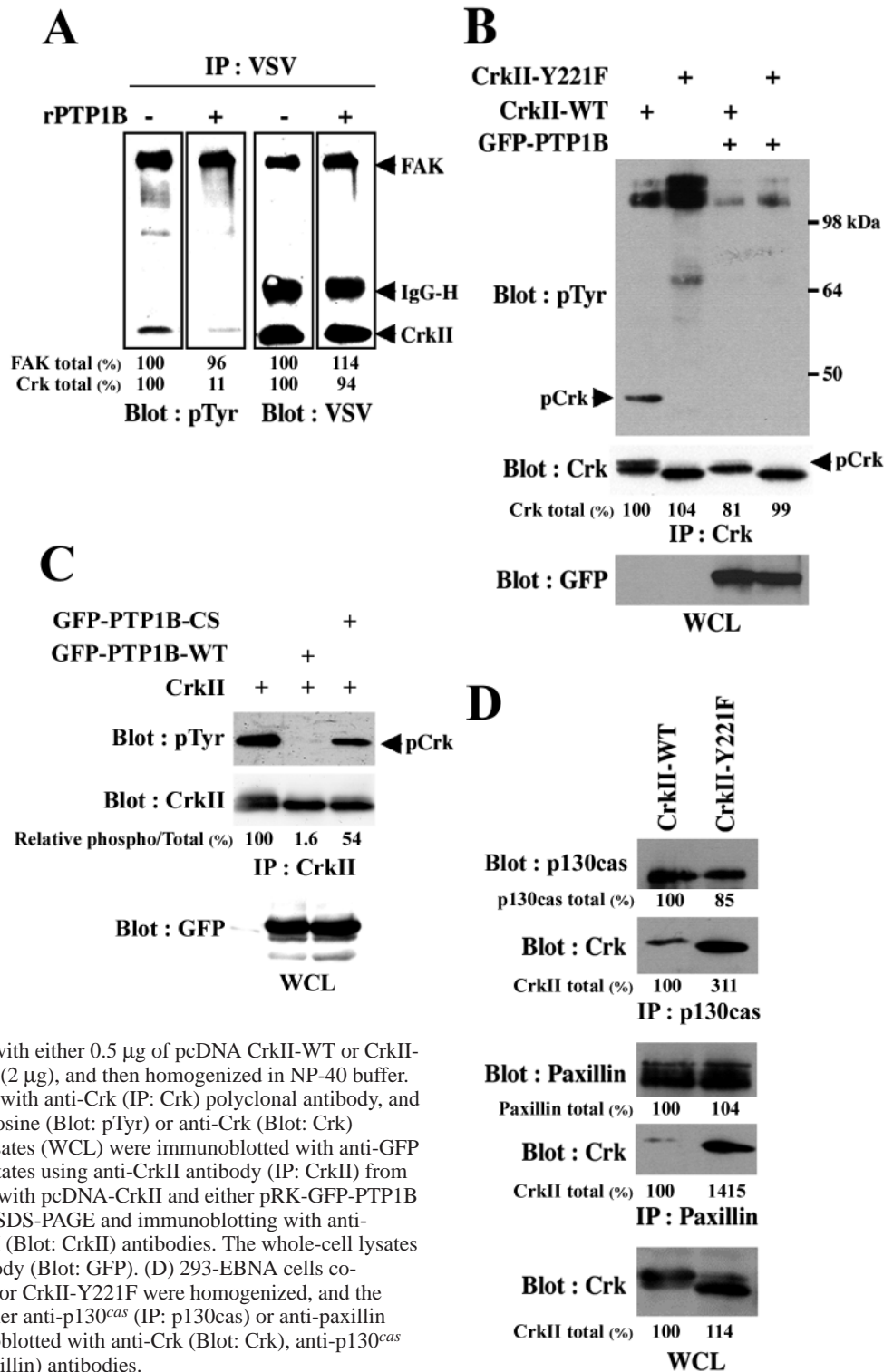


Fig. 2. PTP1B directly dephosphorylates CrkII. (A) An in-blot tyrosine phosphatase assay was performed as described in Materials and Methods. Immunoprecipitates using anti-VSV antibody (IP: VSV) from lysates of 293-EBNA cells co-transfected with VSV-FAK and VSV-CrkII were subjected to SDS-PAGE and electroblotting. The membranes were incubated with recombinant human PTP1B (20 U/ml) at 37°C for 30 minutes, then immunoblotted with anti-phosphotyrosine (Blot: pTyr) or anti-VSV antibodies (Blot: VSV).

(B) 293-EBNA cells were co-transfected with either 0.5 µg of pcDNA CrkII-WT or CrkII-Y221F with or without pRK-GFP-PTP1B (2 µg), and then homogenized in NP-40 buffer. The cell lysates were immunoprecipitated with anti-Crk (IP: Crk) polyclonal antibody, and then immunoblotted with anti-phosphotyrosine (Blot: pTyr) or anti-Crk (Blot: Crk) monoclonal antibodies. The whole-cell lysates (WCL) were immunoblotted with anti-GFP antibody (Blot: GFP). (C) Immunoprecipitates using anti-CrkII antibody (IP: CrkII) from lysates of 293-EBNA cells co-transfected with pcDNA-CrkII and either pRK-GFP-PTP1B or GFP-PTP1B-C215S were subjected to SDS-PAGE and immunoblotting with anti-phosphotyrosine (Blot: pTyr) or anti-CrkII (Blot: CrkII) antibodies. The whole-cell lysates were immunoblotted with anti-GFP-antibody (Blot: GFP). (D) 293-EBNA cells co-transfected with either pcDNA-CrkII-WT or CrkII-Y221F were homogenized, and the lysates were immunoprecipitated with either anti-p130^{cas} (IP: p130cas) or anti-paxillin (IP: paxillin) antibodies, and then immunoblotted with anti-Crk (Blot: Crk), anti-p130^{cas} (Blot: p130cas), or anti-paxillin (Blot: paxillin) antibodies.

phosphotyrosine from PTP1B; paxillin was less effectively protected.

PTP1B dephosphorylates CrkII

We tested the hypothesis that PTP1B can directly dephosphorylate CrkII using an in-blot phosphatase assay. Incubation with recombinant human PTP1B significantly reduced the tyrosine phosphorylation of CrkII compared with controls (Fig. 2A). By contrast, PTP1B did not dephosphorylate FAK, indicating that PTP1B can directly and specifically dephosphorylate CrkII in vitro. To determine whether expression of PTP1B could also reduce the tyrosine-phosphorylation level of CrkII in vivo, either 0.5 μ g of CrkII-WT or CrkII-Y221F was co-expressed in 293-EBNA cells with or without 2 μ g of GFP-PTP1B, and both CrkII tyrosine phosphorylation and the tyrosine-phosphorylated proteins co-precipitating with CrkII were analyzed (Fig. 2B). Although overexpressed CrkII-WT was tyrosine phosphorylated, co-expression with GFP-PTP1B abrogated tyrosine phosphorylation of CrkII. In parallel with the loss of phosphorylated CrkII, the electrophoretic mobility of CrkII-WT in cells co-expressing GFP-PTP1B shifted to a single band with faster migration, due to loss of the more-slowly migrating

band of a doublet; this result is consistent with dephosphorylation. CrkII-Y221F migrated with slightly higher mobility, and it did not stain with anti-phosphotyrosine antibody; it remained as a single band regardless of GFP-PTP1B co-expression. Thus, PTP1B could dephosphorylate CrkII and shift its mobility, unless CrkII was mutated at Y221.

Overexpression of the catalytically inactive mutant of PTP1B (PTP1B-C215S) had no effect on CrkII phosphorylation (Fig. 2C), further supporting a role for PTP1B as a phosphatase for CrkII in vivo. Tyrosine-phosphorylated proteins of approximately 130 kDa and 67 kDa (same sizes as p130^{cas} and paxillin) were found to co-precipitate substantially more with CrkII-Y221F than with CrkII-WT (Fig. 2B). The identity of these proteins co-precipitating with CrkII-Y221F was confirmed by immunoprecipitation using anti-p130^{cas} and anti-paxillin antibodies. As shown in Fig. 2D, CrkII-Y221F was considerably more effectively co-precipitated with both p130^{cas} and paxillin compared with CrkII-WT. These data suggest a negative role for Y221 phosphorylation in these CrkII interactions.

Fibronectin promotes partial CrkII dephosphorylation

Although the adhesion of cells to fibronectin is associated with well-known increases in tyrosine phosphorylation, including phosphorylation of FAK and p130^{cas}, our findings with the CrkII-Y221F mutant suggested that there might actually be a concomitant loss of phosphorylation of CrkII. As shown in Fig. 3A, attachment of 293-EBNA and HT1080 cells to fibronectin induced tyrosine phosphorylation and binding of p130^{cas} to CrkII compared with detached cells in suspension. Concomitantly, however, CrkII

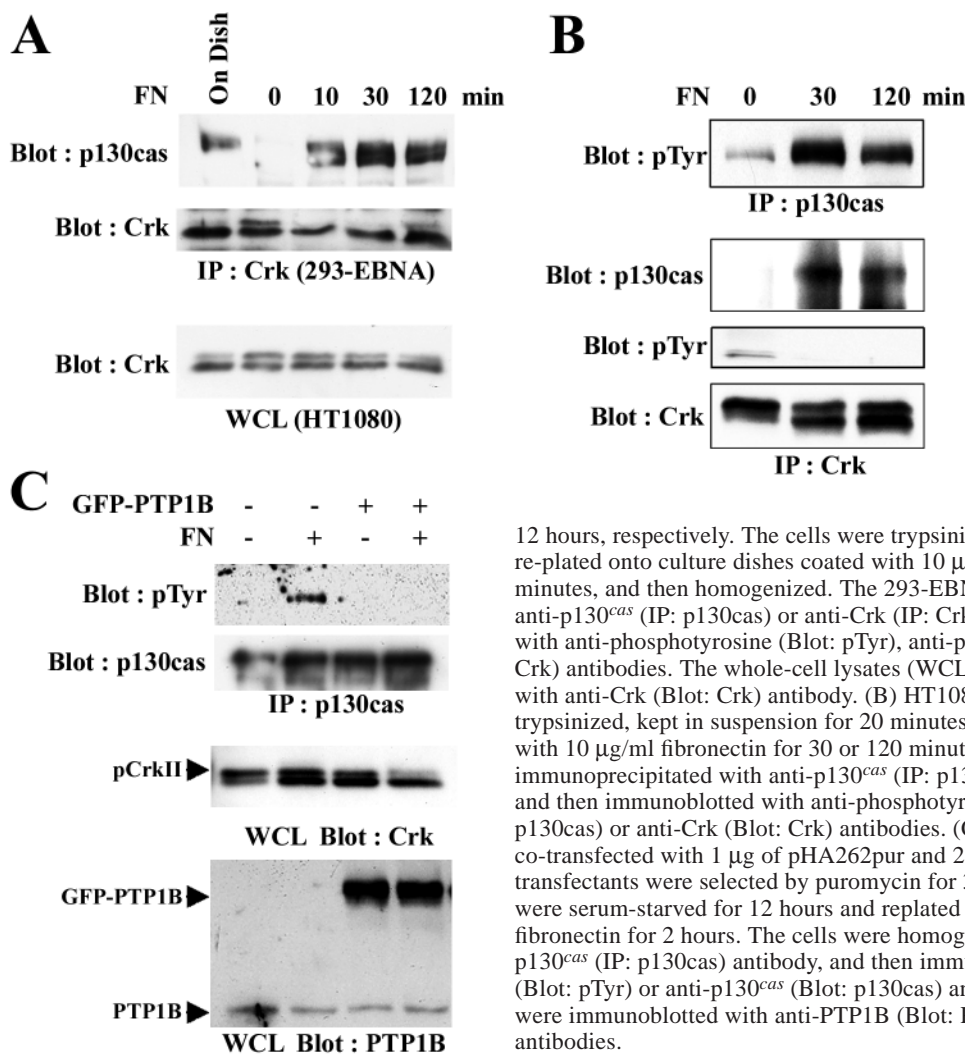


Fig. 3. Fibronectin promotes dephosphorylation of CrkII. (A) 293-EBNA and HT1080 cells were serum-starved for 6 hours or

12 hours, respectively. The cells were trypsinized, kept in suspension for 20 minutes, re-plated onto culture dishes coated with 10 μ g/ml fibronectin for 10, 30 or 120 minutes, and then homogenized. The 293-EBNA lysates were immunoprecipitated with anti-p130^{cas} (IP: p130cas) or anti-Crk (IP: Crk) antibodies, and then immunoblotted with anti-phosphotyrosine (Blot: pTyr), anti-p130^{cas} (Blot: p130cas) or anti-Crk (Blot: Crk) antibodies. The whole-cell lysates (WCL) from HT1080 cells were immunoblotted with anti-Crk (Blot: Crk) antibody. (B) HT1080 cells were serum-starved for 12 hours, trypsinized, kept in suspension for 20 minutes, then replated onto culture dishes coated with 10 μ g/ml fibronectin for 30 or 120 minutes. The cells were homogenized and immunoprecipitated with anti-p130^{cas} (IP: p130cas) or anti-Crk (IP: Crk) antibodies, and then immunoblotted with anti-phosphotyrosine (Blot: pTyr), anti-p130^{cas} (Blot: p130cas) or anti-Crk (Blot: Crk) antibodies. (C) HT1080 cells in 100 mm dishes were co-transfected with 1 μ g of pHA262pur and 2 μ g of pRK-GFP-PTP1B, and transfectants were selected by puromycin for 36 hours. After puromycin selection, cells were serum-starved for 12 hours and replated onto culture dishes coated with 10 μ g/ml fibronectin for 2 hours. The cells were homogenized and immunoprecipitated with anti-p130^{cas} (IP: p130cas) antibody, and then immunoblotted with anti-phosphotyrosine (Blot: pTyr) or anti-p130^{cas} (Blot: p130cas) antibodies. The whole-cell lysates (WCL) were immunoblotted with anti-PTP1B (Blot: PTP1B) or anti-Crk (Blot: Crk) antibodies.

was dephosphorylated as indicated by western immunoblotting. This loss of phosphorylation was accompanied by an electrophoretic mobility shift of CrkII, which formed a doublet band with a faster-migrating component lacking tyrosine phosphorylation (Fig. 3A,B,C). In HT1080 cells transfected with PTP1B, this fibronectin-induced mobility shift involved nearly all CrkII molecules (as indicated by the loss of nearly the entire upper band of the doublet), and the tyrosine phosphorylation of p130^{cas} induced by fibronectin stimulation was reduced (Fig. 3C).

PTP1B is essential for fibronectin-induced CrkII dephosphorylation

Our results showing that dephosphorylation of CrkII is induced by PTP1B expression and fibronectin stimulation suggests that PTP1B may be involved in fibronectin-promoted dephosphorylation of CrkII. To test the ability of PTP1B to dephosphorylate CrkII with fibronectin stimulation, we used a short interfering dsRNA (siRNA) RNA interference approach to achieve knockdown of endogenous PTP1B levels. PTP1B was downregulated in the cells transfected with siRNA for PTP1B but not with actin siRNA (Fig. 4A). By contrast, actin was decreased in the cells transfected with siRNA for actin but with PTP1B siRNA, confirming specific downregulation of PTP1B by siRNA transfection. As shown in Fig. 4B, attachment of 293-EBNA cells to fibronectin induced dephosphorylation of CrkII compared with detached cells in suspension, which was consistent with the previous results in Fig. 3. Concomitant with the decreased levels of PTP1B, the faster-migrating component (dephosphorylated CrkII) was decreased in PTP1B siRNA knockdown cells compared with control cells, both when cells were kept in suspension and when plated onto fibronectin (Fig. 4B).

Subcellular localization of PTP1B

PTP1B contains two proline-rich domains, which are consensus sequences for SH3 domain-binding motifs, and it selectively binds to the SH3 domains of Grb2, Crk and p130^{cas} (Liu et al., 1996). We next investigated the subcellular distribution of PTP1B and CrkII in cells stimulated with fibronectin. HT1080 cells transfected with GFP-PTP1B were plated on fibronectin-coated coverslips and analyzed by confocal immunofluorescence microscopy. As shown in Fig. 5, CrkII was mainly distributed in the cytoplasm with some membrane-associated staining. Consistent with a previous study, PTP1B was not only strongly localized to the endoplasmic reticulum, but also extended to the cell periphery. This pattern of PTP1B localization was at times parallel to microtubules, and it ended at focal adhesions. Co-localization between CrkII and PTP1B was observed faintly at plasma membrane-associated sites (Fig. 5).

Effects of CrkII phosphorylation on cell migration

The association of CrkII with p130^{cas} has been shown by Klemke et al. (Klemke et al., 1998) to regulate FG pancreatic carcinoma cell migration on fibronectin. HT1080 cells were transfected with high or low amounts of plasmids encoding CrkII-WT or CrkII-Y221F, and the dominant-negative p130^{cas}

(p130^{cas}DSD) and the central SH3 mutant of CrkII (CrkII-SH3M) were tested for effects on rates of cell migration on fibronectin. Both p130^{cas}DSD and CrkII-SH3M reduced cell migration to 60% of control cells ($P < 0.001$). High but not low levels of CrkII-WT overexpression enhanced HT1080 cell migration by 35% compared with control cells ($P < 0.001$). By contrast, low but not high levels of nonphosphorylated CrkII-Y221F expression significantly increased the average rate of cell migration 47% above controls (Fig. 6A; $P < 0.001$). Fig. 6B shows the morphology of the transfected cells at 12 hours after replating onto fibronectin. Concomitant with the increase of migration, the cells expressing high levels of CrkII-WT (high) and low levels of CrkII-Y221F (low) shows pseudopodial extension and membrane ruffling compared with control and low-level expressors of CrkII-WT (low). By contrast, high

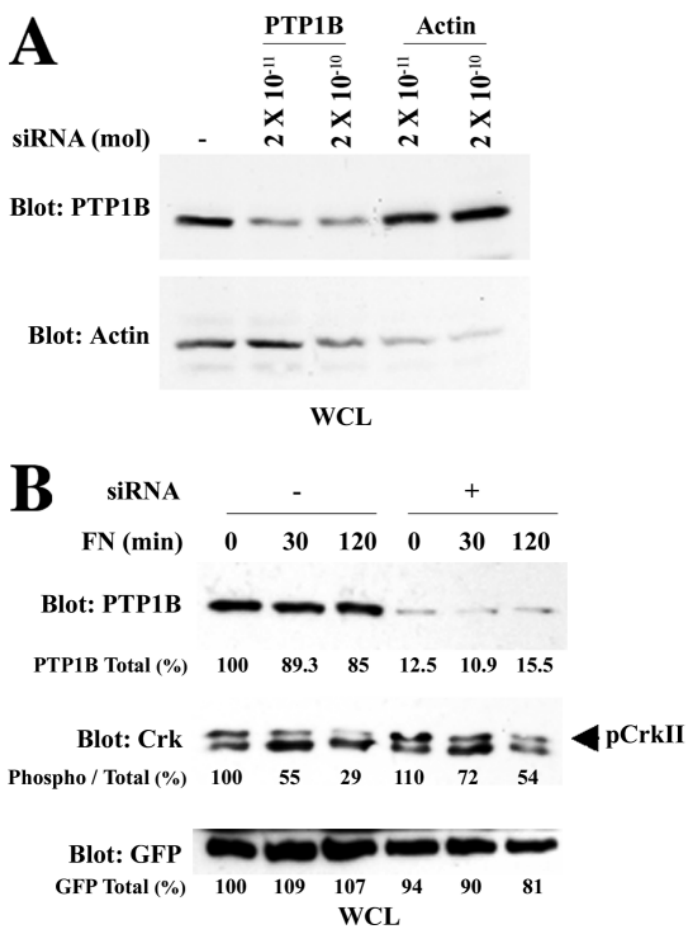


Fig. 4. PTP1B is essential for effective fibronectin-induced CrkII dephosphorylation. (A) 293-EBNA cells were co-transfected with siRNA (2×10^{-11} or 2×10^{-10} mol) for either PTP1B or β -actin and pRK-GFP and pcDNA3 plasmids. The cells were homogenized at 36 hours after transfection and immunoblotted with anti-PTP1B (Blot: PTP1B) or anti-actin (Blot: actin) antibodies. (B) 293-EBNA cells were co-transfected with siRNA for PTP1B, and pRK-GFP and pcDNA3 plasmids. At 36 hours after transfection, cells were trypsinized, kept in suspension for 20 minutes, and replated onto culture dishes coated with 10 μ g/ml fibronectin for 30 or 120 minutes. The cells were homogenized and immunoblotted with anti-PTP1B (Blot: PTP1B), anti-Crk (Blot: Crk) or anti-GFP (Blot: GFP) antibodies.

levels of CrkII-Y221F expression were accompanied by random pseudopodial extension and a flattened, more-spread morphology consistent with increased cell-substrate adhesion.

Co-expression of CrkII and PTP1B promotes cell migration

Although our mutational analysis of CrkII at residue Y221 strongly suggested that lack of phosphorylation of this site mimicking fibronectin-induced reduction of CrkII phosphorylation can regulate migration, the direct experimental test would be to induce CrkII dephosphorylation by PTP1B and to examine its effects on cell migration. HT1080

cells were co-transfected with CrkII and various concentrations of the GFP-PTP1B expression plasmid. After 36 hours of puromycin selection, the cells were serum-starved for 12 hours and replated onto culture dishes coated with fibronectin for 2 hours; tyrosine phosphorylation of p130^{cas} and paxillin were analyzed by immunoprecipitation and immunoblotting. As shown in Fig. 7A, the co-expression of PTP1B with CrkII significantly decreased the binding of CrkII to paxillin, but not its binding to p130^{cas}. Although the tyrosine phosphorylation of paxillin was attenuated by PTP1B regardless of co-expression with CrkII, tyrosine phosphorylation of p130^{cas} was protected by CrkII expression, suggesting that CrkII dephosphorylated by PTP1B preferentially bound to and protected tyrosine-phosphorylated p130^{cas}, but not paxillin. As shown in Fig. 7B, HT1080 cell migration rates on fibronectin were increased in the cells co-expressed with CrkII and PTP1B, and CrkII-Y221F with or without PTP1B (to 119%, 125% and 121% of controls, respectively; $P < 0.05$), but not in the cells expressed CrkII or PTP1B alone. These results indicate that CrkII dephosphorylated by PTP1B preferentially binds to and protects tyrosine-phosphorylated p130^{cas} (but not paxillin) associated with promotion of cell migration.

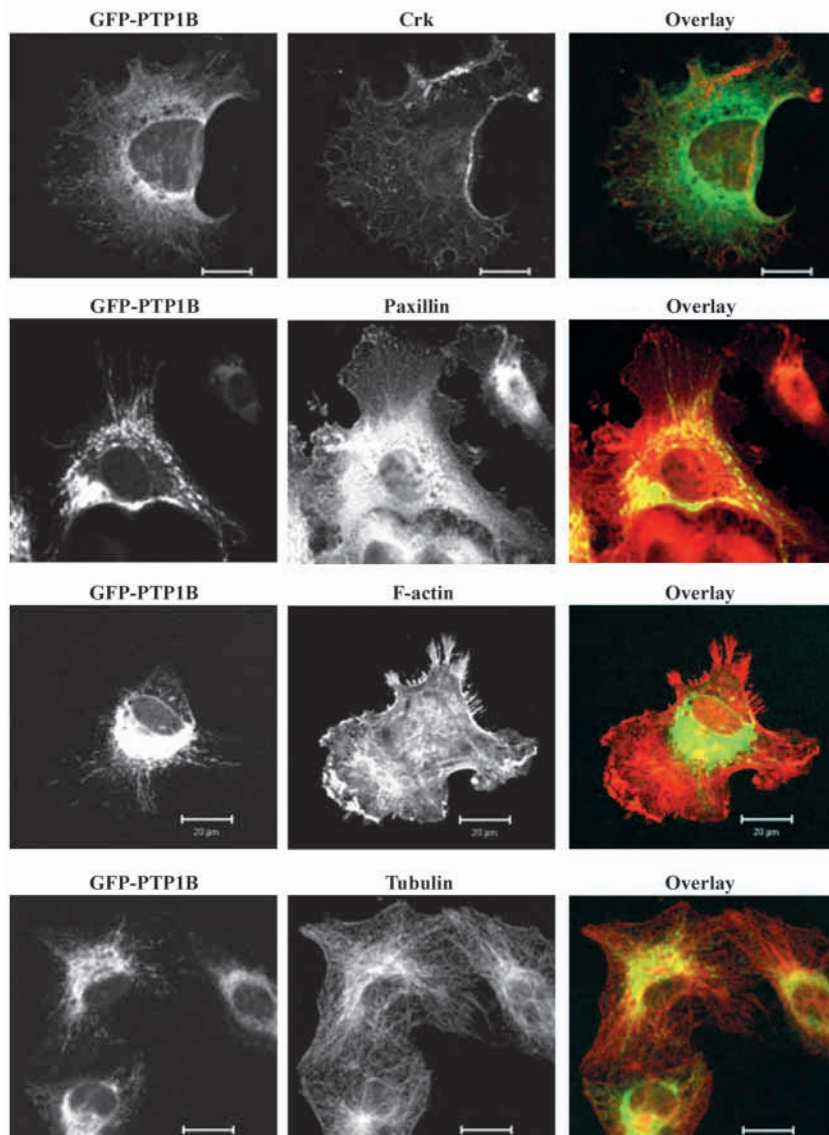


Fig. 5. Subcellular localization of PTP1B and CrkII. HT1080 cells in 60 mm dishes were co-transfected with 0.5 μ g of pHA262pur and 1 μ g of pRK-GFP-PTP1B, and transfectants were selected by puromycin for 36 hours. After puromycin selection, cells were replated on glass coverslips coated with 10 μ g/ml fibronectin for 2 hours. The cells were analyzed by immunofluorescence confocal microscopy using anti-Crk (Crk), anti-paxillin (Paxillin), or anti- β -tubulin (Tubulin) antibodies, or rhodamine-phalloidin (F-actin). Bars, 20 μ m.

Discussion

In this study we have established the following novel points: first, PTP1B directly dephosphorylates CrkII in vitro and in vivo; second, CrkII is tyrosine-phosphorylated in cells in suspension, and it is dephosphorylated by attachment to fibronectin and PTP1B expression; third, nonphosphorylated CrkII-Y221F can increase HT1080 cell migration on fibronectin, whereas wild-type CrkII cannot at moderate expression levels; and fourth, CrkII dephosphorylated by PTP1B preferentially binds to and protects tyrosine-phosphorylated p130^{cas}, but not paxillin, in association with accelerated cell migration.

The CrkII adaptor protein has been implicated in signal transduction cascades by its association with SH2- and SH3-binding partner proteins. Tyrosine dephosphorylation of p130^{cas} was effectively prevented by binding of CrkII (Fig. 1 of this study). CrkII itself is phosphorylated at residue tyrosine 221 after many types of stimuli (Matsuda and Kurata, 1996). Abl tyrosine kinase, which binds to the N-terminal SH3 domain of CrkII, is a negative regulator of cell migration through its ability to regulate the p130^{cas}/CrkII complex accompanied by phosphorylation of the tyrosine 221 residue of CrkII, suggesting the importance of CrkII tyrosine phosphorylation in cell migration (Kain and Klemke, 2001). By mutational analysis, we show in this study that tyrosine 221 can be directly linked to the rate

of cell migration (Figs 6, 7). The mechanism by which CrkII might be dephosphorylated and its binding activated has not been clear. Here, we provide, to the best of our knowledge, the first evidence that PTP1B can directly dephosphorylate CrkII both in vitro and in intact cells (Figs 2-4).

PTP1B is a ubiquitous endoplasmic reticulum-associated enzyme, which is also localized at focal adhesions (Frangioni et al., 1992; Arregui et al., 1998). We also found that PTP1B was mainly localized at the endoplasmic reticulum, but also extended to the cell periphery. This peripheral population was parallel to some microtubules, ended at focal adhesions, and was accompanied by faint co-localization between CrkII and PTP1B when the cells were plated onto fibronectin (Fig. 5). PTP1B has been implicated in the negative regulation of cell growth, differentiation and transformation (Byon et al., 1997). Liu et al. (Liu et al., 1998) reported that overexpression of PTP1B in Rat-1 fibroblasts resulted in markedly reduced migration on fibronectin. By contrast, several lines of evidence suggest an opposite role for PTP1B in integrin-mediated signaling. Expression of a catalytically inactive mutant of PTP1B in L cells decreases fibronectin-mediated cell spreading and FAK phosphorylation, whereas wild-type PTP1B had no

effect (Arregui et al., 1998). We also could not show any negative effect of overexpressing PTP1B alone on fibronectin-induced cell migration and spreading in two other cell types, U87-MG and HT1080 cells (Tamura et al., 1998) (Fig. 7B). Furthermore, overexpression of PTP1B in breast cancer cells (MDA-MB-435S) and HEK293 cells decreases phosphorylation of the c-Src inhibitory site (Tyr-527), resulting in an increase in Src kinase activity, which is essential for adhesion-dependent p130^{cas} phosphorylation (Bjorge et al., 2000). It is likely, therefore, that the different phenotypes resulting from PTP1B expression may be due to differences in cell types and/or expression levels of PTP1B. Interestingly, embryonic fibroblasts from PTP1B knockout mice display significant delays in p130^{cas} phosphorylation and cell spreading induced by attachment to fibronectin (Cheng et al., 2001). Our findings that CrkII phosphorylation is reduced by fibronectin stimulation and further reduced by PTP1B expression, and that 293-EBNA cells with PTP1B knockdown by siRNA show decreased CrkII dephosphorylation induced by attachment to fibronectin suggest a positive role of PTP1B in integrin-mediated signaling (Figs 3, 4). Indeed, Src Tyr-527 is hyperphosphorylated in PTP1B-deficient fibroblasts compared with wild-type, but only when the cells are held in suspension and not when plated onto fibronectin (Cheng et al., 2001). We found that detachment of cells (cells maintained in suspension) can induce CrkII phosphorylation, which is also elevated in PTP1B knockdown cells and is reduced by PTP1B expression (Figs 3, 4). These data suggest that the high levels of phosphorylation of CrkII in cells kept in suspension may result in the attenuation of fibronectin-mediated events. However, PTP1B knockdown did not increase cell migration (data not shown), implying the involvement of other phosphatases or small remnants of PTP1B in dephosphorylation of CrkII. In fact, a recent study has reported that even though PTP1B^{-/-} cells can exhibit enhanced tyrosine phosphorylation of epidermal growth factor (EGF) receptor or platelet-derived growth factor (PDGF) receptor in response to EGF or PDGF

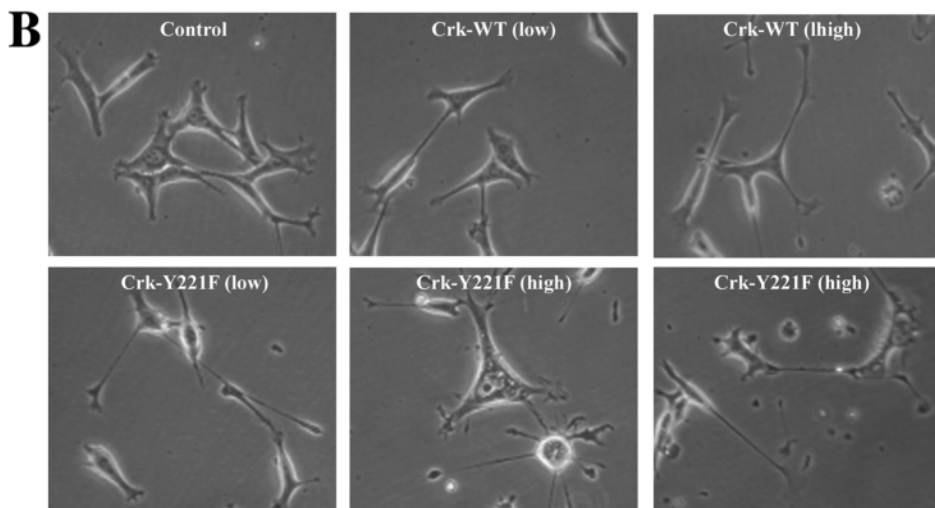
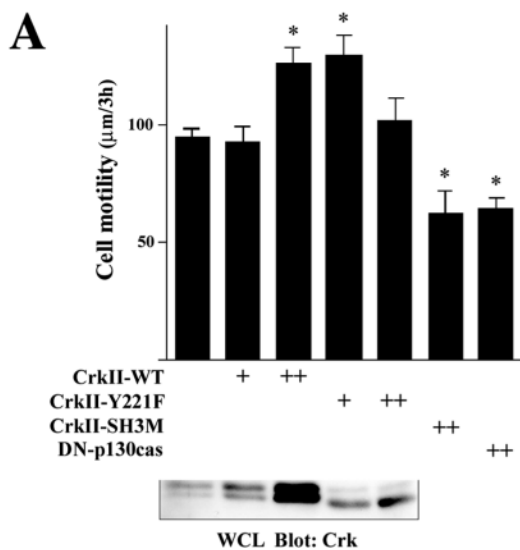


Fig. 6. CrkII-Y221F accelerates cell motility on fibronectin.

(A) HT1080 cells were co-transfected with 0.5 µg of pHA262pur, 0.5 µg of pRK-GFP, and 0.5 µg or 2 µg of pcDNA-CrkII-WT and pcDNA-CrkII-Y221F, or 2 µg of CrkII-W169L, psSRα-DN-p130^{cas} or control plasmids, and transfectants were selected by puromycin

for 36 hours. After puromycin selection, cells were replated on 35 mm glass dishes coated with 10 µg/ml fibronectin, and cultured overnight in serum-free DMEM containing 1 mg/ml BSA. Cell movements were monitored for 3 hours by time-lapse video microscopy. Error bars indicate s.d. for at least 30 cells per condition (* $P < 0.001$ versus controls). Parallel cultures of these cells were homogenized and subjected to immunoblotting with anti-Crk (WCL Blot: Crk) antibody. (B) Phase contrast microscopy of HT1080 cells co-transfected with 0.5 µg of pHA262pur, pRK-GFP, and 0.5 µg (low) or 2 µg (high) of pcDNA-CrkII-WT, CrkII-Y221F or control plasmids 12 hours after replating onto fibronectin. Magnification, $\times 200$.

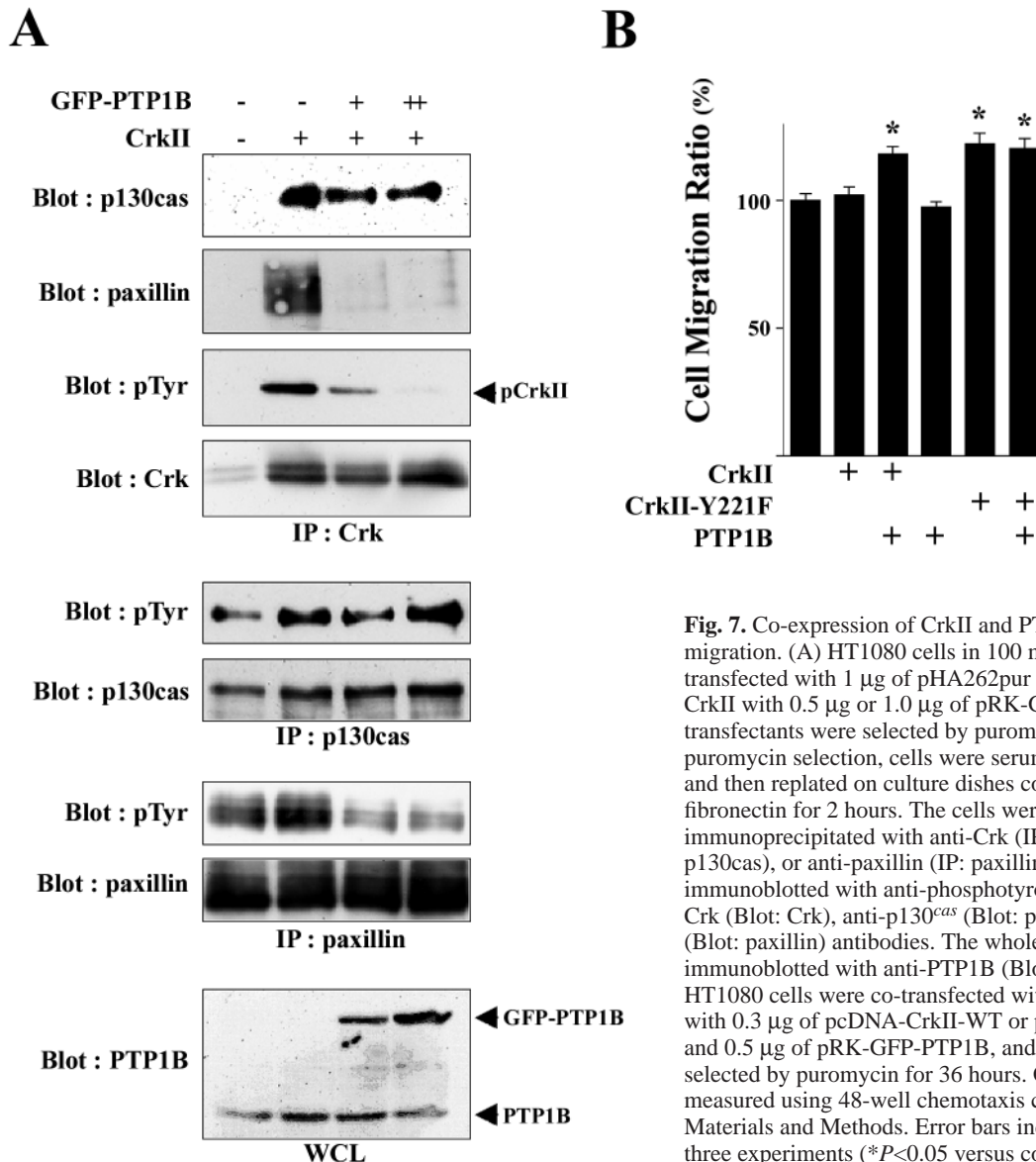


Fig. 7. Co-expression of CrkII and PTP1B promotes cell migration. (A) HT1080 cells in 100 mm dishes were co-transfected with 1 μ g of pHA262pur and 0.5 μ g of pcDNA-CrkII with 0.5 μ g or 1.0 μ g of pRK-GFP-PTP1B, and transfectants were selected by puromycin for 36 hours. After puromycin selection, cells were serum-starved for 12 hours and then replated on culture dishes coated with 10 μ g/ml fibronectin for 2 hours. The cells were homogenized and immunoprecipitated with anti-Crk (IP: Crk), anti-p130^{cas} (IP: p130cas), or anti-paxillin (IP: paxillin) antibodies, and then immunoblotted with anti-phosphotyrosine (Blot: pTyr), anti-Crk (Blot: Crk), anti-p130^{cas} (Blot: p130cas), or anti-paxillin (Blot: paxillin) antibodies. The whole-cell lysates (WCL) were immunoblotted with anti-PTP1B (Blot: PTP1B) antibody. (B) HT1080 cells were co-transfected with 0.5 μ g of pHA262pur with 0.3 μ g of pcDNA-CrkII-WT or pcDNA-CrkII-Y221F, and 0.5 μ g of pRK-GFP-PTP1B, and transfectants were selected by puromycin for 36 hours. Cell migration was measured using 48-well chemotaxis chambers as described in Materials and Methods. Error bars indicate s.d. for at least three experiments (* P <0.05 versus controls).

stimulation, Akt and ERK activation are only minimally or not enhanced (Haj et al., 2003). The authors suggest that even though PTP1B plays a role in regulating EGFR and PDGFR, other regulatory mechanisms come into play when it is missing; they speculate that this finding might explain why no classical protein tyrosine phosphatases have been found to be tumor suppressor genes. PTP1B might be important in cell migration by activating CrkII and Src in a process dependent on their relative intracellular concentrations.

CrkII has been identified as a mediator of cell migration through its association with p130^{cas} and paxillin (Klemke et al., 1998). The p130^{cas}/CrkII/DOCK180 pathway is reported to promote cell migration and to activate Rac1 (Kiyokawa et al., 1998a; Kiyokawa et al., 1998b). As documented in Fig. 2, CrkII-Y221F can bind more effectively to paxillin and p130^{cas} than wild-type CrkII, suggesting that the expression of CrkII-Y221F might facilitate this pathway. At a relatively low expression level where wild-type CrkII could not stimulate cell migration, nonphosphorylated CrkII-Y221F could readily

promote HT1080 cell migration on fibronectin. By contrast, high expression of CrkII-Y221F did not elevate rates of cell migration in these cells, which displayed enhanced random pseudopodial extension and a flattened, more-spread, apparently more-adhesive morphology. This result may be explained by Crk activation of C3G by tyrosine phosphorylation and C3G-dependent Rap1 activation that promotes cell adhesion and spreading, but repression of cell migration (Ohba et al., 2001). The authors also suggest that CrkII-C3G may function downstream of paxillin, but not p130^{cas}, to suppress cell migration. CrkII-Y221F fails to induce JNK activation and cell migration on fibronectin in COS-7 cells (Abassi et al., 2002). Girardin and Yaniv (Girardin and Yaniv, 2001) reported that the p130^{cas}/CrkII complex serves as a scaffolding structure for JNK signaling pathway. With scaffold proteins for mitogen-activated protein kinase cascades, even though the presence of an optimal scaffold concentration can increase the signaling output, if the scaffold concentration is greater than optimal, a significant decrease in signaling can occur (Levchenko et al.,

2000). A well-studied scaffold for the JNK signaling cascade is the JNK interacting protein (JIP) family. Indeed, JIP-1 was originally identified as an inhibitor of the JNK signaling pathway, whereas the JIP family can function as scaffolding for the JNK signaling cascade (Dickens et al., 1997; Ito et al., 1999). Overexpression of the JNK binding domain of JIP-1 can inhibit JNK activation (Harding et al., 2001). Our results that a relatively low expression level, but not high expression, of CrkII-Y221F promoted HT1080 cell migration on fibronectin is consistent with the ability of CrkII to function as a scaffold structure binding p130^{cas} in cell migration. Consequently, it appears that the differing roles of Y221F-CrkII in cell migration may reflect differing expression and optimal concentration in different cell types.

Recent research has revealed that tyrosine phosphorylation of paxillin reduces cell migration, whereas tyrosine phosphorylation of p130^{cas} facilitates cell migration (Yano et al., 2000). In this study, we found that co-expression of CrkII and PTP1B preferentially protects against dephosphorylation of p130^{cas}, but not of paxillin, concomitant with promoting cell migration on fibronectin. The promotion of cell migration by PTP1B associated with dephosphorylation of paxillin and CrkII might involve a change in the balance of tyrosine phosphorylation between p130^{cas} and paxillin. v-Crk has been implicated in the activation of Rho and phosphatidylinositol 3-kinase, which are required for focal adhesion formation and cell migration (Altun-Gultekin et al., 1998; Akagi et al., 2000), although it is not known whether nononcogenic CrkII also participates in Rho and phosphatidylinositol 3-kinase activation. In general, CrkII may act as a molecular switch for cell migration after binding to p130^{cas} or paxillin and activation of Rac1 (Klemke et al., 1998).

The data presented here provide the first evidence that PTP1B directly dephosphorylates CrkII in vitro and in vivo, with tyrosine residue 221 of CrkII serving as a regulatory element in binding to tyrosine-phosphorylated proteins and transducing a signal to induce cell migration on fibronectin. We conclude that the regulation of CrkII activity by tyrosine kinases and phosphatases can be an important factor in regulating cell migration mediated by the FAK/p130^{cas}/CrkII pathway.

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