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Transformation Suppression by Protein Tyrosine Phosphatase 1B Requires a Functional SH3 Ligand

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We have recently shown that protein tyrosine phosphatase 1B (PTP1B) associates with the docking protein p130^{Cas} in 3Y1 rat fibroblasts. This interaction is mediated by a proline-rich sequence on PTP1B and the SH3 domain on p130^{Cas}. Expression of wild-type PTP1B (WT-PTP1B), but not a catalytically competent, proline-to-alanine point mutant that cannot bind p130^{Cas} (PA-PTP1B), causes substantial tyrosine dephosphorylation of p130^{Cas} (F. Liu, D. E. Hill, and J. Chernoff, J. Biol. Chem. 271:31290–31295, 1996). Here we demonstrate that WT-, but not PA-PTP1B, inhibits transformation of rat 3Y1 fibroblasts by v-*crk*, -*src*, and -*ras*, but not by v-*raf*. These effects on transformation correlate with the phosphorylation status of p130^{Cas} and two proteins that are associated with p130^{Cas}, Paxillin and Fak. Expression of WT-PTP1B reduces formation of p130^{Cas}-Crk complexes and inhibits mitogen-activated protein kinase activation by Src and Crk. These data show that transformation suppression by PTP1B requires a functional SH3 ligand and suggest that p130^{Cas} may represent an important physiological target of PTP1B in cells.

The regulation of protein tyrosine phosphorylation is a key process associated with cell growth, differentiation, and transformation (17, 45, 47). Tyrosine phosphorylation levels are maintained by a dynamic balance between competing kinases and phosphatases. While the involvement of protein tyrosine kinases in this process has been well studied, the precise functions of protein tyrosine phosphatases (PTPs) are still unclear.

Most (but not all) PTPs are thought to act as negative regulators of signaling pathways. For example, PTP1B, a ubiquitous, endoplasmic reticulum (ER)-associated enzyme, has been implicated as a potential negative regulator of cell growth, differentiation, and transformation (8). Microinjection of PTP1B into *Xenopus* oocytes delays insulin-induced maturation and blocks insulin-stimulated S6 peptide phosphorylation (10, 43). Overexpression of wild-type PTP1B (WT-PTP1B), but not a catalytically inactive version, inhibits insulin-stimulated receptor autophosphorylation (21). When overexpressed in cultured cells, PTP1B dephosphorylates a wide range of receptors, such as those for epidermal growth factor, insulin-like growth factor 1, platelet-derived growth factor (α and β), insulin, and colony-stimulating factor 1, as well as the c-kit kinases (23). NIH 3T3 cells that stably overexpress PTP1B are resistant to subsequent transformation by an oncogenic form of the human *neu* gene (6). Similarly, v-*src*-transformed mouse 3T3 fibroblasts are partially reverted by overexpression of rat PTP1B (46). Interestingly, TC-PTP, which is structurally related to PTP1B and also localized in the ER, does not reverse transformation of rat2 cells by v-*fms* (48). However, a truncated form of TC-PTP, in which an 11-kDa carboxy-terminal extension has been removed, causes dramatic changes in cell morphology, loss of anchorage-independent growth, and reduction of tumor formation in nude mice (48). Therefore, despite their strong structural homology, the functions of PTP1B and TC-PTP may be distinct.

We have recently shown that p130^{Cas} is likely to be a physiological substrate for PTP1B (25). p130^{Cas} was initially identified as a highly tyrosine-phosphorylated molecule in v-*src*-, -*crk*-, and -*abl*-transformed cells (4, 19, 20, 27, 36). p130^{Cas} is thought to function as a docking protein and contains numerous sequence motifs predicted to be involved in mediating protein-protein interactions. These include an N-terminal *src* homology 3 (SH3) domain, proline-rich regions that may serve as SH3 ligands, numerous *src* homology 2 (SH2) binding sites, and a C-terminal region that appears to direct homo- and heterodimerization (reviewed in reference 15). Several potential partners of p130^{Cas} have been identified, such as Crk, which binds to phosphotyrosine sites (7); Src, which binds to a proline-rich sequence in the C-terminal region (30); and focal adhesion kinase (Fak), which binds to the SH3 domain (16). Human ornithine decarboxylase-transformed NIH 3T3 and Rat-1 cells display increased tyrosine phosphorylation levels of p130^{Cas}, as do *ras*-transformed cells. Treatment with herbimycin A, a potent inhibitor of Src family kinases, or other inhibitors of protein tyrosine kinases causes such cells to phenotypically revert. This reversion correlates with a marked reduction in the tyrosine phosphorylation level of p130^{Cas}. In addition, the expression of antisense mRNA for p130^{Cas} results in reversion of the transformed phenotype of ornithine decarboxylase-, v-*ras*-, and v-*src*-transformed cell lines, indicating that p130^{Cas} is involved in cell transformation by these and perhaps other agents (2). These data raise the intriguing possibility that p130^{Cas} may be generally required for transformation.

We have recently shown that PTP1B associates with p130^{Cas} in 3Y1 rat fibroblasts. This interaction is mediated by a proline-rich sequence on PTP1B and the SH3 domain on p130^{Cas}. Expression of WT-PTP1B, but not of a catalytically competent, proline-to-alanine double point mutant that cannot bind p130^{Cas} (PTP1B$^{P309A,\ P310A}$, termed PA-PTP1B), causes substantial tyrosine dephosphorylation of p130^{Cas} (25). Here we demonstrate that WT-, but not PA-PTP1B, inhibits in vitro transformation of 3Y1 cells by v-*crk*, -*src*, and -*ras*. These data indicate that suppression of transformation by PTP1B is mediated by interactions with one or more SH3-containing proteins. Furthermore, they suggest that one of these SH3-containing proteins is p130^{Cas}, which may represent an important physiological target of PTP1B in cells.

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MATERIALS AND METHODS

Materials. The monoclonal antihemagglutinin (anti-HA) antibody 12CA5 was obtained from BabCo. Monoclonal anti-PTP1B antibody FG6 was obtained from Oncogene Science. Monoclonal antiphosphotyrosine (PY20), anti-p130^{Cas}, anti-Fak, anti-Paxillin, and anti-Crk antibodies were purchased from Transduction Laboratories, and polyclonal anti-p130^{Cas} antibody was from Santa Cruz Biotechnology, Inc. Polyclonal anti-phospho-mitogen-activated protein kinase (MAPK) antibodies were obtained from Promega. G418 and puromycin were purchased from Sigma.

Expression plasmids. A catalytically inactive, cysteine-215-to-serine mutant of PTP1B (PTP1B^{C215S}; CS-PTP1B) and a proline-309-to-alanine, proline-310-to-alanine mutant of PTP1B (PTP1B$^{P309A,\ P310A}$; PA-PTP1B) were constructed as described previously (25). Mutations were confirmed by sequence analysis. pJ3H-PTP1B constructs were made as described elsewhere (41). These plasmids express an N-terminal HA-tagged form of PTP1B. pMS v-*crk* was constructed by digesting pCT10 (a gift from H. Hanafusa) with *Aat*II and *Eco*RV, ligating the resulting 1.9-kb v-*crk* insert with *Sal*I linkers, and then subcloning it into *Sal*I-cut pMSE (39). pMSE v-*src* and pMS v-*ras* EJ were kindly provided by M. Schuermann (39).

Recombinant glutathione *S*-transferase (GST) fusion proteins. WT-, CS-, and PA-PTP1B were subcloned as *Bam*HI-*Eco*RI fragments into pGEX-2T, and GST-PTP1B fusion proteins were made and purified by standard methods (42). The p130^{Cas} SH3 domain was subcloned as a *Bam*HI-*Eco*RI fragment into pGEX-2TK. ^{32}P-labeled GST-SH3 (p130^{Cas}) protein was made and purified as described by Kaelin et al. (18).

Transient transfection. 3Y1 and 3Y1-v-*crk* cells were grown to 40% confluence in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum (FBS) and transfected with expression plasmids by a calcium phosphate precipitation method (9). Forty-eight hours posttransfection, the cells were harvested for analysis.

Overlay assay. One, five, and 10 μg of recombinant GST–WT-PTP1B, GST–CS-PTP1B, GST–PA-PTP1B, or GST alone were spotted onto nitrocellulose filters and incubated for 30 min at room temperature in a blocking buffer containing 5% FBS, 1 M glycine, and 5% dry skim milk. The filters were washed twice with a solution containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 5 mM $MgCl_2$ and then incubated for 30 min at 4°C, with [γ-^{32}P]ATP-labeled recombinant GST-SH3 (p130^{Cas}). After being washed twice with a solution containing 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 5 mM $MgCl_2$, the filters were dried and exposed to Kodak XAR film.

Immunoprecipitation and immunoblotting. 3Y1-v-*crk* cells were stably transfected with either pJ3H alone or pJ3H bearing PTP1B, CS-PTP1B, or PA-PTP1B. Cells were lysed in Nonidet P-40 lysis buffer. For immunoprecipitation, 1 mg of cell lysates was immunoprecipitated with 2 μg of anti-HA antibody or anti-PTP1B antibody, or 250 μg of cell lysates was immunoprecipitated with 2 μg of anti-p130^{Cas} (polyclonal), anti-p130^{Cas} (monoclonal), anti-Fak, or anti-Paxillin antibodies at 4°C for 2 h. Immunocomplexes were washed three times with Nonidet P-40 lysis buffer and boiled for 5 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The samples were fractionated by SDS–8% PAGE and transferred to polyvinylidene fluoride membranes. The membranes were probed with antiphosphotyrosine, anti-p130^{Cas}, or anti-Crk antibodies. The blots were then stripped and reprobed with anti-p130^{Cas}, anti-Fak, or anti-Paxillin antibodies.

Construction of 3Y1 cell lines expressing oncogenes and PTP1B. 3Y1 cells and their derivatives were maintained in DMEM plus 10% FBS. 3Y1-v-*crk*-transformed cells were provided by Gary Kruh (Fox Chase Cancer Center). 3Y1-v-*src* and -v-*ras* cells were made by transfecting 3Y1 cells with pMSE-v-*src* or pMS-*ras* EJ plasmids, by a calcium phosphate method (9). Forty-eight hours after transfection, cells were diluted 1:5 in DMEM containing 10% FBS and 300 μg of G418 per ml. Media were changed once every 3 days until colonies appeared. Twenty colonies were picked with cloning cylinders. The expression level of v-Src and v-Ras was determined by immunoblotting. Cells expressing high levels of these oncogenes were then transfected with either pJ3H, pJ3H-PTP435, pJ3H-CS-PTP1B, or pJ3H-PA-PTP1B together with a plasmid encoding a puromycin resistance marker. Single clones were isolated by using 2 μg of puromycin per ml plus 300 μg of G418 per ml. The expression of PTP1B and oncogenes was determined by immunoblotting.

Transformation assays. (i) Focus formation. Cells were grown in triplicate in 60-mm dishes for 14 days with a complete medium change (10% FBS–DMEM with 2 μg of puromycin per ml and 300 μg of G418 per ml) every 3 days, and the transformed foci were counted.

(ii) Anchorage independence. Cells were assessed for anchorage-independent growth by colony formation in soft agar. A total of 2×10^4 cells were seeded in DMEM containing 10% FBS, 2 μg of puromycin per ml, 300 μg of G418 per ml, and 0.3% soft agar. Cells were fed once a week with DMEM containing 10% FBS, 2 μg of puromycin per ml, 300 μg of G418 per ml, and 0.3% agar. Two weeks after seeding, colonies larger than 0.1 mm in diameter were scored as positive for growth.

(iii) Serum independence. Clonal cell lines selected in the presence of puromycin and G418 were seeded at a density of 10^4 cells in 35-mm dishes containing DMEM with 10% FBS. Replicate dishes were counted 24 h later to confirm that all the plates contained approximately the same initial cell number, and they were designated day 0 in the time course curve. The medium was removed and replaced with culture medium containing 1% FBS. The culture medium was changed every 2 days, and cell counts were performed.

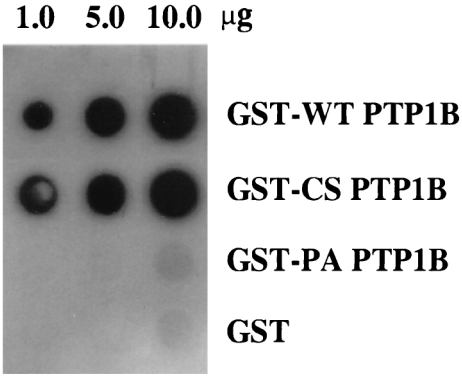

FIG. 1. PTP1B binds to p130^{Cas}. GST-PTP1B fusion proteins were purified by affinity chromatography with glutathione-Sepharose beads. Quantities (1.0, 5.0, or 10.0 μg) of GST–WT-PTP1B, –CS-PTP1B, –PA-PTP1B, and GST alone were each spotted on a nitrocellulose filter, which was then probed with a ^{32}P-labeled GST-SH3 domain derived from p130^{Cas}.

RESULTS

PTP1B binds directly to p130^{Cas}. We recently reported that overexpression of WT-PTP1B, but not of a proline-to-alanine mutant form of this enzyme (PA-PTP1B), in which a potential SH3 ligand is disrupted by alanine residues, causes decreased tyrosine phosphorylation of p130^{Cas} in 3Y1 cells transformed with v-*crk* (25). Moreover, WT-PTP1B, but not PA-PTP1B, binds to p130^{Cas}, suggesting that this protein may be a physiologic target for PTP1B in cells (25). To determine whether PTP1B is capable of binding p130^{Cas} directly, we performed an overlay assay. Defined amounts of GST–WT-PTP1B, GST–PA-PTP1B, and catalytically inactive PTP1B (GST–CS-PTP1B) were each spotted on a nitrocellulose filter, which was then probed with a ^{32}P-labeled GST-SH3 domain derived from p130^{Cas}. The results of this experiment show that recombinant PTP1B binds to the purified p130^{Cas} SH3 domain and that this binding is mediated by the proline-rich motif located near the C terminus of PTP1B (Fig. 1). The presence or absence of PTP catalytic activity does not detectably influence binding to the p130^{Cas} SH3 domain, as evidenced by the similar binding properties of WT- and CS-PTP1B.

Overexpression of WT-PTP1B, but not of a mutant form that cannot bind SH3-containing proteins, causes phenotypic reversion of v-*crk*-transformed cells. In a transient transfection system, we have found that p130^{Cas} tyrosine phosphorylation levels are substantially decreased in cells overexpressing WT-PTP1B, but not PA- or CS-PTP1B (25). These results, combined with the binding data described above, suggest that p130^{Cas} may be a direct substrate for PTP1B. To test the physiological significance of this interaction, we examined the effects of PTP1B expression on v-*crk* oncogene-mediated transformation of 3Y1 cells.

When transfected with v-*crk*, 3Y1 fibroblasts become highly transformed. We constructed stable 3Y1-v-*crk* cell lines that overexpress WT-, PA-, or CS-PTP1B. The expression levels of PTP1B in these cell lines were monitored by immunoblotting and represent about a three- to fivefold excess over endogenous PTP1B (not shown). The growth properties of these cell lines were first studied by a focus formation assay. As shown in Table 1, the number of foci in 3Y1-v-*crk* cells expressing WT-

TABLE 1. Focus formation and anchorage-independent growth of 3Y1-v-*crk* cell lines overexpressing PTP1B

PTP1B overexpressor	Clone no.	Mean no. of transformed foci ± SD[b]	Colony formation in soft agar[c]
C[a]	1	169 ± 12	++++
	2	178 ± 9	+++++
	3	200 ± 15	+++++
WT	4	9 ± 5	+
	5	4 ± 2	+
	10	11 ± 4	+
PA	1	141 ± 30	++++
	2	111 ± 10	++++
	5	100 ± 12	+++++
CS	2	128 ± 15	++++
	7	121 ± 12	+++
	8	147 ± 10	++++

[a] C, control.
[b] Values are means and standard deviations of three plates of each stable cell line.
[c] Colony number was assessed semiquantitatively, based on numbers of colonies of >0.1 mm in size per microscopic field, + to +++++, fewest to most colonies, respectively.

PTP1B is dramatically decreased compared to that in cells expressing PA-PTP1B, CS-PTP1B, or controls. The appearance of a confluent culture of these cell lines is shown in Fig. 2A. Parental 3Y1 cells display a flattened cobblestone cell morphology (Fig. 2A, subpanel A), whereas v-*crk*-transformed 3Y1 cells lose their contact inhibition and become spindle shaped (subpanel E). Cells expressing WT-PTP1B revert completely to the cobblestone shape representative of the original nontransformed 3Y1 cell line (subpanel B). In contrast, 3Y1-v-*crk* cells expressing CS- or PA-PTP1B retained a transformed morphology (subpanels C and D). As PA-PTP1B retains full catalytic activity in vitro (25), the inability of this PTP1B mutant to effect reversion may reflect changes in its ability to interact with specific physiologic targets.

To determine if these stable cell lines retain the property of anchorage independence, cells were plated in medium containing 0.3% agar, and colony formation was assessed at 4 weeks (Table 1). 3Y1-v-*crk* cells expressing CS-PTP1B and PA-PTP1B displayed typical colony formation in soft agar, similar to the vector control (data not shown). On the other hand, few colonies were observed with 3Y1-v-*crk* cells expressing WT-PTP1B. These experiments demonstrate that cells expressing WT-PTP1B, but not those expressing PA- or CS-PTP1B, became anchorage dependent.

Since anchorage dependence is not always coupled to serum dependence (40), we also determined the growth characteristics of each cell line with respect to serum requirements, by comparing the growth rates of these cell lines in 1% FBS (Fig. 2B). Like parental 3Y1 cells, the 3Y1-v-*crk* cells expressing WT-PTP1B were unable to proliferate in culture medium containing 1% FBS. Similar cells, expressing PA- or CS-PTP1B, displayed substantially increased growth rates, similar to those seen in 3Y1-v-*crk* control cell lines.

We also repeated these experiments by reversing the order of transfection, first constructing PTP1B-expressing stable 3Y1 cell lines and then transfecting these with a v-*crk* expression vector. The results from these experiments mirror those described above: WT-, but not PA- or CS-PTP1B, inhibits transformation by v-*crk*, as assessed by focus formation, anchorage dependence, and serum dependence assays (not shown). Thus, overexpression of WT-PTP1B can suppress the phenotype of previously established transformed cells as well as prevent transformation by v-*crk* de novo.

Overexpression of WT-PTP1B, but not a mutant form that cannot bind SH3-containing proteins, induces tyrosine dephosphorylation of p130^{Cas}, Paxillin, and Fak in v-*crk*-transformed cells. We examined the tyrosine phosphorylation levels of cellular proteins in cell expressing WT-PTP1B or mutant forms of PTP1B in order to evaluate the signal transduction molecules that may be involved in the suppression of transformation by this enzyme. Cell lysates were separated on SDS-PAGE gels and probed with antiphosphotyrosine antibody. Seven prominent phosphotyrosyl proteins, which migrate at about 130, 120, 90, 85, 68, 65, and 52 kDa on SDS-PAGE gels, are apparent in lysates derived from 3Y1-v-*crk* cells (Fig. 3). In 3Y1-v-*crk* cells expressing WT-PTP1B, the tyrosine phosphorylation levels of all of these proteins are substantially reduced. As expected, there is no reduction in tyrosine phosphorylation levels of any of these proteins in cells expressing catalytically inactive PTP1B (CS-PTP1B). Cells expressing PA-PTP1B show no reduction in tyrosine phosphorylation of p130, p125, p68, or p65 but do show diminished tyrosine phosphorylation of p90, p85, and p52. These results are consistent with our previous data showing that PA-PTP1B is catalytically competent (25) and indicate that interactions with SH3 proteins are required for the tyrosine dephosphorylation of some, but not all, proteins by PTP1B in cells.

To determine if the p130 phosphotyrosyl protein that is affected by WT-PTP1B expression is in fact p130^{Cas}, the phosphorylation state of p130^{Cas} was determined directly by immunoprecipitating this protein from these cell lines, followed by antiphosphotyrosine and anti-p130^{Cas} immunoblotting (Fig. 4, top panel). Expression of WT-PTP1B resulted in substantial (approximately threefold, by densitometry) tyrosine dephosphorylation of p130^{Cas}, while PA-PTP1B or CS-PTP1B did not affect this protein. Since we have demonstrated that PTP1B directly binds to p130^{Cas} via a proline-rich ligand–SH3 domain interaction, these data suggest that p130^{Cas} might be one of the physiological targets of PTP1B in cells.

Since Fak and Paxillin are proteins of 125 and 68 kDa, respectively, and are known to associate in complexes with p130^{Cas} and become tyrosine phosphorylated in v-*crk*-transformed cells (7, 16), we examined the tyrosine phosphorylation levels of these proteins in such cells expressing PTP1B. As with p130^{Cas}, expression of WT-PTP1B reduced the tyrosine phosphorylation of Paxillin about threefold (Fig. 4, middle panel). The PA- and CS-PTP1B mutants had little effect. Similarly, expression of WT-PTP1B substantially reduced (approximately fourfold) the tyrosine phosphorylation of Fak, while the PA-PTP1B mutant had little effect (Fig. 4, lower panel). These results suggest that the ≈120- and 68-kDa proteins that are dephosphorylated in cells expressing WT-PTP1B may be Fak and Paxillin.

Expression of PTP1B in 3Y1-v-*crk* cells induces loss of association between p130^{Cas} and Crk. In 3Y1-v-*crk* cells, the SH2 domain of v-Crk has been shown to mediate binding to tyrosine-phosphorylated p130^{Cas} (7, 36). To investigate the effect of expressing PTP1B on association between v-Crk and p130^{Cas}, p130^{Cas} was immunoprecipitated from these cell lines and the amount of v-Crk in the immunocomplexes was determined by immunoblotting (Fig. 5A). In the PA- or CS-PTP1B-expressing cells, approximately the same amount of v-Crk was associated with p130^{Cas} as in control cells. In cells expressing WT-PTP1B, much less v-Crk was associated with p130^{Cas}. Densitometric studies show that the extent of decreased ty-

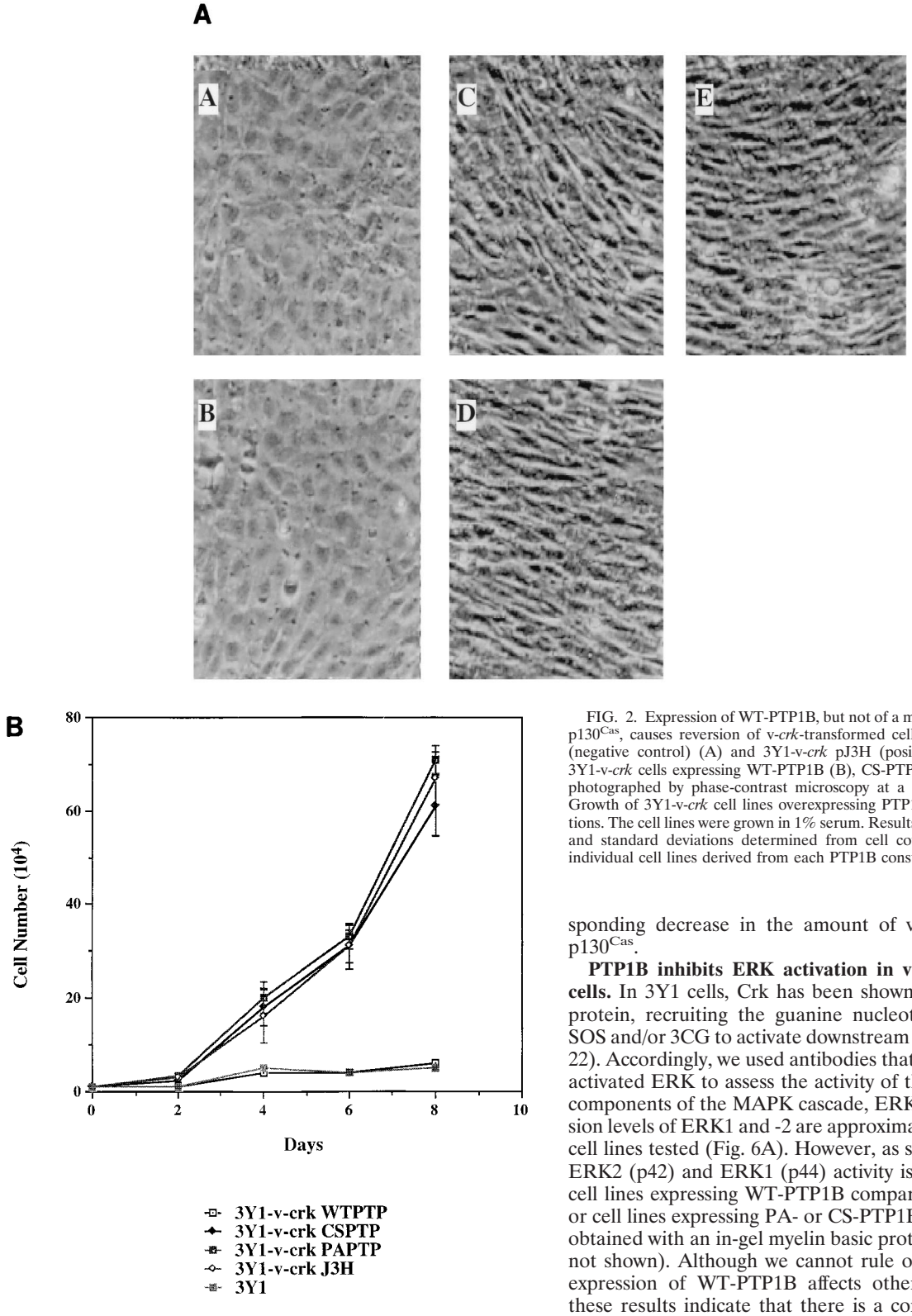

FIG. 2. Expression of WT-PTP1B, but not of a mutant form that cannot bind $p130^{Cas}$, causes reversion of v-*crk*-transformed cells. (A) Morphology of 3Y1 (negative control) (A) and 3Y1-v-*crk* pJ3H (positive control) (E) cells and 3Y1-v-*crk* cells expressing WT-PTP1B (B), CS-PTP1B (C), or PA-PTP1B (D), photographed by phase-contrast microscopy at a 100-fold magnification. (B) Growth of 3Y1-v-*crk* cell lines overexpressing PTP1B in low serum concentrations. The cell lines were grown in 1% serum. Results are expressed as the means and standard deviations determined from cell counts of two wells of three individual cell lines derived from each PTP1B construct.

rosine phosphorylation of $p130^{Cas}$ corresponds to the decreased amount of v-Crk associated with $p130^{Cas}$. The total amount of $p130^{Cas}$ in each immunoprecipitate is approximately equal (Fig. 5B). These data show that the dephosphorylation of $p130^{Cas}$ induced by WT-PTP1B is accompanied by a corresponding decrease in the amount of v-Crk associated with $p130^{Cas}$.

PTP1B inhibits ERK activation in v-*crk*-transformed 3Y1 cells. In 3Y1 cells, Crk has been shown to act as an adapter protein, recruiting the guanine nucleotide exchange factors SOS and/or 3CG to activate downstream signaling pathways (5, 22). Accordingly, we used antibodies that specifically recognize activated ERK to assess the activity of the major downstream components of the MAPK cascade, ERK1 and -2. The expression levels of ERK1 and -2 are approximately equal in all of the cell lines tested (Fig. 6A). However, as shown in Fig. 6B, both ERK2 (p42) and ERK1 (p44) activity is markedly reduced in cell lines expressing WT-PTP1B compared to that in controls or cell lines expressing PA- or CS-PTP1B. Similar results were obtained with an in-gel myelin basic protein kinase assay (data not shown). Although we cannot rule out the possibility that expression of WT-PTP1B affects other signaling pathways, these results indicate that there is a correlation between the ability of PTP1B to cause morphologic reversion of 3Y1-v-*crk* cells and its ability to deactivate ERK1 and -2.

Overexpression of WT-PTP1B, but not of PA-PTP1B, causes reversion of v-*src*- and v-*ras*-, but not v-*raf*, transformed cells. $p130^{Cas}$ is a highly tyrosine-phosphorylated protein in v-*src*- and v-*crk*-transformed cells and has been suggested to be a key

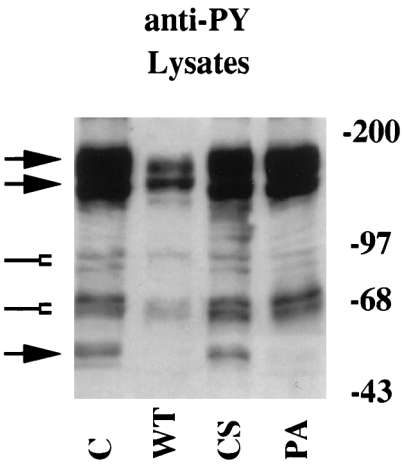

FIG. 3. Overexpression of WT-, but not a proline mutant, PTP1B in 3Y1-v-*crk* cells causes dephosphorylation of a 130-kDa protein that comigrates with p130^{Cas}. Lysates derived from representative 3Y1-v-*crk* stable cell lines expressing vector alone (C; clone 1), WT-PTP1B (clone 10), CS-PTP1B (cysteine to serine, enzymatically inactive; clone 2), and PA-PTP1B (proline-to-alanine mutation, defective in p130^{Cas} SH3 binding; clone 5) were separated by SDS–7% PAGE and immunoblotted with antiphosphotyrosine (anti-PY) antibodies. Numbers at right are molecular masses in kilodaltons. Arrows and forks indicate prominent phosphotyrosyl proteins.

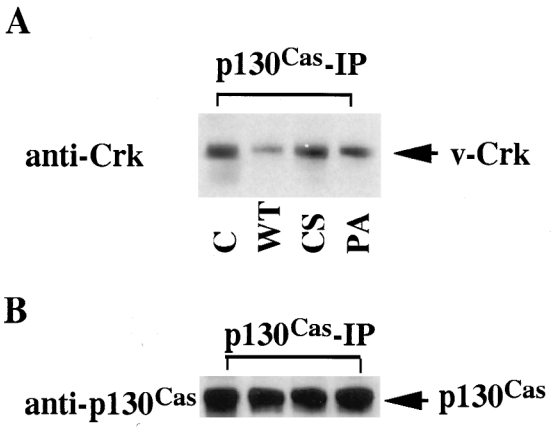

FIG. 5. Expression of WT-, but not PA-, PTP1B decreases the amount of v-Crk associated with p130^{Cas}. (A) Lysates derived from representative 3Y1-v-*crk* stable cell lines expressing vector alone, WT-PTP1B, CS-PTP1B, and PA-PTP1B were immunoprecipitated with polyclonal anti-p130^{Cas} sera. Immunocomplexes were separated by SDS–10% PAGE and immunoblotted with monoclonal anti-Crk. C, control. (B) The same blot was stripped and immunoblotted with monoclonal anti-p130^{Cas}.

molecule in regulating cell transformation (2, 7, 36). Since we have found that WT-PTP1B can reverse or inhibit transformation of v-*crk*, we wondered whether this enzyme could inhibit transformation by other oncogenes, such as v-*src*, v-*ras*, and v-*raf*. Accordingly, 3Y1-v-*src*, -v-*ras*, and v-*raf* cell lines were established. The expression of these oncogenes was monitored by immunoblotting (data not shown). These cell lines were then transfected with an empty vector or expression vectors bearing WT-, PA-, or CS-PTP1B. Stable cell lines were established as outlined in Materials and Methods. The transformation potential of these cell lines was assayed by focus formation and anchorage independence assays. In control cells, all three oncogenes induced focus formation and anchorage-independent growth (Table 2). Expression of WT-, but not PA-PTP1B, resulted in morphological reversion of v-*src* (Fig. 7A)- and v-*ras* (not shown)-transformed cells, as well as marked inhibition of focus formation and growth in soft agar (Table 2). However, WT-PTP1B had little effect on the morphology (data not shown), focus-forming ability (Table 2), or anchorage independence of v-*raf*-transformed cells. The parental 3Y1-v-*src*

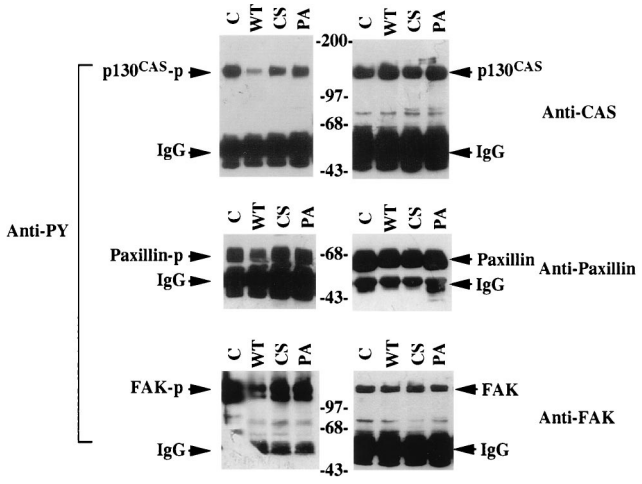

FIG. 4. Expression of PTP1B reduces tyrosine phosphorylation of p130^{Cas}, Paxillin, and Fak. Lysates derived from representative 3Y1-v-*crk* stable cell lines expressing vector alone (C; clone 1), WT-PTP1B (clone 10), CS-PTP1B (cysteine to serine, enzymatically inactive mutant; clone 2), and PA-PTP1B (proline-to-alanine mutation, defective in p130^{Cas} SH3 binding; clone 5) were immunoprecipitated with anti-p130^{Cas}, anti-Paxillin, or anti-Fak antibodies. Immunocomplexes were separated by SDS–7% PAGE and immunoblotted with antiphosphotyrosine (PY) antibodies or anti-p130^{Cas}, anti-Paxillin, or anti-Fak antibodies as indicated. P indicates the tyrosine-phosphorylated form of protein. IgG, immunoglobulin G. Numbers between panels show molecular mass in kilodaltons.

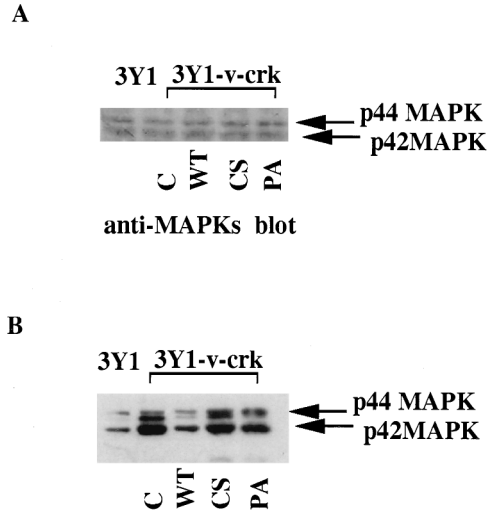

FIG. 6. Expression and enzymatic deactivation of MAPK by WT-PTP1B but not mutant forms of PTP1B. Protein lysates were prepared from 3Y1-v-*crk* stable lines bearing: vector alone (C; clone 1), WT-PTP1B (clone 10), CS-PTP1B (enzymatically inactive; clone 2), and PA-PTP1B (proline-to-alanine mutation, defective in p130^{Cas} SH3 binding; clone 5). Immunodetection of MAPK, ERK2 (p42), and ERK1 (p44) was performed with either anti-ERK sera (A) or anti-phospho-MAPK antibodies (B).

cells and 3Y1-v-*src* cells expressing PA-PTP1B were able to grow in 1% FBS, while cells expressing WT-PTP1B were not (Fig. 7B). Similar data were obtained for 3Y1-v-*ras* cells but not 3Y1-v-*raf* cells (data not shown). These data demonstrate that transformation by v-*src* and v-*ras* can be reversed by WT-PTP1B, but not by PA-PTP1B, and that this reversion is specific to particular oncogenes. Interestingly, like WT-PTP1B, expression of catalytically inactive PTP1B (CS-PTP1B) in 3Y1-v-*ras* cells partially inhibited transformation. This paradoxical effect may be due to sequestration of phosphotyrosine residues in signaling proteins required for transformation by Ras (44). However, CS-PTP1B expression has no detectable effect on transformation by v-*src* or v-*raf*.

Transformation of 3Y1 cells with v-*src* results in a modest activation of ERK1 and -2, as detected by anti-phospho-ERK antibodies (Fig. 8). Coexpression of WT-PTP1B reduces this Src-induced activation to control levels. In contrast, Ras potently activates ERK1 and -2, but coexpression of PTP1B has no detectable effect on this activation. ERK1 and -2 expression was equal in all cell lysates (data not shown). Thus, cells expressing oncogenic Ras plus WT-PTP1B are phenotypically normal and yet have constitutive, sustained ERK activation.

Interestingly, the phosphotyrosine levels of $p130^{Cas}$ are substantially elevated in Ras-transformed cells (Fig. 9). Coexpression of WT-PTP1B causes an approximately 50% reduction in this tyrosine phosphorylation, whereas PA- and CS-PTP1B are without notable effect. These data imply that Ras, either directly or indirectly, activates one or more tyrosine kinases that tyrosine phosphorylate $p130^{Cas}$ and suggest that this phosphorylation may be required for transformation by Ras. The reduction in $p130^{Cas}$ tyrosine phosphorylation in WT-PTP1B-expressing cells is consistent with the hypothesis that $p130^{Cas}$ represents an important target for PTP1B in cells. If this theory is correct, one must also assume that PTP1B dephosphorylates specific key residues in $p130^{Cas}$, since this protein remains substantially tyrosine phosphorylated in (reverted) cells expressing Ras and WT-PTP1B.

TABLE 2. Focus formation and anchorage-independent growth of 3Y1-v-*src* and eY1-v-*ras* cell lines overexpressing PTP1B

Oncogene and PTP1B overexpressor	Clone no.	Mean no. of transformed foci ± SD[b]	Colony formation in soft agar[c]
v-*src*			
C[a]	1	122 ± 11	++++
	2	163 ± 16	+++++
	3	155 ± 21	+++++
WT	1	12 ± 2	+
	2	5 ± 2	+
	4	6 ± 3	+
PA	1	135 ± 15	++++
	2	147 ± 18	+++++
	4	163 ± 14	+++++
CS	3	111 ± 10	++++
	6	107 ± 9	+++
	9	98 ± 18	++++
v-*ras*			
C	3	132 ± 11	+++++
	5	121 ± 7	+++++
	8	99 ± 8	++++
WT	1	6 ± 4	+
	5	11 ± 2	+
	6	7 ± 3	+
PA	1	141 ± 16	+++++
	7	167 ± 15	+++++
	8	171 ± 17	+++++
CS	2	26 ± 14	+++[d]
	4	31 ± 12	++
	9	27 ± 3	+++
v-*raf*			
C	1	146 ± 12	+++++
	2	176 ± 20	++++
WT	4	122 ± 19	+++
	6	120 ± 14	+++
PA	7	136 ± 3	++++
	14	114 ± 11	+++
CS	3	152 ± 16	+++
	10	118 ± 10	+++

[a] C, control.
[b] Values are means and standard deviations of three plates for each stable cell line.
[c] Colony number was assessed semiquantitatively, based on number of colonies of >0.1 mm in size per microscopic field. + to +++++, fewest to most colonies, respectively.
[d] These colonies are smaller in size than control colonies.

DISCUSSION

PTP1B has been implicated as a negative regulator of cell growth and differentiation (1, 3, 10, 21, 43). While it has been reported that the expression of PTP1B in NIH 3T3 cells suppresses v-*src*- and *neu*-mediated transformation (6, 46), the molecular mechanism(s) by which this repression occurs is unknown. PTP1B is a notoriously promiscous enzyme in vitro; thus, it has been difficult to ascribe its negative effects on cell growth to interruption of specific signaling pathways.

Given its abundant expression, broad tissue distribution, and robust catalytic capabilities, it is clear that the activity of PTP1B, and/or its ability to access substrates, must be tightly regulated in cells. In an effort to determine the identity of such substrates, we noted that PTP1B has two potential SH3 binding sequences in its C terminus, and therefore, we tested whether this phosphatase binds to SH3-containing proteins. We showed that PTP1B can associate with a variety of SH3-containing proteins in vitro and with at least one of these, $p130^{Cas}$, in cultured cells (25). In this report, we show that the ability of PTP1B to suppress or inhibit transformation is abolished by mutation of prolines 309 and 310 within a proline-rich sequence in its C terminus. This region is predicted to form a class II SH3-binding ligand and is required for binding to $p130^{Cas}$. A trivial explanation for these findings—that mutations in this region destabilize the enzyme, rendering it catalytically defective—is unlikely, as the kinetic properties of the WT enzyme and the PA-PTP1B mutant are indistinguishable in vitro (25). It is therefore most likely that the contrasting effects of WT- and PA-PTP1B on transformation are related to differences in the abilities of these proteins to interact with SH3-containing proteins.

Overexpression of WT-, but not PA-PTP1B, can inhibit transformation of 3Y1 cells by v-*crk*, -*src*, and -*ras*. In v-*crk*-transformed cells, these effects on transformation correlate with the tyrosine phosphorylation state of at least four distinct proteins, which migrate at about 130, 120, 68, and 65 kDa on SDS-PAGE gels. These proteins could each represent direct, SH3-containing substrates for PTP1B or, alternatively, could represent proteins whose phosphorylation depends on a common, tyrosine-phosphorylated signaling molecule, which is targeted by PTP1B. In this latter case, dephosphorylation of a protein (for example, a tyrosine kinase or a docking protein that is required for assembly of a signaling complex) might also affect tyrosine phosphorylation of one or more additional proteins. The 130-, 125-, and 68-kDa species appear to represent

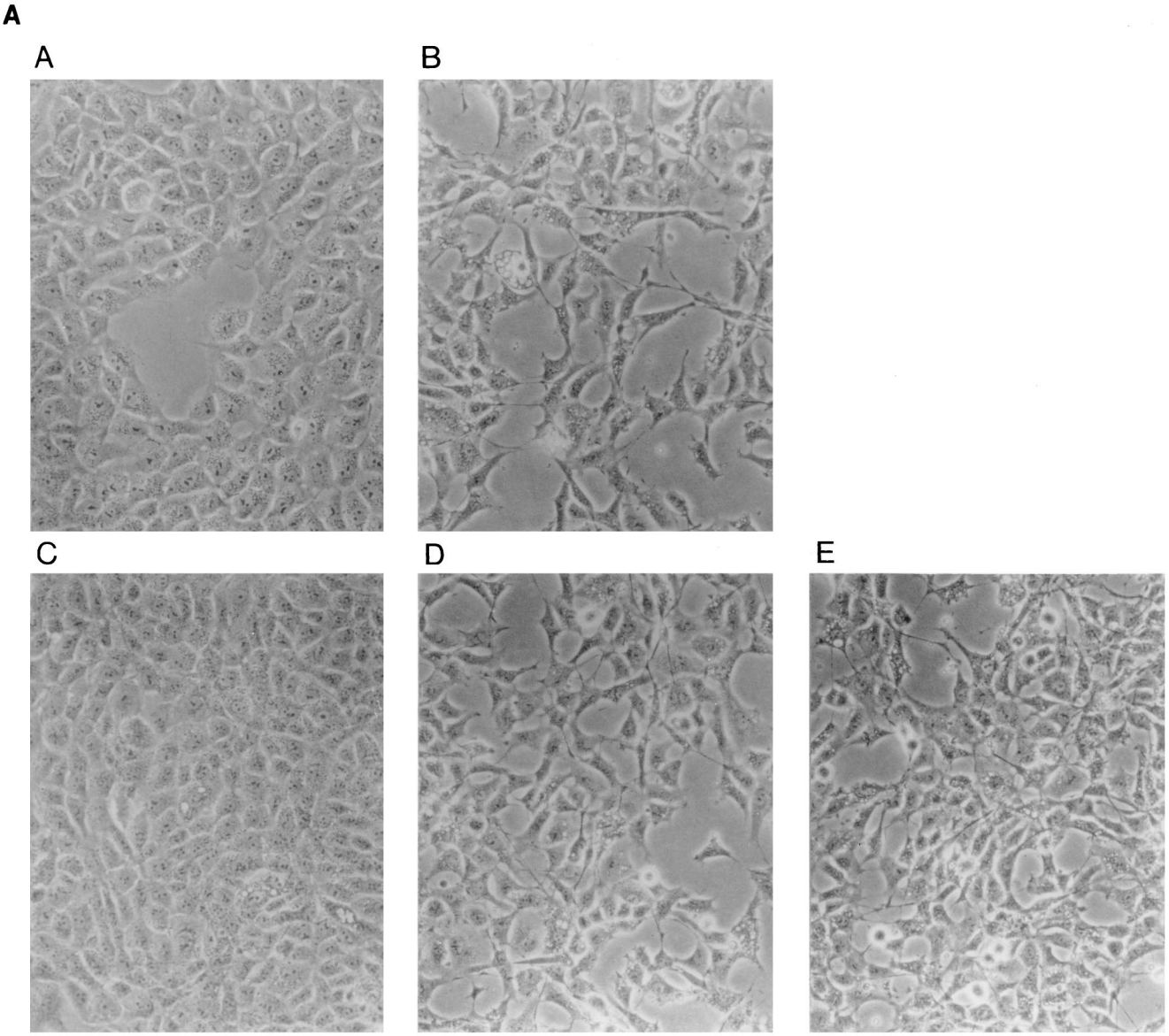

FIG. 7. Expression of WT-PTP1B, but not PA-PTP1B, suppresses transformation by Src. (A) Morphological reversion, demonstrated by morphology of 3Y1 cells (A); 3Y1-v-*src* cells expressing CS-PTP1B (B), WT-PTP1B (C), or PA-PTP1B (D); or control 3Y1 v-*src* cells (E). (B) Restoration of serum dependence. The indicated cell lines were grown in 1% serum. Results are expressed as the means and standard deviations determined from cell counts of two wells of three individual cell lines derived from each PTP1B construct.

$p130^{Cas}$ and two other proteins (Fak and Paxillin) that are associated with $p130^{Cas}$ in cells. Although Fak, like $p130^{Cas}$, contains an SH3 domain, we have not been able to show direct association of Fak or Paxillin with PTP1B in vitro or in cells (data not shown). Since Fak and Paxillin associate with $p130^{Cas}$, it seems reasonable to assume that PTP1B is brought into close proximity with these proteins when bound to $p130^{Cas}$ and subsequently dephosphorylates them. The inability of PA-PTP1B to reverse or inhibit transformation, or to bind or dephosphorylate $p130^{Cas}$, suggests that WT-PTP1B specifically down-regulates $p130^{Cas}$, a pivotal molecule regulating cell growth.

PTP1B is located in the ER (14), whereas $p130^{Cas}$ has been reported to reside primarily in focal adhesions, where it is

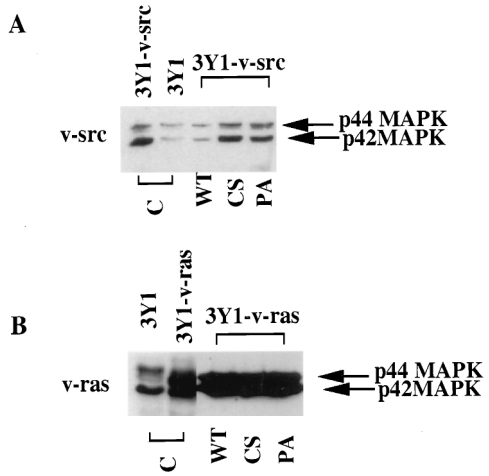

FIG. 8. PTP1B suppresses MAPK activation by Src but not by Ras. Protein lysates were prepared from parental 3Y1 cells or 3Y1-v-*src* (A) or 3Y1-v-*ras* (B) stable cell lines expressing WT-, CS-, or PA-PTP1B. Immunodetection of the MAPKs ERK2 (p42) and ERK1 (p44) was performed with anti-phospho-MAPK antibodies. C, control.

thought to play a role in integrin signaling (16, 26, 31–33, 37). How then do these proteins interact? One possibility is that a subset of PTP1B is located in focal adhesions, as suggested by Mauro and Dixon (28). However, subcellular fractionation and immunofluorescence data do not thus far support an extra-ER location for any significant fraction of PTP1B (14, 25). A second possibility is that a subpopulation of $p130^{Cas}$ is located in or near the ER. In 3Y1 and 3Y1-v-*crk* cells, we found that substantial amounts of $p130^{Cas}$ cosediment with the 1.2-2.0 M sucrose fraction, which is highly enriched for ER membranes and contains nearly all the cellular PTP1B. Thus, it is possible that the PTP1B-$p130^{Cas}$ interaction occurs at or near the ER. In this context, it should be noted that the membranes of the ER are extensive, contiguous with the nuclear envelope, and in contact with the cytoskeleton. It is therefore not inconceivable that ER-bound PTP1B has access to cytoplasmic or cytoskeletal substrates. Thus, there is ample opportunity for PTP1B to contact signaling proteins that affect cell growth and adhesion. Whatever the exact site of interaction between PTP1B and $p130^{Cas}$, the strict correlation among $p130^{Cas}$ binding, phosphorylation state, and transformation strongly suggests that the negative growth effects of PTP1B can in large part be attributed to its interactions with SH3-containing proteins such as $p130^{Cas}$. It remains to be seen whether interactions with other SH3-containing proteins such as Crk or Shc are also important to the biological functions of PTP1B.

The dephosphorylation of $p130^{Cas}$ and its binding partners could well account for many of PTP1B's effects on cell growth. Cells lacking $p130^{Cas}$ (due to antisense expression) resist transformation by a variety of oncogenes, whereas overexpression of $p130^{Cas}$ induces transformation (2). $p130^{Cas}$ dephosphorylation by PTP1B may affect the functions of Crk and Src, either because these oncoproteins signal through $p130^{Cas}$ or because they too are dephosphorylated by PTP1B. However, how does Ras fit into this scheme? Unlike Crk and Src, Ras is not known to associate with either $p130^{Cas}$ or PTP1B. Why, then, should PTP1B interfere with transformation by Ras? Interestingly, Auvinen et al. recently showed that reducing $p130^{Cas}$ levels by antisense expression inhibits transformation by Ras (2), though the molecular details underlying this phenomenon were not addressed by these authors. Here, we show that overexpression of PTP1B inhibits transformation by Ras, without impeding the ability of Ras to activate MAPKs. We also show that the tyrosine phosphorylation level of $p130^{Cas}$ is markedly elevated in Ras-transformed cells and that coexpression of WT- (but not mutant) PTP1B reduces this phosphorylation. In addition to supporting the argument that PTP1B inhibits transformation by its action on $p130^{Cas}$ or other SH3-containing proteins, these data also imply that, either directly or indirectly, Ras activates one or more tyrosine kinases that phosphorylate $p130^{Cas}$. To our knowledge, the only prior demonstration of Ras-induced tyrosine kinase activity was reported by Cuadrado (12). Using a dexamethasone-dependent expression system, this author showed that tyrosine phosphorylation of cellular proteins correlated with Ras expression levels. Because tyrosine phosphorylation preceded manifestations of the transformed phenotype, this author argued that the effects of Ras on tyrosine phosphorylation were unlikely to represent secondary changes due to Ras-dependent expression of autocrine growth factors. Our data, which are derived from stable expression of Ras plus or minus PTP1B, cannot address the issue of primary versus secondary stimulation of tyrosine kinases by Ras. However, they are consistent with the findings of both Auvinen et al. (2) and Cuadrado (12) and suggest that, for transformation, Ras must activate a tyrosine kinase(s) that phosphorylates $p130^{Cas}$.

While overexpression of PTP1B suppresses transformation by Crk, Src, and Ras, it has little effect on transformation of Raf. These results may appear surprising, since PTP1B does not affect MAPK activation by Ras, which is presumably mediated by endogenous c-Raf. However, it is likely that the signals generated from overexpressed v-Raf differ, either in intensity or in specificity, from those of Ras-activated endogenous c-Raf. The v-Raf construct we used is driven by a strong promoter and lacks the entire N-terminal regulatory domain; thus, it might be expected to affect different pathways than endogenous c-Raf, even when the latter is maximally stimulated by exogenous oncogenic Ras.

Assuming that $p130^{Cas}$ is in fact a major physiological target for PTP1B, we can link these data together by a model that places $p130^{Cas}$ downstream of Ras (Fig. 10). This model posits

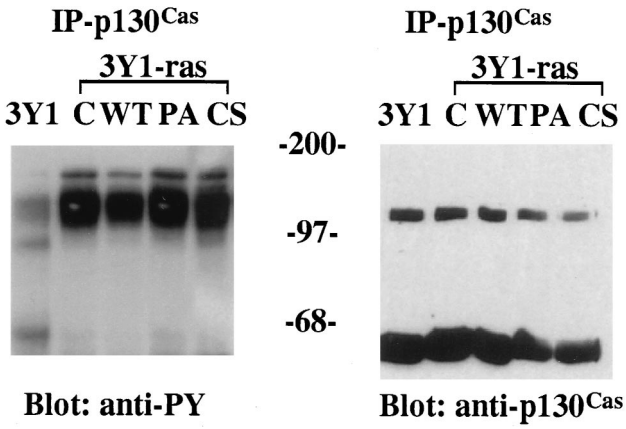

FIG. 9. PTP1B suppresses $p130^{Cas}$ tyrosine phosphorylation by Ras. Protein lysates were prepared from parental 3Y1 cells or 3Y1-v-*ras* stable cell lines expressing WT-, CS-, or PA-PTP1B. Cell lysates were immunoprecipitated with anti-$p130^{Cas}$, and immunocomplexes were separated by SDS–7% PAGE and immunoblotted with anti-phosphotyrosine (PY) antibodies or anti-$p130^{Cas}$ as indicated. Densitometric analysis indicates a ≈50% reduction in phosphotyrosine content of $p130^{Cas}$ in cells expressing WT-PTP1B relative to that of controls (left panel, compare lanes 2 and 3). C, control. Numbers between panels show molecular mass in kilodaltons.

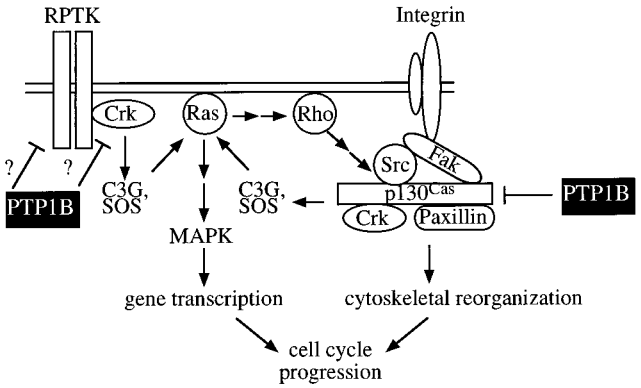

FIG. 10. Model for suppression of growth by PTP1B. In rat fibroblasts, certain RPTKs, such as the epidermal growth factor receptor, signal to Ras via the adapter Crk. Similarly, adhesion signals may be transmitted to Ras via Crk. Ras activates the MAPK cascade and may also contribute a signal to $p130^{Cas}$ via Rho. Activation of both these pathways may be required for cell cycle progression and full transformation. Reducing the level of tyrosine-phosphorylated $p130^{Cas}$, either by dominant negative Rho, by PTP1B-catalyzed tyrosine dephosphorylation, or by reduction of $p130^{Cas}$ expression levels by antisense techniques, may therefore suppress transformation by Ras and other oncogenes. PTP1B may also affect proliferation via interactions with the adapter protein Crk or with RPTKs.

that for full transformation, Ras must activate both the MAPK cascade and at least one additional pathway that includes $p130^{Cas}$. The pathway from Ras to $p130^{Cas}$ may well involve Rho, which is known to stimulate $p130^{Cas}$ tyrosine phosphorylation (13). This model is also consistent with the observation that Rho is required for transformation by Ras (34, 35). However, it is likely that the relationship between Ras and $p130^{Cas}$ is not simply that of upstream activator to downstream effector. For example, adhesion-dependent MAPK activation (which may flow through $p130^{Cas}$) requires Ras (11, 37, 38). Therefore, Ras and $p130^{Cas}$ signaling pathways are unlikely to be linear but, rather, complex and mutually reinforcing. In this view, interruption of the circuit at any point could terminate the growth signal. PTP1B may also interfere with mitogenic signal transduction via interactions with Crk or other SH3-containing adapters such as Shc, Grb2, or Nck. Alternatively, PTP1B might suppress growth pathways by direct binding to receptor protein tyrosine kinases (RPTKs) (3, 23, 24, 29). However, direct binding and subsequent dephosphorylation of such receptors by PTP1B are unlikely to represent a major mechanism by which this phosphatase down-regulates mitogenic signal transduction, since PA-PTP1B, which should bind normally to RPTKs, has minimal effects on cell growth. Instead our results suggest that such RPTK dephosphorylation may be mediated by binding of PTP1B to an SH3-containing protein that also associates with RPTKs (e.g., an adapter protein) (Fig. 10). Whatever the exact identity of PTP1B's key targets, it is clear that interaction with SH3-containing proteins is required for growth suppression by this enzyme. The identification of such additional SH3-containing binding partners for PTP1B should add considerably to our understanding of the regulation of mitogenic signal transduction.

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