Combination of Gene Targeting and Substrate Trapping to Identify Substrates of Protein Tyrosine Phosphatases Using PTP-PEST as a Model†

Jean-François Côté,‡ Alain Charest,§ John Wagner,‡ and Michel L. Tremblay*,‡

Department of Biochemistry, McGill University, 3655 Drummond Street, Montreal, Quebec, Canada, H3G 1Y6, and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received May 27, 1998; Revised Manuscript Received July 16, 1998

ABSTRACT: Identification of physiological substrates of protein tyrosine phosphatases is a key step in understanding the function of these enzymes. We have generated fibroblast cell lines having a gene-targeted PTP-PEST in order to identify potential substrates with the premise that specific substrates of this enzyme would exist in a hyperphosphorylated state. Analysis of the profile of the phosphotyrosine proteins in the PTP-PEST −/− cells revealed the presence of hyperphosphorylated proteins of 180, 130, and 97 kDa when compared to control cells. The p130 was identified as p130^{Cas}, and direct immunoprecipitates of p130^{Cas} demonstrate that this protein is constitutively hyperphosphorylated in cells lacking PTP-PEST. In addition, p130^{Cas} was also isolated by the substrate-trapping mutant of PTP-PEST in the PTP-PEST −/− cell lysates. Interestingly, we have demonstrated for the first time that PTP-PEST, through its first proline-rich sequence ^{332}PPKPPR^{337}, interacts with other members of the p130^{Cas} family (Hef1 and Sin) via their SH3 domain in vitro. This result suggests that Hef1 and Sin could also be potential substrates of PTP-PEST. In conclusion, we have combined genetic and biochemical strategies to allow the identification of PTP-PEST substrates. This experimental approach could potentially be used to identify substrates of other PTPases. Furthermore, the Cas-like molecules Hef1 and Sin associate via their SH3 domains with a proline-rich motif found on PTP-PEST, suggesting the possibility that PTP-PEST could be a general modulator of the Cas family of proteins.

Phosphorylation on tyrosine residues is an important mechanism for transmitting extracellular stimuli in cellular events such as cell attachment, mitogenesis, differentiation, and migration (for review, see ref *1*). Protein tyrosine phosphorylation levels are regulated by the activity of two protein families: the protein tyrosine kinases (PTK)[1] and the protein tyrosine phosphatases (PTP). All PTP have a conserved catalytic domain characterized by the signature motif [I/V]HCxAGxxR[S/T]G. Biochemical and kinetic studies have demonstrated that the cysteine residue found in this signature motif is essential for the catalytic activity of PTPs since mutation of this cysteine completely abolishes PTPase activity (*2*). Interestingly, mutation of cysteine 215 to serine or aspartic acid 181 to alanine within the catalytic domain of PTP1B results in substrate-trapping intermediates (*2*), revealing that such mutants are important tools to elucidate the function of PTPs. Unfortunately, before performing substrate-trapping experiments, the phosphotyrosine content of the protein sources must be elevated. To achieve this, two approaches are currently used: first, physiological stimulation of cells, such as with EGF, which triggers a cascade of tyrosine phosphorylation events (*3*) but results in tyrosine phosphorylation of only a limited number of proteins; second, treatment of cells with pervanadate to inhibit intracellular PTP (*4*) results in a very high number of proteins being tyrosine phosphorylated, however, not always on physiologically relevant sites thus giving rise to potential artifacts. Overall, it is unlikely that these approaches will result in the identification of all genuine substrates of a given PTPase and alternative strategies should be envisaged.

PTP-PEST is a soluble PTP that is ubiquitously expressed throughout mouse embryonic development and in adult tissues (*5*). The N-terminal portion of the enzyme encodes for the catalytic domain while the C-terminus portion is composed of five proline-rich domains (*3*) and a binding site for the adaptor protein Shc (*6*). During the search for PTP-PEST targets, the adaptor protein p130^{Cas} was identified as a substrate for PTP-PEST by trapping experiments (*4*). Indeed, two "substrate trap" mutants, PTP-PEST D199A and C231S, have been used to isolate p130^{Cas} from extracts of pervanadate treated Hela cells. We have also observed in a murine context that ligand-dependent activation of the EGF receptor provides a way to induce the binding of PTP-PEST C231S "substrate trap" mutant with a 130 kDa protein later identified as p130^{Cas} (*3*). The recognition of tyrosine

* Corresponding author. Phone: (514) 398-7290. Fax: (514) 398-7384. E-mail: tremblay@med.mcgill.ca.

† This work was supported by a grant from the Medical Research Council of Canada (MRC) to M.L.T. J.F.C. is a recipient of a Cancer Research Society studentship (CRS). A.C. is a recipient of a NCI postdoctoral fellowship. M.L.T. is a Chercheur boursier from Les Fonds De La Recherche En Santé Du Québec (FRSQ).

‡ McGill University.

§ Massachusetts Institute of Technology.

[1] Abbreviations: PTP, protein tyrosine phosphatase; SH, Src homology; PTK, protein tyrosine kinase; aa, amino acids; GST, glutathione S-transferase; HA, hemagluttinin epitope; Myc, Myc epitope; WT, wild-type; TCL, total cell lysate; IP, immunoprecipitation; PAGE, polyacrylamide gel electrophoresis.

S0006-2960(98)01259-8 CCC: $15.00
Published on Web 08/29/1998

phosphorylated p130^{Cas} in pervanadate-treated cell extracts by PTP-PEST D199A was recently shown to be severely impaired by the mutational inactivation of a proline-rich region (P337A), and the authors proposed a mechanism for PTP-PEST substrate recognition via the SH3 domain of p130^{Cas} (*7*).

The PTP-PEST noncatalytic tail contains proline-rich regions that can potentially serve as ligands for SH3 (*8*), WW (*9*), or LIM (*10*) domain containing proteins. Analysis of SH3 domain binding sites has revealed two classes of consensus sequences for proline-rich regions: 1-RxxPxxP and 2-PxxPxR. In this respect, the first and fourth proline-rich domains of PTP-PEST belong to the class 2 consensus sequence whereas the three other proline-rich domains have PxxP motifs that do not belong to either class 1 or class 2 consensus sequences and could thus represent novel protein−protein interaction domains (*3*). We have previously reported that PTP-PEST interacts with the SH3 domains of Grb2 and v-Src (*3*). Following EGF stimulation, PTP-PEST forms a molecular complex with the activated EGF receptor through an SH3 domain mediated interaction with the adaptor protein Grb2 (*3*).

PTP-PEST is a member of the PEST family of PTPases which also includes PTP-PEP (11), PTP-HSCF/PTP-K1/PTP20 (*12*−*14*) and PTP BDP1 (15). The homology between the members of the PEST family of PTPases resides mostly in the catalytic domain and also in a 20 aa stretch rich in basic residues at the C-terminus of these proteins. The conserved C-terminus of PTP-HSCF, as well as corresponding peptides of PTP-PEST and PTP-PEP, was shown to associate with a novel phosphotyrosine containing protein named PSTPIP which localizes to cleavage furrow sites and has been shown in vitro to be a substrate for PTP-HSCF (16).

p130^{Cas} is an adaptor protein composed of an SH3 domain, two proline-rich regions, a YxxP domain containing 15 putative tyrosine phosphorylation sites (*17*−*19*), and a binding site for the SH2 domain of the Src kinases (YDYV motif). The SH3 domain of p130^{Cas} has been shown to interact with proline-rich sequences found in FAK, FAK-related nonkinase (FRNK), RAFTK, and PTP1B (*18*−*21*). In a recent study, the SH3 domain of p130^{Cas} was found essential for targeting p130^{Cas} to focal adhesions in non-transformed cells (22). The YxxP domain of p130^{Cas} can interact in a phosphotyrosine dependent manner with the SH2 domains of v-Crk (23), Crk (24), Crk-II (25), CRKL (26), Nck (27), and several other yet unidentified SH2 domain containing proteins (23). p130^{Cas} has been shown to become tyrosine phosphorylated following cell treatment with EGF (24), NGF (25), and PDGF (28) and also following integrin activation (29). The YDYV motif and the proline-rich region at the C-terminus of p130^{Cas} are binding sites for the SH2 and SH3 domains of Src family of kinases resulting in phosphorylation of other tyrosine residues on p130^{Cas} following its association with these kinases (30).

Two other proteins share a high degree of homology with p130^{Cas}: Hef1/Cas-L (*31*, *32*) and Efs/Sin (*33*, *34*). On the basis of their similar structural organization, Hef1 and Sin have been grouped into the p130^{Cas} family of adaptor proteins. The SH3 domains of Hef1 and Sin are, respectively, 74 and 64% identical to the SH3 domain of p130^{Cas} and the YxxP domains of Hef1 and Sin contain 13 and 8 tyrosine phosphorylation sites, respectively. Several of these tyrosine phosphorylation motifs differ from those found in p130^{Cas}, suggesting that Hef1 and Sin interact with different SH2 domain containing proteins. Hef1 and Sin expression is restricted to specific cell populations, whereas p130^{Cas} is ubiquitously expressed. Indeed, Hef1 is mainly expressed in hematopoietic cells (32) but little is known about Sin expression, although its mRNA is found at high levels in the mouse embryo (33).

Identification of physiological substrates of protein tyrosine phosphatases is a key element in understanding the biological functions of this family of enzymes. The principal aim of this study is to describe a novel experimental approach to identify PTP substrate(s) by combining the technology of gene targeting and substrate-trapping experiments using PTP-PEST as a paradigm. Using these approaches, p130^{Cas} was isolated in high amounts by a PTP-PEST-trapping mutant, thus supporting this strategy. A second objective of the present study was to demonstrate for the first time that the other members of the p130^{Cas} family of proteins, Hef1 and Sin, interact in a similar manner with a proline-rich region found on PTP-PEST through their SH3 domains, suggesting they too could be potential substrates.

MATERIALS AND METHODS

Generation of PTP-PEST Gene Targeted Cell Lines. PTP-PEST +/− and −/− embryonic fibroblast cell lines were established from primary embryonic fibroblast cultures isolated from PTP-PEST heterozygous (+/−) and homozygous (−/−) mouse embryos, respectively.[2] Briefly, embryos were isolated 8.5 days postcoitum from the mating of PTP-PEST (+/−) mice, and then trypsinized for 30 min at 37 °C. Cells in suspension were isolated and cultured. After one passage, cells were immortalized by stably transfecting, using Lipofectamine (Gibco-BRL), a dominant negative mutant p53 (C153S) gene (35) along with adenovirus E1A/E1B genes (obtained from Dr. Branton, McGill University) and a vector conferring puromycin resistance. Cells surviving puromycin selection were pooled and genotyped by Southern and northern blot analyses. For Southern blotting, genomic DNA was digested with *Bam*HI and separated on a 0.8% agarose gel. After transfer to Hybond N+ (Amersham), the blot was probed with a ^{32}P-radiolabeled *Kpn*I/*Sac*I fragment of the PTP-PEST cDNA. For northern blotting, total RNA was separated on a 1% formaldehyde agarose gel and following transfer to Hybond N+, the blot was probed with a ^{32}P-radiolabeled probe generated by PCR from the PTPase domain of PTP-PEST. The blot was stripped and reprobed with a GAPDH probe to verify equal loading of total RNA.

Constructs. p130^{Cas} mouse cDNA in pBluescript was a generous gift from Dr. Steven Hanks (Vanderbilt School of Medicine, Tennessee). The p130^{Cas} cDNA was cloned into pJFTAG, pBluescript II KS (Stratagene), and pCDNA3 (Invitrogen) at the *Eco*RI and *Sal*I sites using standard recombinant DNA technology. pJFTAG is a pCDNA3 derivative in which six copies of the c-myc epitope were cloned in the *Hin*dIII and *Eco*RI sites. The SH3 domain of p130^{Cas} was cloned in the *Eco*RI and *Apa*I sites of pJFTAG.

[2] A.C., and M.L.T., unpublished data.

The GST-SH3 domain of p130^{Cas} and Sin were constructed by amplifying the region encoding for the SH3 domains by PCR using oligonucleotides containing engineered *Bam*HI and *Eco*RI sites followed by cloning of these products in the *Bam*HI and *Eco*RI sites of pGEX 2TK (Pharmacia). The GST SH3 domain of Hef1 was cloned in pGEX1LambdaT by Dr. Yuzhu Zhang and was a kind gift from Dr. Erica Golemis (Fox Chase Cancer Center, Philadelphia, PA).

Cell Culture, Transfections, and Coimmunoprecipitation. 293T, COS-1, PTP-PEST +/−, and PTP-PEST −/− cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin. 293T cells were transiently cotransfected with 10 μg of PTP-PEST and 10 μg of p130^{Cas} plasmids using the calcium phosphate precipitation method as previously described (*6*). Exponentially growing COS-1 cells from a 15 cm tissue culture dish were also transfected with 40 μg of pACTAG PTP-PEST by electroporation as previously described (*5*). For coimmunoprecipitations, 293T cells transiently coexpressing HA-PTP-PEST and Myc-p130^{Cas} proteins were harvested and washed with PBS. Cells were lysed in HNMETG containing 500 μM sodium vanadate and protease inhibitors as described earlier (*6*). Protein complexes were immunoprecipitated using anti-PTP-PEST 1075 antibody (*5*) and protein G-agarose (Gibco-BRL) for 90 min at 4 °C. Immune complexes were washed three times in HNTG and proteins were eluted from beads in SDS sample buffer by boiling for 5 min. Proteins were separated on a 11% SDS−PAGE, transferred onto PVDF membrane (Immobilon-P, Millipore), and analyzed for the presence of the Myc tagged p130^{Cas} proteins using the anti-Myc epitope monoclonal antibody 9E10. COS-1 cells were treated with pervanadate in order to increase protein tyrosine phosphorylation levels. Briefly, 360 μL of freshly prepared pervanadate (10 mM sodium orthovanadate in 50 mM hydrogen peroxide) were mixed with 15 mL of DMEM and added to the cells for 15 min before harvesting.

Substrate-Trapping Experiments. One microgram of GST PTP-PEST aa 1−453 or GST PTP-PEST C231S aa 1−453 prebound to glutathione sepharose (Pharmacia) was incubated with 1 mg of HNMETG lysates from the indicated cell extracts for 90 min as described previously (*3*). The beads were washed extensively in HNTG buffer and proteins were eluted in SDS-sample buffer. After SDS−PAGE and transfer to PVDF membrane, immunoblotting was performed with the antiphosphotyrosine monoclonal antibody 4G10 or a rabbit polyclonal anti-p130^{Cas} B+F (a generous gift from Dr. Amy H. Bouton, University of Virginia).

Binding Studies. GST fusion proteins encoding the different regions of PTP-PEST have been described elsewhere (*3*). The GST fusion proteins were purified by glutathione Sepharose (Pharmacia) according to manufacturer's protocol. For in vitro binding studies, 1 μg of GST alone or GST-SH3 domain of p130^{Cas}, Hef1 or Sin prebound to glutathione Sepharose was incubated for 90 min at 4 °C with 1 mg of COS-1 cell lysates expressing HA-PTP-PEST. After washing three times in HNTG, the proteins were eluted in sample buffer and separated by SDS−PAGE, and the presence of HA-PTP-PEST was analyzed by immunoblotting with an anti-HA antibody. For farwestern assays, glutathione Sepharose purified PTP-PEST fusion proteins were separated on SDS−PAGE and transferred onto PVDF membrane. Proteins were allowed to renature overnight at 4 °C in binding buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, and 0.2% BSA). Blots were probed with ^{32}P-labeled GST-SH3 domains fusion proteins of either p130^{Cas}, Hef1, or Sin (labeled according to manufacturer's instruction, Pharmacia) for 8−16 h in binding buffer at 4 °C. The blots were washed extensively in binding buffer at room temperature and exposed to X-ray film (Kodak) for 20 min.

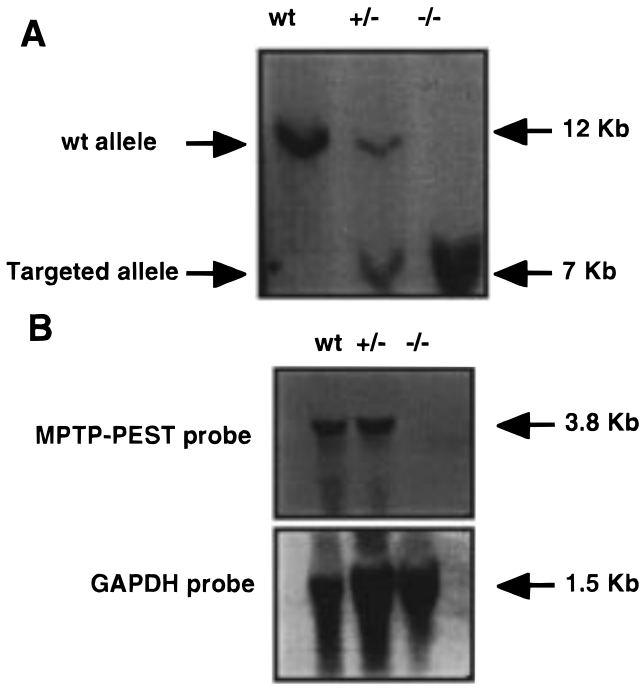

FIGURE 1: Southern blot and northern blot analyses of DNA and total RNA isolated from the PTP-PEST +/+, +/−, and −/− cell lines established from primary cultures of embryos isolated from the PTP-PEST knock-out mice. (A) Genomic DNA was digested with *Bam*HI and probed with a *Kpn*I/*Sac*I fragment of the PTP-PEST cDNA. The 12 kb band corresponds to the wt allele while the 7 kb band corresponds to the targeted allele. (B) A transcript of 3.8 kb was detected in PTP-PEST +/+ and +/− cell lines but was absent in the PTP-PEST −/− cell lines by northern blotting. The blot was also probed with GAPDH (lower panel) to ensure equal loading.

RESULTS

Generation of Gene Targeted PTP-PEST Cell Lines (PTP-PEST −/−). To study the phosphotyrosine protein profile of cells lacking PTP-PEST with the aim of identifying novel substrates for this PTP, we generated mouse embryonic fibroblasts from 8.5 day post-coitum embryos isolated from the mating of PTP-PEST +/− animals.[2] These primary cultures were immortalized by an intermediate transforming C135S mutant of p53 (35) and E1A/E1B proteins as described in the Materials and Methods. The genotypes of the established cell lines were determined by Southern blotting. Figure 1A shows that one of the cell lines has both the 12 kb wt and 7 kb targeted allele (PTP-PEST +/−) while the other cell line has only the targeted allele (PTP-PEST −/−). As a positive control, DNA isolated from WT embryonic fibroblasts was included and only the 12 kb wt allele was observed. To verify the absence of PTP-PEST mRNA in the PTP-PEST −/− cell line, northern blot

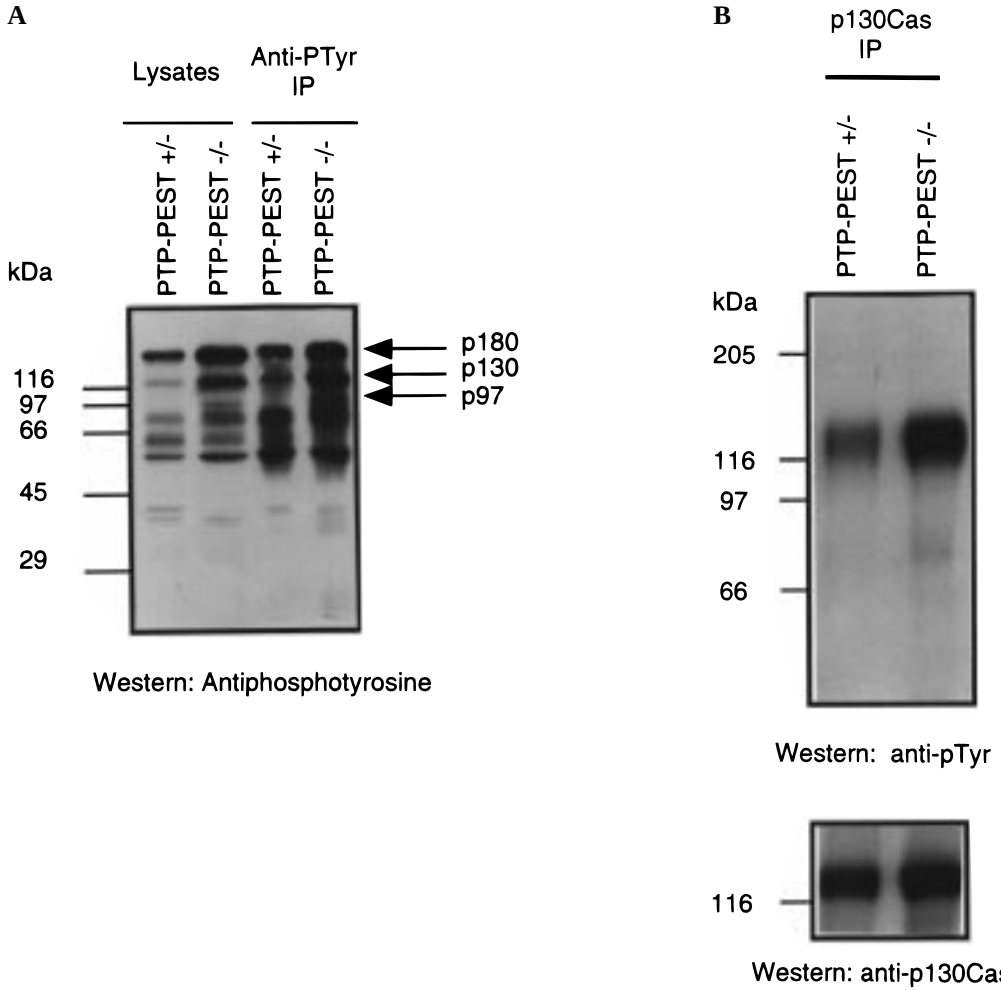

FIGURE 2: Analysis of the phosphotyrosine profile of the PTP-PEST +/− and −/− cell lines. (A) Fifteen micrograms of lysate of PTP-PEST +/− and −/− were analyzed by antiphosphotyrosine immunoblotting (first two lanes). Antiphosphotyrosine immunoprecipitates from the PTP-PEST +/− and −/− cell lines were also analyzed by antiphosphotyrosine immunoblotting. Hyperphosphorylated proteins of 180, 130, and 97 kDa are detected in the PTP-PEST −/− cells. (B) $p130^{Cas}$ is hyperphosphorylated in the PTP-PEST deficient cell line (PTP-PEST −/−). (Upper panel) $p130^{Cas}$ was immunoprecipitated from PTP-PEST +/− and −/− cell lines and the phosphorylation level was analyzed by antiphosphotyrosine immunoblotting using the 4G10 monoclonal antibody. (Lower panel) Equal amounts of $p130^{Cas}$ in the immunoprecipitates were confirmed by stripping the blot and reprobing with a rabbit polyclonal anti-$p130^{Cas}$ B+F.

analysis was performed. In Figure 1B, the 3.8 kb mRNA species of PTP-PEST is present in the PTP-PEST +/+ and +/− cells but absent from the −/− cells (upper panel). As a loading control, the blot was stripped and probed with GAPDH (Figure 1B, lower panel). The phenotypic characterization of the PTP-PEST deficient cell lines is currently under progress.[3]

Analysis of the Phosphotyrosine Protein Profile of the PTP-PEST −/− Cells. To identify putative substrate(s) of PTP-PEST, we have analyzed the phosphotyrosine protein profile of the PTP-PEST −/− cells. Comparison of antiphosphotyrosine immunoblots of the total cell lysate (TCL) of the +/− and −/− revealed the presence of hyperphosphorylated proteins of approximately 180, 130, and 97 kDa in the −/− cells (Figure 2A, first two lanes). When the TCL of the +/− and −/− cells were subjected to immunoprecipitation using an antiphosphotyrosine antibody and analyzed by antiphosphotyrosine western, the same hyperphosphorylated proteins were detected (Figure 2A, last two lanes).

To identify these hyperphosphorylated proteins, an in vivo direct immunoprecipitation of candidate proteins were performed. $p130^{Cas}$ was immunoprecipitated both from the PTP-PEST +/− and −/− cell lines, and its phosphorylation status was analyzed by antiphosphotyrosine immunoblotting using the 4G10 antibody. In the cells lacking PTP-PEST, $p130^{Cas}$ was found to be hyperphosphorylated when compared to $p130^{Cas}$ immunoprecipitated from PTP-PEST +/− cell (Figure 2B, upper panel). Equal amounts of $p130^{Cas}$ protein was found in the immunoprecipitates as verified by stripping the blot and reprobing with a rabbit polyclonal anti-$p130^{Cas}$ as shown in Figure 2B (lower panel). Since we have previously demonstrated that PTP-PEST forms a complex with the EGF receptor via Grb2 (*3*), the amount of EGF receptor in antiphosphotyrosine immunoprecipitates from +/− and −/− cells was compared. Equal amounts of tyrosine-phosphorylated EGF receptor were found in both cell lines (data not shown). To determine the identity of the 97 kDa protein(s), the level of the $p130^{Cas}$-like proteins Hef1 and Sin were also analyzed in antiphosphotyrosine IP from the +/− and −/− cells. Hef1 and Sin were not detected in these IPs, and we have further demonstrated by

[3] A. Angers-Loustau, J.F.C., and M.L.T., unpublished data.

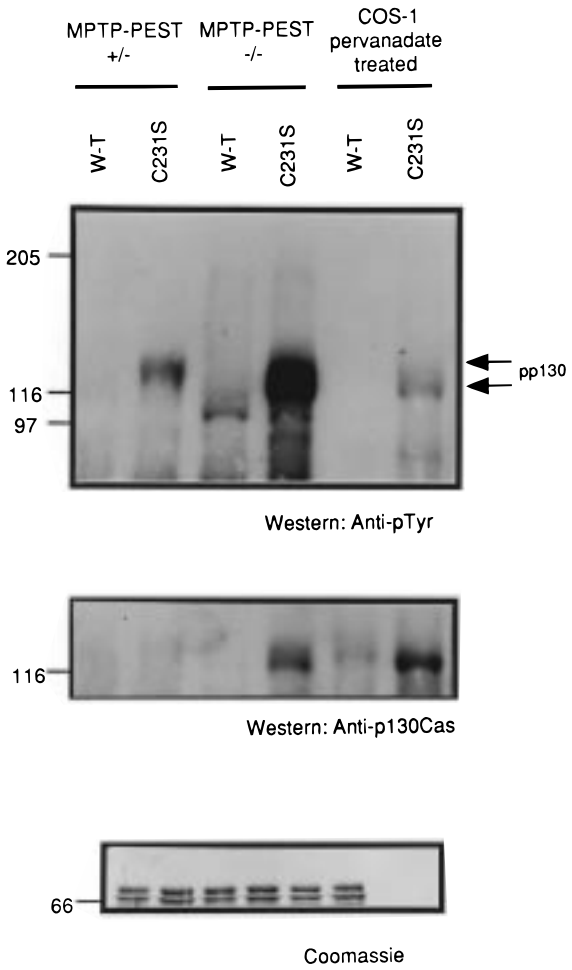

FIGURE 3: Substrate-trapping experiment in PTP-PEST −/− cell lysates demonstrates $p130^{Cas}$ and a p97 as physiological substrates for PTP-PEST. The substrate-trapping experiments were performed by incubating 1 mg of cell lysate from PTP-PEST +/−, −/−, and pervanadate-treated COS-1 cells with 1 μg of either GST-PTP-PEST WT (aa 1−453) or GST-PTP-PEST C231S (1−453). The bound proteins were analyzed by antiphosphotyrosine western blotting using 4G10 monoclonal antibody (top panel) or, after stripping the blot, with anti-$p130^{Cas}$ B+F (middle panel). Interestingly, a tyrosine phosphorylated p97 was bound to the WT and C231S GST fusion proteins in the PTP-PEST −/− cell lysate (upper panel). A Coomassie Blue stained gel of the GST fusion proteins used in the substrate trapping is shown in the bottom panel to show the integrity of products and as a loading control. Two arrows are drawn next to the pp130 (phosphorylated p130 proteins) to emphasize the diffuse band.

direct IPs that Hef1 and Sin are not expressed in the PTP-PEST +/− and −/− fibroblast cell lines used in the present study (data not shown).

Combination of Gene Targeting of PTP-PEST and Substrate-Trapping Experiments to Identify Physiological Substrates. The inactivation of the PTP-PEST gene (PTP-PEST −/− cell line) was hypothesized to render substrates that are highly specific to PTP-PEST in an hyperphosphorylated state and that these substrates could subsequently be isolated using a "substrate trap" mutant of PTP-PEST (C231S mutant). An important aspect of this novel approach is that prior physiological (growth factor) or artificial (pervanadate) treatments of cells is not required. A substrate-trapping experiment was thus performed with PTP-PEST −/− and +/− cell lysates using a GST PTP-PEST C231S mutant. As shown in Figure 3 (upper panel), the GST PTP-PEST WT was unable to bind phosphorylated substrates in the PTP-PEST +/− while the C231S mutant isolated a small amount of a p130 protein. However, in the PTP-PEST −/− cell lysates, a substantially elevated amount of a phosphorylated p130 protein was bound to the C231S mutant of PTP-PEST but not to the wild-type. Two arrows are drawn next to the p130 to emphasize the diffuse band. Interestingly, a tyrosine-phosphorylated p97 was bound to both PTP-PEST WT and C231S GST fusion proteins in the PTP-PEST −/− cell extracts. The p97 isolated by the C231S fusion protein of PTP-PEST in the PTP-PEST −/− cell lysates was slightly more tyrosine phosphorylated than the one isolated by the WT counterpart. Since a hyperphosphorylated p97 was also observed in the TCL of the PTP-PEST −/− cells (Figure 2A), this protein is most likely a direct substrate of PTP-PEST. The hyperphosphorylated p180 observed in the TCL of the PTP-PEST −/− cells was not bound to the trapping mutant of PTP-PEST. Hence, this tyrosine-phosphorylated protein is probably an indirect substrate of PTP-PEST or is bound weakly to the trapping mutant of PTP-PEST and is thus not detectable using this assay. The same substrate-trapping experiment was performed using lysates of pervanadate treated COS-1 to compare both approaches. A detectable amount of a p130 protein was bound to the PTP-PEST C231 mutant but not to the WT. Figure 3 (middle panel) shows the same blot stripped and analyzed for the presence of $p130^{Cas}$ protein using a polyclonal rabbit anti-$p130^{Cas}$ B+F. $p130^{Cas}$ protein was found mostly in the lanes of PTP-PEST C231S mutant in PTP-PEST −/− and pervanadate-treated COS-1 cell lysate. Interestingly, the same amount of $p130^{Cas}$ protein was affinity purified from the PTP-PEST −/− cell lysate and the pervanadate-treated COS-1 cell lysate by the C231S mutant of PTP-PEST (Figure 3, middle panel), but the tyrosine phosphorylation level of $p130^{Cas}$ trapped from the PTP-PEST deficient cell line is approximately 20 times greater, as estimated using densitometry, when compared to $p130^{Cas}$ trapped from the pervanadate treated extract. The bottom panel of Figure 3 shows that equal amounts of PTP-PEST GST fusion proteins were used in the trapping assay.

PTP-PEST Associates with the SH3 Domains of the p130Cas-Like Proteins Hef1 and Sin. The SH3 domain of p130Cas has been reported to associate with PTP-PEST in vitro (*7*). The presence of SH3 domains in the $p130^{Cas}$-like proteins Sin and Hef1 have prompted us to investigate if these SH3 domains could interact with PTP-PEST. The SH3 domains of $p130^{Cas}$ and Sin were cloned in pGEX-2TK, and the SH3 domain of Hef1 was cloned in pGEX1LambdaT vector and expressed as GST fusion proteins in order to perform a binding assay with PTP-PEST. The integrity of the fusion proteins used in the binding assay is shown on a Coomassie Blue stained gel in the right panel of Figure 4. In the binding assay (Figure 4, left panel), GST alone did not bind the HA-PTP-PEST as expected. As a positive control, the SH3 domain of $p130^{Cas}$ bound to HA-PTP-PEST. To a similar extent, HA-PTP-PEST was specifically bound by the purified SH3 domains of Hef1 and Sin even after extensive washing of the complex.

Mapping of the First Proline-Rich Region of PTP-PEST (Amino Acids 316−346) as the Binding Site for the SH3 Domains of Sin and Hef1. Five proline-rich motifs are found in PTP-PEST (*3*), and SH3 domains are known to bind to

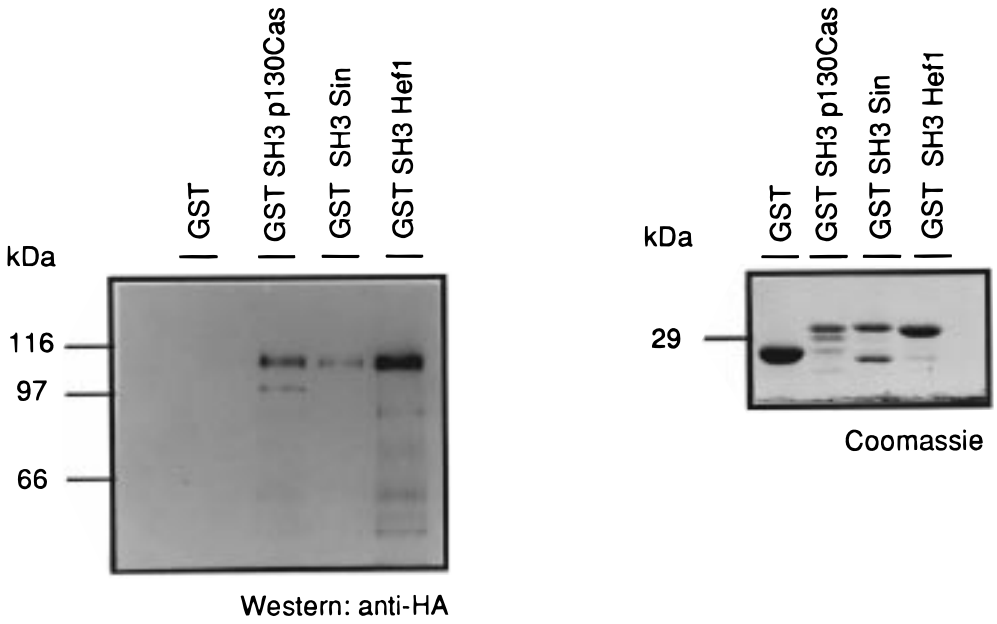

FIGURE 4: PTP-PEST associates with the SH3 domains of p130^{Cas}, Hef1, and Sin in vitro. HA-PTP-PEST transfected COS-1 cell lysates (1 mg) were incubated with 1 μg of either the GST linked SH3 domains of p130^{Cas}, Hef1, Sin or the GST alone prebound to glutathione sepharose. Following extensive washing, the proteins were eluted, separated by SDS−PAGE and analyzed by western blotting for the presence of HA-PTP-PEST using the 12CA5 monoclonal antibody (left panel). In the right panel, a Coomassie Blue stained gel of the GST fusion proteins used in the in vitro binding assay is shown to verify integrity of the products.

proline-rich motifs. We have investigated which one of these PTP-PEST proline-rich motifs mediates the interaction with the SH3 domains of Hef1 and Sin. A series of GST fusion proteins encoding the five different proline-rich regions of PTP-PEST (Pro 1−5) was generated in order to map the essential region on PTP-PEST for the interaction with the SH3 domains of the Cas-like proteins Sin and Hef1 using a farwestern binding assay. A schematic representation and a Coomassie Blue stained gel of the fusion proteins showing integrity of the products used in the farwestern assay are shown in Figure 5, panels A and B, respectively. For the farwestern assay, the PTP-PEST GST-fusion proteins were separated by SDS−PAGE and then transferred onto PVDF membranes and after renaturation of the proteins and blocking, the blots were probed with ^{32}P-radiolabeled GST SH3 domains of either Sin, Hef1, or p130^{Cas}. The SH3 domains of Sin and Hef1 were found to associate with high affinity to the Pro1 region of PTP-PEST (aa 316−346) and also to the complete C-terminus (aa 276−775) and to the N-terminus (aa 1−453) fusion proteins of PTP-PEST as seen in Figure 5, panels D and E. These three constructs have in common the Pro1 region of PTP-PEST. No detectable levels of binding of these SH3 domains to the Pro 2−5 of PTP-PEST and GST alone (negative control) were noted even after a longer exposure. The SH3 domains of p130^{Cas} was used as a positive control to probe an identical blot and the same result was obtained (Figure 5A). These results demonstrate that the SH3 domains of the family of adaptor proteins p130^{Cas}, Sin, and Hef1 interact with high affinity and selectively with the Pro1 region of PTP-PEST.

PTP-PEST and p130^{Cas} Associate in Vivo. The association between PTP-PEST and p130^{Cas} has only been investigated in vitro using either GST fusion proteins (present work) or bacculovirus produced proteins (*4*, *7*). To evaluate the binding between p130^{Cas} and PTP-PEST in vivo, we performed a coimmunoprecipitation experiment. 293T cells were cotransfected by the calcium phosphate method with HA-PTP-PEST WT or C231S (10 μg) and either Myc-p130^{Cas} or Myc p130^{Cas} SH3 domain (10 μg). A schematic representation of the p130^{Cas} proteins is shown in Figure 6A. Cell lysates were immunoprecipitated with anti-PTP-PEST 1075 and analyzed for the presence of Myc p130^{Cas} proteins (Figure 6B). The Myc-p130^{Cas} and the Myc-p130^{Cas} SH3 domain were found in the HA-PTP-PEST IPs (Figure 6B). More Myc p130^{Cas} was found in the PTP-PEST C231S IP when compared to the WT-PTP-PEST IP probably due to a substrate-trapping effect. As a negative control, the Myc p130^{Cas} and Myc p130^{Cas} SH3 domain were cotransfected with HA-T-cell PTP (TC-PTP). Figure 6B demonstrate that neither Myc p130^{Cas} nor Myc p130^{Cas} SH3 domain were found in the HA-T cell PTP (HA-TC-PTP) immunoprecipitates clearly showing that this interaction is specific for PTP-PEST. These results demonstrate that the association of PTP-PEST occurs in vivo with the full-length p130^{Cas} or specifically with the SH3 domain of p130^{Cas}. Figure 6C shows that the PTP-PEST and p130^{Cas} proteins were expressed to similar level.

DISCUSSION

The identification of protein tyrosine phosphatase substrates is one of the key steps in understanding the biological function of this family of enzymes. Recent mutagenesis experiments of invariant amino acids within the conserved catalytic domain of PTPs, in conjunction with the analysis of the crystal structure of PTP1B, have yielded two interesting mutants for substrate trapping: First, a cysteine to serine mutant in the VHCSAG signature motif (C215S in PTP1b), and second, a mutation of a catalytically active aspartic acid to alanine (D199A in PTP 1B) (*2*). The present data proposes a novel approach by combining substrate-trapping experiments with the gene targeting of a PTP, PTP-PEST, to identify putative physiological substrates. Fibroblast cell

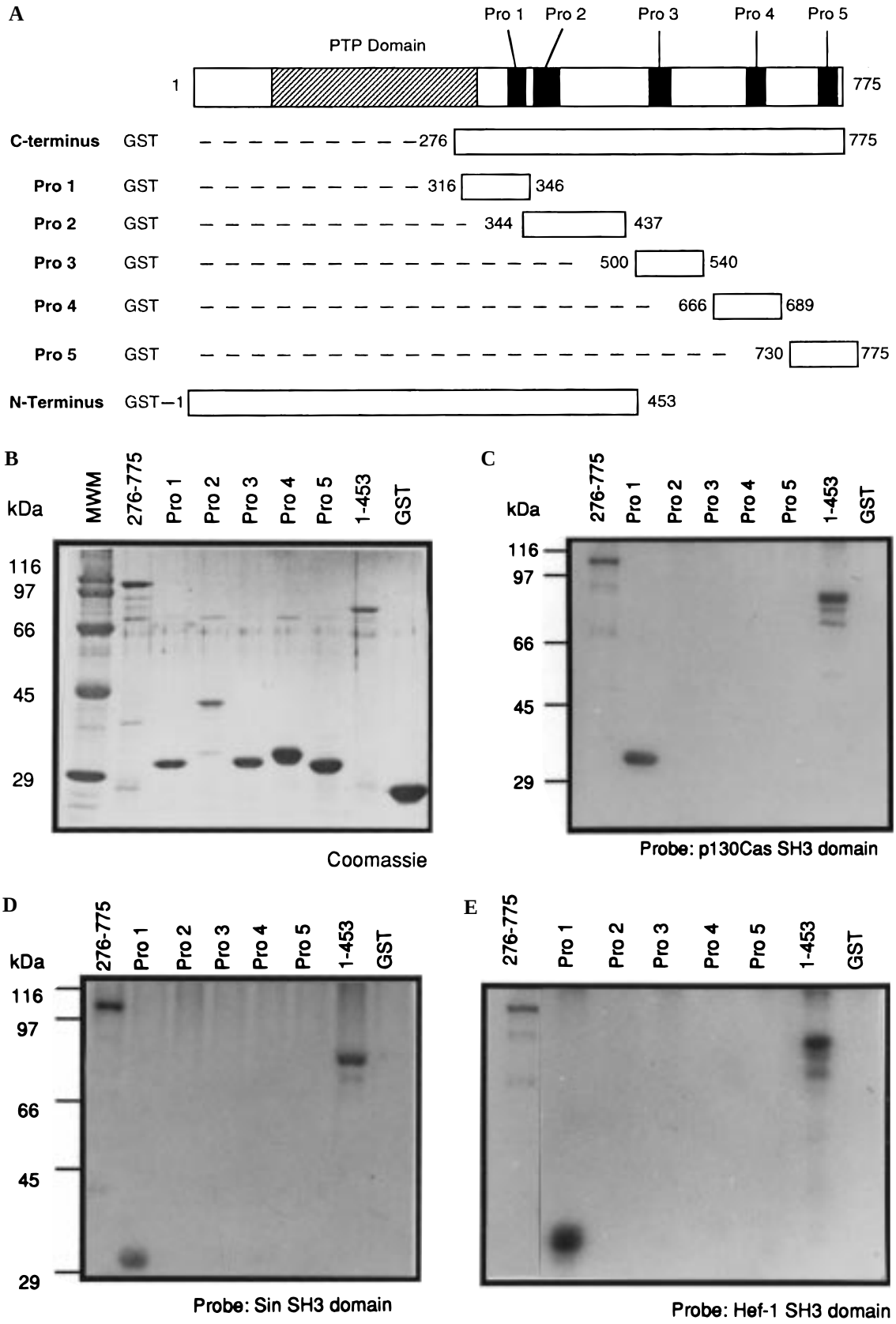

FIGURE 5: PTP-PEST proline-rich region 1 (Pro1) is responsible for the interaction with the SH3 domains of the family of adaptor molecules $p130^{Cas}$, Sin and Hef1. (A) Schematic representation of the PTP-PEST GST fusion proteins encoding for the different proline-rich regions of PTP-PEST (Pro1−5) used in the farwestern binding assay, (B) Coomassie blue stained gel of 100 ng of the PTP-PEST fusion proteins used in the farwestern assay and is also representative of the protein found on the PVDF membrane. Three blots containing the PTP-PEST fusion proteins were respectively incubated overnight with ^{32}P-radiolabeled GST SH3 domains of $p130^{Cas}$ (C), Sin (D), and Hef1 (E), and following extensive washing, the blots were exposed 20 min to X-ray film.

lines lacking PTP-PEST were generated from the PTP-PEST knock-out[2] and used as protein extracts in subsequent substrate-trapping experiments. Using such an approach, $p130^{Cas}$ was the major protein isolated by a PTP-PEST

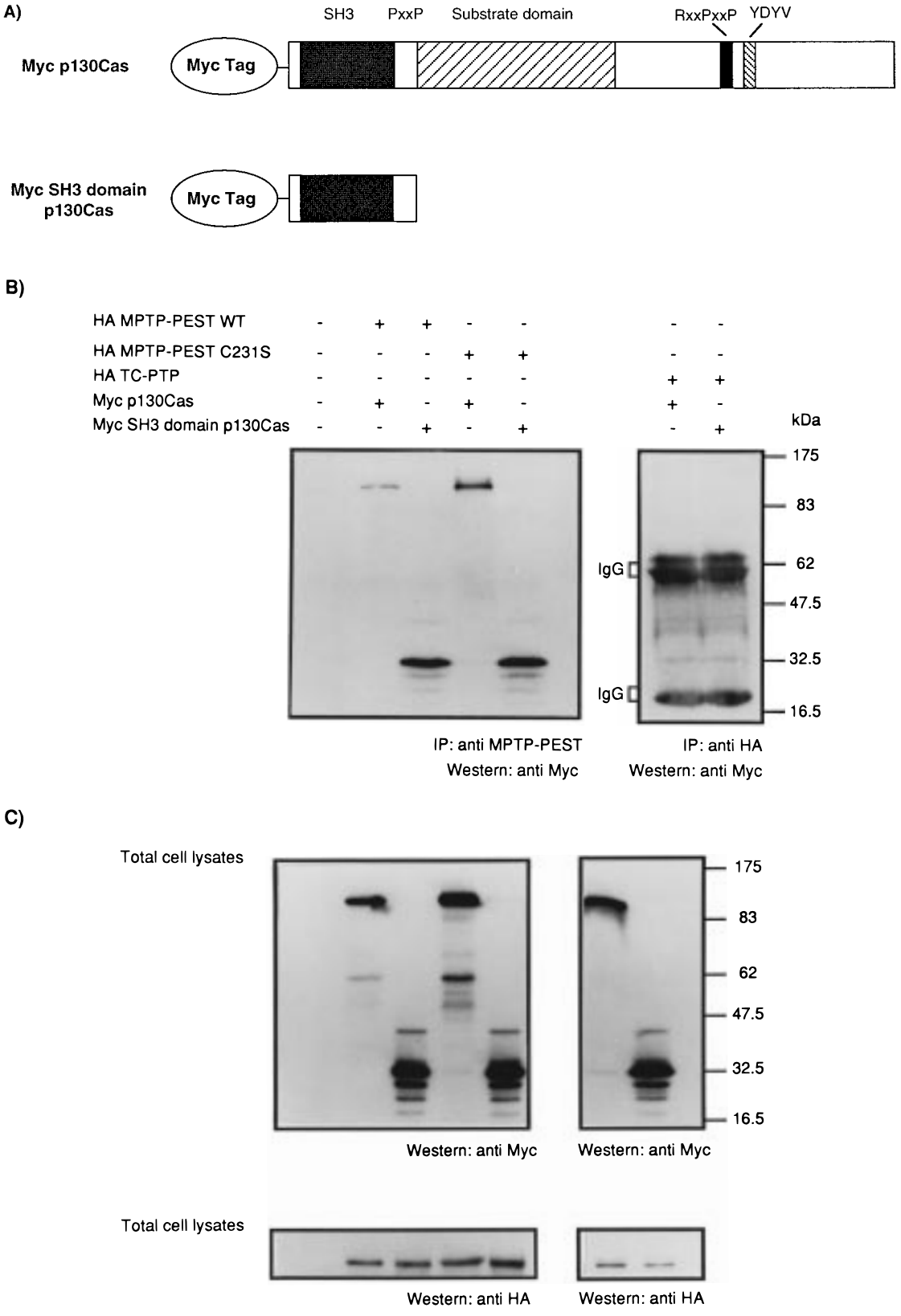

FIGURE 6: PTP-PEST and p130^{Cas}associate in vivo. Myc-tagged p130^{Cas} vector or a Myc SH3 domain p130^{Cas} vector were cotransfected with HA-PTP-PEST, WT, or C231S, in 293T cells and the presence of the various Myc tagged p130^{Cas} proteins were analyzed in anti PTP-PEST immunoprecipitates. (A) Schematic representation of the myc-tagged p130^{Cas} proteins used in the coimmunoprecipitation experiment. (B) Following coimmunoprecipitation and extensive washing of the complexes, the bound proteins were resolved on SDS−PAGE, transferred to PVDF and analyzed by western blotting using anti-Myc 9E10 monoclonal for the presence of the various Myc tagged p130^{Cas} proteins. As a negative control, HA-tagged T-cell PTP (HA TC-PTP) was cotransfected with the Myc p130^{Cas} construct and was immunoprecipitated with anti-HA 12CA5. The full-length Myc-p130^{Cas} proteins migrates just above 130 kDa and the Myc-SH3 domain of p130^{Cas} migrates at 30 kDa. (C) Western blotting of the TCL with 9E10 and 12CA5 antibodies to visualize the different Myc-p130^{Cas} proteins, the HA-PTP-PEST and HA-TC-PTP, respectively.

Table 1: Proline-Rich Domains Found on the PEST Family of Enzyme PTP-PEST (*5*), PTP-PEP (*37*), PTP-HSCF (*13*), and PTP-BDP (*15*)[a]

proline-rich motifs	PTP-PEST	PTP-PEP	PTP-HSCF	PTP-BDP
class 1 RxxPxxP	none	none	none	^{197}RGVPSSP^{203}
class 2 PxxPxR	^{333}**PPKPRR**^{338} ^{675}PPLPER^{680}	^{614}PPLPER^{619} ^{690}PPLPER^{695}	none	none
nonclassical PxxP	^{355}PPEPHPVPP^{363} ^{520}PPRPDCLP^{527} ^{764}PKGPREPP^{771}	^{790}PKGPRNPP^{797}	^{440}PKGPRDPP^{447}	^{444}PKGPRDPP^{451}

[a] The class 2 motif ^{333}PPKPRR^{338} mediating the interaction with the SH3 domains of p130^{Cas}, Sin, and Hef1 is found only on PTP-PEST (bold). A conserved nonclassical proline-rich sequence having the consensus PKGPRxPP (x represents E, N, or D) is present in this family of enzyme and is implicated in the binding of the novel actin associated protein PSTPIP (*16*). PTP-PEST is the only member of the family having a proline-rich motif which is a ligand for the SH3 domains of the p130^{Cas} family members.

substrate-trapping mutant. A second substrate of approximately 97 kDa was also trapped but is yet to be identified. The advantage of this novel approach is that only physiological substrates that are highly specific for the PTP of interest would be hyperphosphorylated in the gene-targeted tissues or cell lines. As a result, such substrates could be isolated in large amounts by the substrate-trapping mutants of the chosen PTP.

Its stands to reason that the removal of a PTPase will result in the hyperphosphorylation of its physiological substrates at the basal level or following specific stimulation. For example, it was shown that when the vaccinia virus dual specificity phosphatase VH1 is deleted from the virion capsule, two encapsidated substrate proteins, p18 and p11, become hyperphosphorylated, and as a result, the life cycle of the virus is impaired (*36*). With the premise that physiological substrates of PTP-PEST would become hyperphosphorylated, we generated a gene targeted PTP-PEST fibroblast cell line, PTP-PEST −/−. In this case, the antiphosphotyrosine profile of the total cell lysate of PTP-PEST −/− cell line was compared to the total cell lysate of PTP-PEST +/−. This enabled the identification of hyperphosphorylated proteins of 180, 130, and 97 kDa in the −/− cells. One of these proteins was identified as p130^{Cas}. Direct analyses by antiphosphotyrosine blotting of p130^{Cas} immunoprecipitates from PTP-PEST +/− and −/− clearly indicate that p130^{Cas} is hyperphosphorylated in cells lacking PTP-PEST. This suggests that even if many PTPs are expressed in these murine fibroblasts and that at least one of them, PTP1B, has been shown to interact with the SH3 domain of p130^{Cas} (*20*), the major regulation of the phosphorylation of p130^{Cas} at the basal level is mediated by PTP-PEST. The identity of the hyperphosphorylated p180 and p97 is still under investigation. We have investigated whether the p180 protein could be the EGF receptor since we have previously shown that PTP-PEST can complex the EGF receptor via Grb2 (*3*). EGF receptor was found in equal amounts in antiphosphotyrosine immunoprecipitates in the PTP-PEST −/− cells and +/− cells. The identity of the hyperphosphorylated p97 is still unknown, but interestingly, a tyrosine phosphorylated p97 was isolated, in addition to p130^{Cas}, in the trapping experiment using the PTP-PEST −/− cell lysate both by the WT and C231S PTP-PEST GST fusion proteins. The tyrosine phosphorylation level of this p97 was greater when bound to the C231S mutant of PTP-PEST than by the WT counterpart. It is thus likely that the p97 is a direct substrate of PTP-PEST. The identification of this protein is currently in progress.

The data presented in this report also describes that the family of p130^{Cas} adaptor proteins p130^{Cas}, Sin and Hef1 interact with PTP-PEST via their SH3 domains. The first proline-rich region (Pro1) of PTP-PEST, ^{332}PPKPPR^{337}, was found to mediate this interaction without contribution of the four other proline-rich motifs (Pro 2−5) found on PTP-PEST. Our data support recent findings by Garton et al. (*7*) suggesting that the SH3 domain mediated association of p130^{Cas} with PTP-PEST potentially plays a crucial role in the substrate specificity. Our results also suggest that both Hef1 and Sin are candidate substrates for PTP-PEST since they also associate with PTP-PEST via their SH3 domains. It would have been interesting to analyze the phosphorylation status of Hef1 and Sin in the PTP-PEST −/− cells, but unfortunately, they are not expressed in the cell lines tested.

The proline-rich region (Pro1) of PTP-PEST, ^{332}PPKPPR^{337}, belongs to the class 2 consensus sequence (PxxPxR) (*8*) for binding SH3 domains. Other proline-rich domains that have been shown to interact with the SH3 domain of p130^{Cas} such as: PRPPKR from PTP1B (*20*), PPKPSR from FAK, and PPKPSR from FRNK (*19*), all belong to the class 2 consensus sequence. Three other enzymes share homology to PTP-PEST: PTP-PEP, PTP-HSCF, and PTP-BDP. Table 1 summarizes the putative proline-rich regions found on this family of PTP. PTP-PEP has two identical proline-rich domains containing the sequence PPLPER and thus belongs to the class 2 consensus sequence (*37*). It is unlikely that the two proline-rich sequences of PTP-PEP could interact with the SH3 domains of p130^{Cas}, Sin, or Hef1 since an identical proline-rich sequence found on PTP-PEST (Pro 4,^{675}PPLPER^{680}) does not interact with these SH3 domains (this paper, Figure 5). Analysis of the PxxP motifs found on PTP-HSCF and PTP-BDP (Table 1) reveals that none of them belong to the class 2 consensus sequence (PxxPxR), and in this respect, these two PTPs probably do not interact with the SH3 domain of p130^{Cas}, Sin, and Hef1. In summary, among the members of PEST-like PTPs, PTP-PEST appears to be the only one that can interact with the SH3 domain containing proteins p130^{Cas}, Sin, and Hef1 via a proline-rich region. Since PTP-PEST is expressed ubiquitously and since p130^{Cas}, Hef1, and Sin have differential expressions, it is reasonable to envisage that PTP-PEST is a potential modulator of the tyrosine phosphorylation level of this family of adaptor proteins in different cell types.

We have clearly demonstrated that PTP-PEST, WT, or C231S is in vivo constitutively associated with p130^{Cas} and that the SH3 domain alone of p130^{Cas} can mediate this interaction. In this respect, the proline-rich/SH3 domain

association is constitutive as usually observed in this kind of interaction and does not terminate when $p130^{Cas}$ becomes dephosphorylated by the catalytic activity of PTP-PEST as previously proposed (*4*). The deletion of the Pro1 domain of PTP-PEST completely abolishes the binding with GST fusion proteins of the SH3 domain of $p130^{Cas}$ (*7*), Hef1 and Sin[4] in vitro thus clearly demonstrating physical interaction between these proteins. Interestingly, the association between PTP-PEST and $p130^{Cas}$ was not abrogated by the deletion of the Pro 1 domain of PTP-PEST and/or the SH3 domain of $p130^{Cas}$ in a coimmunoprecipitation experiment performed in transfected 293T cells.[4] These results suggest that PTP-PEST and $p130^{Cas}$ might also associate via an SH3 independent mechanism, and this is presently under investigation.

In summary, the combination of gene targeting of a PTP and substrate-trapping experiments is a powerful approach to identify relevant physiological substrate(s) of a given PTP. Using such an approach, $p130^{Cas}$ is confirmed as a physiological substrate of the murine PTP-PEST. We have also identified a 97 kDa protein that is likely a PTP-PEST substrate. We believe that this approach reduces the risk of artifacts since no artificial treatment of the cells is required. The SH3 domains of the $p130^{Cas}$-like molecules, Sin and Hef1, were found to interact with high affinity with a proline-rich region of PTP-PEST (Pro1, aa 332−338). These data also suggest that Hef1 and Sin could be candidate substrates for PTP-PEST. This hypothesis is also supported by the fact that an effective dephosphorylation of $p130^{Cas}$ by PTP-PEST requires an intact Pro 1 region. Finally, the in vivo association of PTP-PEST and $p130^{Cas}$ has been demonstrated to be stable and constitutive.

ACKNOWLEDGMENT

We are thankful to Dr. Steven Hanks for the generous gift of the murine $p130^{Cas}$ cDNA, Dr. Konstantina Alexandropoulos for the template DNA of the Sin SH3 domain and the Sin antiserum, Dr. Yuzhu Zhang, Dr. Susan Law, and Dr. Erica Golemis for the GST SH3 Hef1 construct and Hef1 antiserum, and to Dr. Amy H. Bouton for the gift of the anti-Cas B+F antibody. We are also indebted to Dr. Louise Larose, Alan Cheng and Alexandre Angers-Loustau (McGill University, Montréal, PQ, Canada) for critical reading of the manuscript.

REFERENCES

1. Neel, B. G., and Tonks, N. K. (1997) *Curr. Opin. Cell Biol. 9*, 193−204.
2. Flint, A. J., Tiganis, T., Barford, D., and Tonks, N. K. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 1680−1685.
3. Charest, A., Wagner, J., Kwan, M., and Tremblay, M. L. (1997) *Oncogene 14*, 1643−1651.
4. Garton, A. J., Flint, A. J., and Tonks, N. K. (1996) *Mol. Cell. Biol. 16*, 6408−6418.
5. Charest, A., Wagner, J., Shen, S. H., and Tremblay, M. L. (1995) *Biochem. J. 308*, 425−432.
6. Charest, A., Wagner, J., Jacob, S., McGlade, C. J., and Tremblay, M. L. (1996) *J. Biol. Chem. 271*, 8424−8429.
7. Garton, A. J., Burnham, M. R., Bouton, A. H., and Tonks, N. K. (1997) *Oncogene 15*, 877−885.
8. Mayer, B. J., and Eck, M. J. (1995) *Curr. Biol. 5*, 364−367.
9. Chen, H. I., Einbond, A., Kwak, S. J., Linn, H., Koepf, E., Peterson, S., Kelly, J. W., and Sudol, M. (1997) *J. Biol. Chem. 272*, 17070−17077.
10. Wu, R. Y., Durick, K., Zhou, S. Y., Cantley, L. C., Taylor, S. S., and Gill, G. N. (1996) *J. Biol. Chem. 271*, 15934−15941.
11. Flores, E., Roy, G., Patel, D., Shaw, A., and Thomas, M. L. (1994) *Mol. Cell. Biol. 14*, 4938−4946.
12. Huang, K., Sommers, C. L., Grinberg, A., Kozak, C. A., and Love, P. E. (1996) *Oncogene 13*, 1567−1573.
13. Cheng, J., Daimaru, L., Fennie, C., and Lasky, L. A. (1996) *Blood 88*, 1156−1167.
14. Aoki, N., Yamaguchi-Aoki, Y., and Ullrich, A. (1996) *J. Biol. Chem. 271*, 29422−29426.
15. Kim, Y. W., Wang, H., Sures, I., Lammers, R., Martell, K. J., and Ullrich, A. (1996) *Oncogene 13*, 2275−2279.
16. Spencer, S., Dowbenko, D., Cheng, J., Li, W. L., Brush, J., Utzig, S., Simanis, V., and Lasky, L. A. (1997) *J. Cell Biol. 138*, 845−860.
17. Sakai, R., Iwamatsu, A., Hirano, N., Ogawa, S., Tanaka, T., Mano, H., Yazaki, Y., and Hirai, H. (1994) *EMBO J. 13*, 3748−3756.
18. Polte, T. R., and Hanks, S. K. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10678−10682.
19. Harte, M. T., Hildebrand, J. D., Burnham, M. R., Bouton, A. H., and Parsons, J. T. (1996) *J. Biol. Chem. 271*, 13649−13655.
20. Liu, F., Hill, D. E., and Chernoff, J. (1996) *J. Biol. Chem. 271*, 31290−31295.
21. Astier, A., Avraham, H., Manie, S. N., Groopman, J., Canty, T., Avraham, S., and Freedman, A. S. (1997) *J. Biol. Chem. 272*, 228−232.
22. Nakamoto, T., Sakai, R., Honda, H., Ogawa, S., Ueno, H., Suzuki, T., Aizawa, S., Yazaki, Y., and Hirai, H. (1997) *Mol. Cell. Biol. 17*, 3884−3897.
23. Burnham, M. R., Harte, M. T., Richardson, A., Parsons, J. T., and Bouton, A. H. (1996) *Oncogene 12*, 2467−2472.
24. Khwaja, A., Hallberg, B., Warne, P. H., and Downward, J. (1996) *Oncogene 12*, 2491−2498.
25. Ribon, V., and Saltiel, A. R. (1996) *J. Biol. Chem. 271*, 7375−7380.
26. Salgia, R., Pisick, E., Sattler, M., Li, J. L., Uemura, N., Wong, W. K., Burky, S. A., Hirai, H., Chen, L. B., and Griffin, J. D. (1996) *J. Biol. Chem. 271*, 25198−25203.
27. Schlaepfer, D. D., Broome, M. A., and Hunter, T. (1997) *Mol. Cell. Biol. 17*, 1702−1713.
28. Casamassima, A., and Rozengurt, E. (1997) *J. Biol. Chem. 272*, 9363−9370.
29. Vuori, K., Hirai, H., Aizawa, S., and Ruoslahti, E. (1996) *Mol. Cell. Biol. 16*, 2606−2613.
30. Sakai, R., Nakamoto, T., Ozawa, K., Aizawa, S., and Hirai, H. (1997) *Oncogene 14*, 1419−1426.
31. Law, S. F., Estojak, J., Wang, B., Mysliwiec, T., Kruh, G., and Golemis, E. A. (1996) *Mol. Cell. Biol. 16*, 3327−3337.
32. Minegishi, M., Tachibana, K., Sato, T., Iwata, S., Nojima, Y., and Morimoto, C. (1996) *J. Exp. Med. 184*, 1365−1375.
33. Alexandropoulos, K., and Baltimore, D. (1996) *Genes Dev. 10*, 1341−1355.
34. Ishino, M., Ohba, T., Sasaki, H., and Sasaki, T. (1995) *Oncogene 11*, 2331−2338.
35. Slingerland, J. M., Jenkins, J. R., and Benchimol, S. (1993) *EMBO J. 12*, 1029−1037.
36. Liu, K., Lemon, B., and Traktman, P. (1995) *J. Virology 69*, 7823−7834.
37. Matthews, R. J., Bowne, D. B., Flores, E., and Thomas, M. L. (1992) *Mol. Cell. Biol. 12*, 2396−2405.

[4] J. F. C., and M.L.T., unpublished data.

BI981259L