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Phosphorylation of receptor protein-tyrosine phosphatase α on Tyr789, a binding site for the SH3-SH2-SH3 adaptor protein GRB-2 *in vivo*

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Receptor protein-tyrosine phosphatase α (RPTPα) is a transmembrane protein with a short extracellular domain (123 amino acids) and two cytoplasmically localized protein-tyrosine phosphatase (PTP) domains. Here we report that RPTPα is constitutively phosphorylated on tyrosine in NIH 3T3 mouse fibroblasts. The *in vivo* tyrosine phosphorylation site was localized to the C-terminus of RPTPα by phosphopeptide mapping experiments using *in vivo* and *in vitro* ^{32}P-labeled RPTPα. The identity of this site as Tyr789, located five residues from the C-terminus, was confirmed by site-directed mutagenesis. Transient overexpression of c-Src together with RPTPα in human embryonic kidney 293 cells increased phosphorylation of Tyr789, suggesting that c-Src may phosphorylate RPTPα *in vivo*. RPTPα had autodephosphorylation activity *in vitro*. When expressed in 293 cells the level of Tyr789 phosphorylation was higher in a non-functional mutant of RPTPα than in wild type RPTPα, indicating that RPTPα may have autodephosphorylation activity *in vivo* as well. The sequence on the C-terminal side of Tyr789 (YANF) fits the consensus binding site for the SH3-SH2-SH3 adaptor protein GRB2 (YXNX). We show that RPTPα, but not a mutant of RPTPα with a Tyr→Phe mutation at position 789, bound to GRB2 *in vitro*. In addition, RPTPα co-immunoprecipitated with GRB2 from NIH 3T3 cells, demonstrating that GRB2 bound to RPTPα *in vivo*. The guanine nucleotide releasing factor for the Ras GTPase, Son of sevenless (Sos), which associates with GRB2 via its SH3 domains, was not detected in RPTPα immunoprecipitates. Our results suggest a role for RPTPα in attenuation of GRB2-mediated signaling.

Key words: GRB2/protein-tyrosine phosphatase/SH2 and SH3 domains/signal transduction/tyrosine phosphorylation

Introduction

Protein phosphorylation on tyrosine residues is one of the main eukaryotic cell signaling mechanisms, and plays a pivotal role in cell proliferation and differentiation. Cellular protein phosphotyrosine (P.Tyr) levels are regulated by protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). The PTKs constitute a well-characterized family of both cytoplasmically localized as well as transmembrane enzymes (Hanks *et al.*, 1988; Schlessinger and Ullrich, 1992). Recent work has led to the recognition of Src homology 2 and 3 (SH2 and SH3) domains as protein modules that play an important role in receptor PTK signal transduction (reviewed by Pawson and Schlessinger, 1993). SH2 domains bind to short P.Tyr-containing sequences in specific phosphoproteins (Songyang *et al.*, 1993), and SH3 domains bind to ~10 residue sequences containing proline and hydrophobic amino acids in their target proteins (Cicchetti *et al.*, 1992). Mammalian GRB2, *Caenorhabditis elegans* Sem-5, and *Drosophila* Drk are homologous 'adaptor' proteins consisting of an SH2 domain, flanked by two SH3 domains (Clark *et al.*, 1992; Lowenstein *et al.*, 1992; Olivier *et al.*, 1993). GRB2 binds to activated, autophosphorylated receptor PTKs through its SH2 domain, and constitutively to Son of Sevenless (Sos), a guanine nucleotide releasing factor for the Ras GTPase, through its SH3 domains (Chardin *et al.*, 1993; Egan *et al.*, 1993; Li *et al.*, 1993; Olivier *et al.*, 1993; Simon *et al.*, 1993). It has been speculated that translocation of the GRB2–Sos complex to activated receptor PTKs at the cell membrane catalyzes activation of Ras into its GTP-bound state, thereby triggering signal transduction pathways downstream of Ras (Pawson and Schlessinger, 1993).

Whereas signal transduction by PTKs has been studied extensively, relatively little is known about signal transduction by their enzymatic counterpart, the PTPs. Over the past few years a number of PTPs have been cloned, based on sequence homology in the domains that contain the catalytic activity (for reviews see Fischer *et al.*, 1991; Charbonneau and Tonks, 1992; Pot and Dixon, 1992). The PTP domain is ~250 amino acids long, and sequence comparison between PTPs has led to the identification of the 'PTP-fingerprint' sequence (consensus: [I/V]HCXAGXXR[S/T]G). Within this sequence is an absolutely conserved cysteine residue that is essential for PTP activity (Streuli *et al.*, 1989, 1990; Guan and Dixon, 1990). This conserved cysteine forms part of the catalytic site of PTPs, and dephosphorylation reactions proceed through a cysteine-phosphate transition state (Guan and Dixon, 1991a; Pot and Dixon, 1991). Both transmembrane as well as cytoplasmically localized PTPs have been identified. Most transmembrane PTPs contain two cytoplasmic catalytic PTP domains. The one closest to the membrane exhibits the majority of the PTP activity, but in some cases the second domain also possesses detectable, but low PTP activity (Wang and Pallen, 1991; Tan *et al.*, 1993). Whether the extracellular domain of transmembrane PTPs can bind ligands, leading to modulation of PTP activity, remains to be determined. Recently, it has been demonstrated that overexpression of RPTPμ or RPTPκ at high levels in insect cells leads to an aggregation of these cells, which is mediated by homophilic interactions of the extracellular domain of these PTPs (Brady-Kalnay *et al.*, 1993; Gebbink *et al.*, 1993; Sap *et al.*, 1994).

Receptor protein-tyrosine phosphatase α (RPTPα) [also known as LCA-related phosphatase, LRP (Matthews *et al.*, 1990)] is a transmembrane PTP with two cytoplasmic PTP domains and a short (123 amino acids) extracellular domain (Sap *et al.*, 1990). Recently we have demonstrated that RPTPα may be involved in neuronal differentiation, since RPTPα mRNA expression is enhanced during neuronal differentiation of three distinct cell lines, and overexpression of RPTPα in pluripotent P19 embryonal carcinoma cells alters the differentiation fate of these cells in favor of neuronal differentiation (den Hertog *et al.*, 1993). The cytoplasmically localized PTK c-Src is activated in the RPTPα-overexpressing P19 cells, as well as in RPTPα-overexpressing Rat-2 cells (Zheng *et al.*, 1992), which may be mediated by direct dephosphorylation of the inhibitory tyrosine phosphorylation site (Tyr527) of c-Src by RPTPα.

Relatively little is known about regulation of PTP activity. A number of studies have indicated that phosphorylation may play an important role. Activation of protein kinase C (PKC) and cAMP-dependent kinase (PKA) leads to an increase in cellular PTP activity (Brautigan and Pinault, 1991). Treatment of T cells with the calcium ionophore ionomycin leads to a decrease in CD45 activity, concomitant with a decrease in CD45 serine phosphorylation (Ostergaard and Trowbridge, 1991). Serine phosphorylation of CD45 and of the cytoplasmically localized PTP1B is enhanced in response to phorbol ester treatment (Yamada *et al.*, 1990; Flint *et al.*, 1993). However, these phosphorylation events do not significantly affect the activity of these two PTPs. In contrast, we have demonstrated recently that phorbol ester treatment of cells leads to an apparent increase in RPTPα activity, concomitant with an increase in serine phosphorylation (J.den Hertog, J.Sap, C.E.G.M.Pals, J.Schlessinger and W.Kruijer, submitted; S.Tracy, P.van der Geer and T.Hunter, submitted). The transmembrane PTP CD45 has been demonstrated to be phosphorylated on tyrosine following treatment of cells with the PTP inhibitor phenylarsene oxide (Stover *et al.*, 1991). In addition, the SH2-containing, cytoplasmically localized PTP Syp (variously known as PTP1D, SH-PTP2 and PTP2C) is phosphorylated on tyrosine in response to growth factor receptor activation (Feng *et al.*, 1993; Vogel *et al.*, 1993). Whether tyrosine phosphorylation affects Syp (PTP1D) activity remains to be established definitively.

Here we report that RPTPα is phosphorylated constitutively on tyrosine *in vivo*. The P.Tyr residue is located at position 789, five amino acid residues from the C-terminus of RPTPα. The sequence on the C-terminal side of Tyr789 (YANF) fits the consensus binding site for the SH3-SH2-SH3 adaptor protein GRB2 (consensus: YXNX). We show that GRB2 binds to RPTPα *in vitro* and *in vivo*, and that this interaction is dependent on RPTPα P.Tyr789.

Results

RPTPα is phosphorylated on serine and tyrosine in vivo

The phosphorylation state of RPTPα was investigated by immunoprecipitation of RPTPα from NIH 3T3 cells labeled overnight with [^{32}P]orthophosphate. For the immunoprecipitations antisera were raised against a glutathione-S-transferase (GST) PTPα fusion protein encompassing the complete cytoplasmic domain of RPTPα, as described in Materials and methods. Following immunoprecipitation the 130 kDa ^{32}P-labeled RPTPα was excised from the gel, eluted and subjected to tryptic phosphopeptide mapping. The tryptic peptides were separated on TLC plates by electrophoresis at pH 1.9 (first dimension) and ascending chromatography (second dimension) (Figure 1A and B); seven major phosphopeptides were detected. The phosphoamino acid content of the individual tryptic peptides was determined (Figure 1C). None of the peptides contained phosphothreonine (P.Thr). Four peptides contained only phosphoserine (P.Ser) (S_A–S_D) and two peptides contained only P.Tyr (Y_A and Y_B). The peptide that contained both P.Ser and P.Tyr (SY) ran

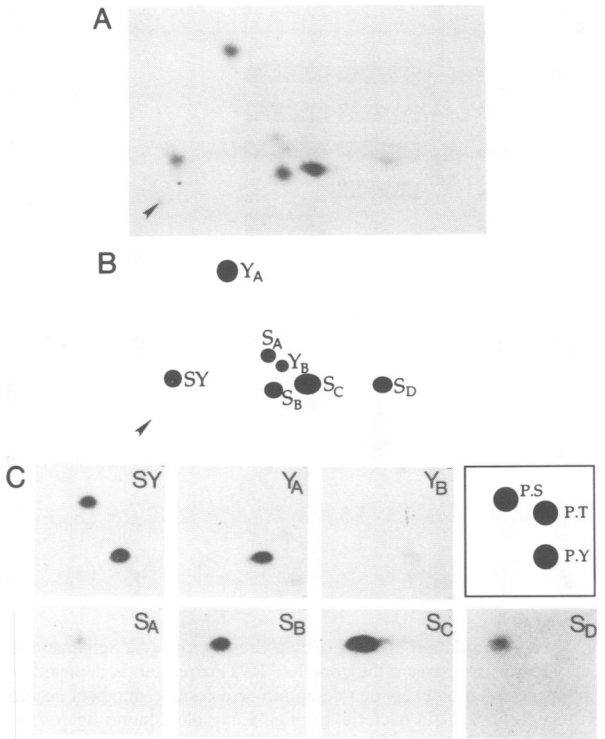

Fig. 1. RPTPα is phosphorylated on serine and tyrosine *in vivo*. **(A)** NIH 3T3 cells were labeled overnight with [^{32}P]orthophosphate (2 mCi/ml). RPTPα was immunoprecipitated using affinity-purified anti-RPTPα antiserum 5478, raised against a GST fusion protein encompassing the complete cytoplasmic domain of RPTPα. The immunoprecipitates were fractionated on a 7.5% SDS–polyacrylamide gel and the 130 kDa RPTPα was visualized by autoradiography. ^{32}P-labeled RPTPα was eluted from the dried gel and digested with trypsin, and the tryptic phosphopeptides were separated in two dimensions on cellulose TLC plates. All phosphopeptide mapping experiments described here (Figures 1–7) were performed identically (electrophoresis at pH 1.9 followed by ascending chromatography), and are depicted similarly (anode at the left, chromatography in the vertical direction with an arrowhead marking the origin). **(B)** Schematic representation of the tryptic phosphopeptide map depicted in (A). The individual peptides are labeled according to their phosphoamino acid content, as determined in C (Y for P.Tyr and S for P.Ser). **(C)** The individual phosphopeptides were isolated from the TLC plate and subjected to phosphoamino acid analysis by acid hydrolysis, followed by two-dimensional separation on a cellulose TLC plate (electrophoresis at pH 1.9 and 3.5 for the first and second dimensions, respectively). The positions of P.Ser (P.S), P.Thr (P.T) and P.Tyr (P.Y) standards that were co-electrophoresed with the samples are indicated schematically in the top right panel. The seven different panels correspond to the individual phosphopeptides in (A) and (B), and are labeled accordingly in the top right corner.

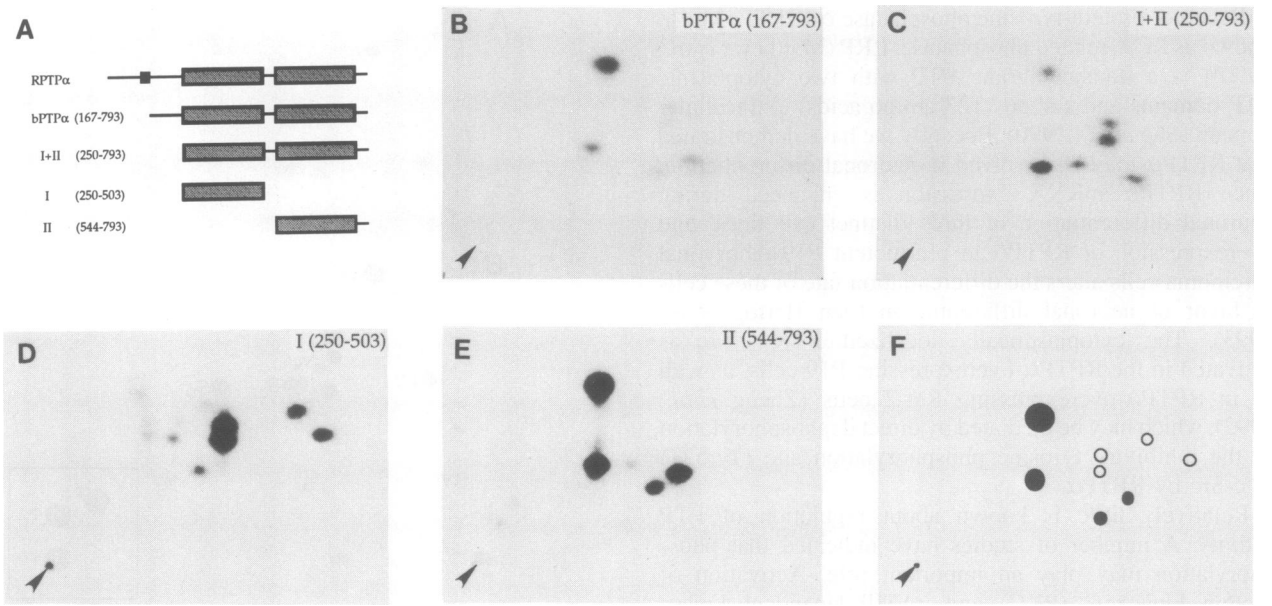

Fig. 2. Phosphorylation of bPTPα by c-Src *in vitro*. (**A**) Schematic representation of the PTPα deletion mutants that were used for the *in vitro* kinase reactions. For comparison the complete RPTPα protein is depicted as well. Solid box: transmembrane domain; hatched boxes: PTP domains. The numbers between brackets denote amino acid residue numbers according to Sap *et al.* (1990). RPTPα and its deletion mutants were expressed as GST fusion proteins in bacteria, purified using glutathione–agarose beads and eluted from the beads with glutathione. The purified RPTPα fusion proteins were incubated with immunoprecipitated, baculovirus-expressed c-Src and [γ-^{32}P]ATP (25 μCi) for 15 min at 30°C. The ^{32}P-labeled fusion proteins were isolated from gel and subjected to tryptic phosphopeptide mapping. The resulting peptide map of bPTPα is depicted in (**B**), I+II in (**C**), I in (**D**) and II in (**E**). (**F**) is a schematic representation of the tryptic phosphopeptides that were found in domain I (open symbols) or domain II (solid symbols).

relatively close to the origin; its intensity varied from experiment to experiment, suggesting that this peptide was a very large partial digestion product. We have previously mapped two major serine phosphorylation sites in RPTPα to Ser180 and Ser204. Peptide S_C contains P.Ser204, and peptides S_A and S_D are derivatives of the P.Ser180 peptide.

The stoichiometry of phosphorylation of RPTPα was determined by parallel equilibrium labeling of NIH 3T3 cells with [^{35}S]methionine and [^{32}P]orthophosphate, and subsequent immunoprecipitation of RPTPα. The amount of radioactivity in the RPTPα bands was determined, and the stoichiometry of phosphorylation of RPTPα was calculated to be 1.2 mol phosphate/mol RPTPα in resting, serum-starved NIH 3T3 cells. Direct scanning of RPTPα tryptic phosphopeptide maps, similar to the one in Figure 1A using a PhosphorImager allowed us to determine the relative amount of P.Tyr in RPTPα. Fifteen to twenty per cent of the total phosphate in RPTPα was contained in the Y_A and Y_B peptides, indicating that ~20% of the RPTPα molecules are phosphorylated on tyrosine in resting, serum-starved NIH 3T3 cells. No gross changes were observed in phosphorylation of the Y_A and Y_B peptides in response to treatment of NIH 3T3 cells with growth factors, including platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), or the phosphatase inhibitor sodium orthovanadate. In addition, treatment with these agents did not lead to additional P.Tyr-containing phosphopeptides in RPTPα (data not shown).

Tyrosine phosphorylation of RPTPα by c-Src in vitro

The ability of the non-receptor PTK c-Src to phosphorylate RPTPα was investigated by *in vitro* kinase assays, using baculovirus-expressed mouse c-Src that was immunoprecipitated using MAb 327 (Lipsich *et al.*, 1983). The cytoplasmic domain of RPTPα (residues 167–793, numbering according to Sap *et al.*, 1990) and deletion mutants were expressed in bacteria as GST fusion proteins. The fusion proteins were purified using glutathione–agarose beads, eluted with glutathione and phosphorylated using immunoprecipitated c-Src and [γ-^{32}P]ATP. Bacterially expressed cytoplasmic domain of RPTPα (bPTPα) phosphorylated by c-Src contained only P.Tyr by phosphoamino acid analysis (data not shown). Tryptic phosphopeptide mapping demonstrated that bPTPα phosphorylated by c-Src *in vitro* contained at least eight major ^{32}P-labeled tryptic peptides (Figure 2B). In order to localize the major *in vitro* phosphorylation sites within the cytoplasmic domain, *in vitro* kinase reactions were performed using deletion mutants of bPTPα (Figure 2). Comparison of the tryptic phosphopeptide maps of ^{32}P-labeled bPTPα (residues 167–793, Figure 2B), domains I and II including the region between the two PTP domains and the C-terminus (I+II, residues 250–793, Figure 2C), domain I (residues 250–503, Figure 2D) and domain II (residues 544–793, Figure 2E), and mixes of the individual ^{32}P-labeled deletion mutants (data not shown) allowed us to localize the major *in vitro* phosphorylation sites to domain I or domain II (see diagram in Figure 2F).

A major in vitro phosphorylation site of bPTPα is located in its extreme C-terminus at position 789

Since one of the major *in vitro* phosphorylation sites of bPTPα is located in the C-terminal domain, domain II, we investigated phosphorylation of a synthetic C-terminal tryptic peptide (VVQEYIDAFSDYANFK) corresponding to residues 778–793. This tryptic peptide was derived by

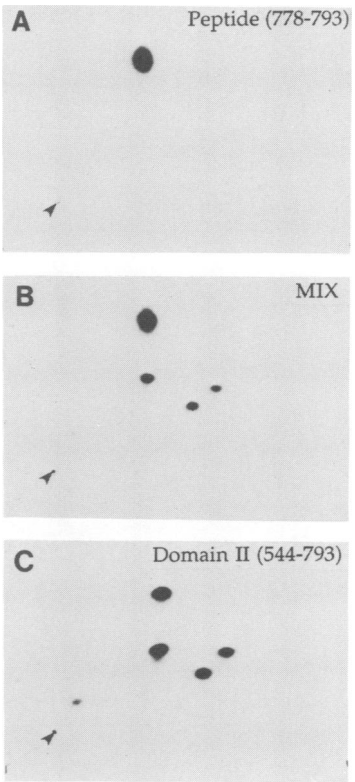

Fig. 3. A major *in vitro* phosphorylation site of bPTPα is located in its extreme C-terminus. (**A**) A C-terminal synthetic peptide of RPTPα, corresponding to residues 776–793 with an additional cysteine residue at the N-terminus (CYKVVQEYIDAFSDYANFK) was phosphorylated *in vitro* by immunoprecipitated c-Src with [γ-^{32}P]ATP (50 μCi). The phosphorylated peptide was digested with trypsin and purified by one-dimensional thin layer electrophoresis at pH 1.9. The ^{32}P-labeled peptide was isolated from the TLC plate and subjected to two-dimensional separation under the conditions described in the legend to Figure 1. (**C**) shows a tryptic phosphopeptide map of RPTPα domain II phosphorylated *in vitro* by c-Src, similar to the one depicted in Figure 2E. (**B**) is a mix of the samples in (A) and (C).

tryptic cleavage from a synthetic peptide that was initially used for immunization of rabbits to generate antisera (CYKVVQEYIDAFSDYANFK). The peptide was phosphorylated *in vitro* using immunoprecipitated, baculovirus-expressed c-Src and purified by one-dimensional TLC. Figure 3A shows the ^{32}P-labeled synthetic peptide corresponding to residues 778–793. The phosphorylated C-terminal synthetic peptide co-migrated with a major phosphopeptide derived from domain II (Figure 3B and C).

The C-terminal tryptic RPTPα peptide contains two tyrosines, at positions 782 and 789. To determine which of these is phosphorylated by c-Src *in vitro*, site-directed mutagenesis was performed. Tyr782 and Tyr789, either individually or together, were replaced by phenylalanine in pGEX-PTPα, the bacterial expression vector for bPTPα. The resulting proteins, bPTPαY782F, bPTPαY789F and bPTPαY782F/Y789F, were expressed in bacteria, purified and phosphorylated *in vitro* by immunoprecipitated c-Src. Tryptic phosphopeptide mapping of the four ^{32}P-labeled fusion proteins demonstrated that the major *in vitro* phosphorylation site of bPTPα is Tyr789 (Figure 4). Mutation of Tyr782 did not affect phosphorylation of this major *in vitro* phosphorylation site (Figure 4B), while replacement of Tyr789 with phenylalanine abolished

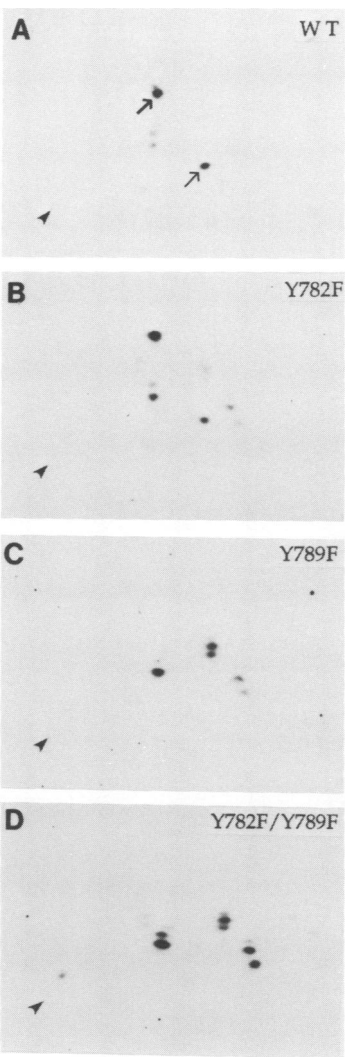

Fig. 4. Tyrosine 789 of bPTPα is phosphorylated by c-Src *in vitro*. Tyr782, Tyr789 or both were mutated by site-directed mutagenesis of pGEX-PTPα, the bacterial expression vector for a GST fusion protein encompassing the complete cytoplasmic domain of RPTPα, as described in Materials and methods. The fusion proteins were expressed in bacteria and purified using glutathione–agarose beads. bPTPα and its mutants, bPTPαY782F, bPTPαY789F and bPTPαY782F/Y789F, were phosphorylated *in vitro* using immunoprecipitated c-Src and [γ-^{32}P]ATP (25 μCi). Following SDS–PAGE the ^{32}P-labeled fusion proteins were subjected to tryptic phosphopeptide mapping. (**A**) Wild type bPTPα; (**B**) bPTPαY782F; (**C**) bPTPαY789F; (**D**) bPTPαY782F/Y789F. The major and the minor phosphopeptides that disappeared upon mutation of Tyr789 are indicated in (A) with a thick and a thin arrow, respectively.

phosphorylation of this peptide (Figure 4C). An additional phosphopeptide disappeared following mutation of Tyr789, but not Tyr782 (thin arrow in Figure 4A). This peptide may be a derivative of the major P.Tyr789 peptide, since it was also sometimes observed as a minor spot in tryptic peptide maps of *in vitro* phosphorylated synthetic peptide (residues 778–793) (data not shown).

Co-migration of phosphopeptides from in vivo labeled RPTPα and in vitro labeled synthetic peptide

The *in vitro* labeled, P.Tyr789-containing peptide and the Y_A peptide from *in vivo* ^{32}P-labeled RPTPα ran in roughly

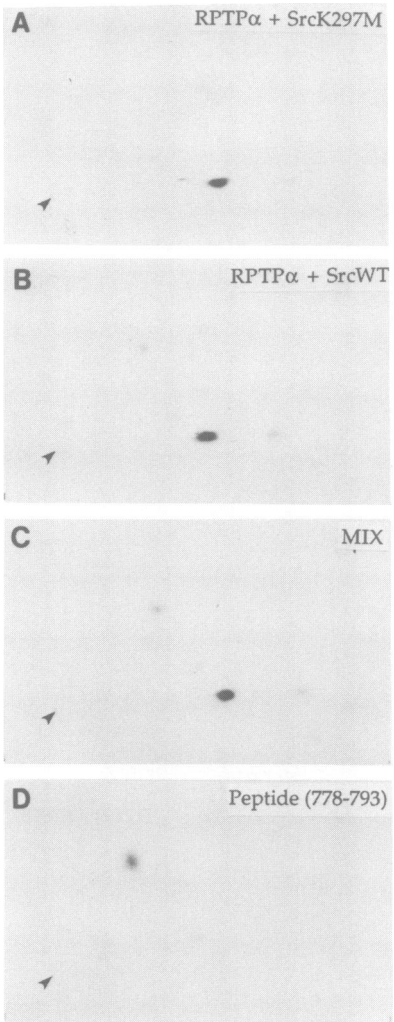

Fig. 5. Co-migration of *in vivo* phosphorylated RPTPα and *in vitro* phosphorylated C-terminal synthetic peptide. Human embryonic kidney (293) cells were transiently co-transfected with pSGRPTPα, an SV40 promoter driven expression vector for RPTPα and CMV promoter driven expression vectors for mutant, kinase inactive c-Src (SrcK297M) (A) or wild type c-Src (B and C). The cells were labeled overnight with [^{32}P]orthophosphate (2.0 mCi/ml), and RPTPα was immunoprecipitated using affinity purified polyclonal antiserum 5478. ^{32}P-labeled RPTPα was eluted from the gel and subjected to tryptic phosphopeptide mapping. (**A**) Tryptic phosphopeptide map of RPTPα immunoprecipitated from 293 cells expressing RPTPα and SrcK297M; (**B**) tryptic phosphopeptide map of RPTPα immunoprecipitated from 293 cells expressing RPTPα and wild type c-Src; (**C**) mix of samples in (B) and (D); (**D**) phosphopeptide map of synthetic C-terminal peptide, phosphorylated *in vitro* and prepared as described in the legend to Figure 3.

the same position in two-dimensional tryptic phosphopeptide maps. In an attempt to identify the *in vivo* labeled peptide, a co-migration experiment was performed. Human embryonic kidney (293) cells were transiently co-transfected with an SV40 early promoter driven expression vector for RPTPα (pSGRPTPα; den Hertog *et al.*, 1993) and CMV promoter driven expression vectors for c-Src, which express either wild type c-Src or a kinase-inactive mutant of c-Src with a point mutation in the ATP binding site at position 297 (SrcK297M). The transfected 293 cells were labeled with [^{32}P]orthophosphate and RPTPα was immunoprecipitated. Subsequent tryptic phosphopeptide mapping of RPTPα demonstrated that the pattern of

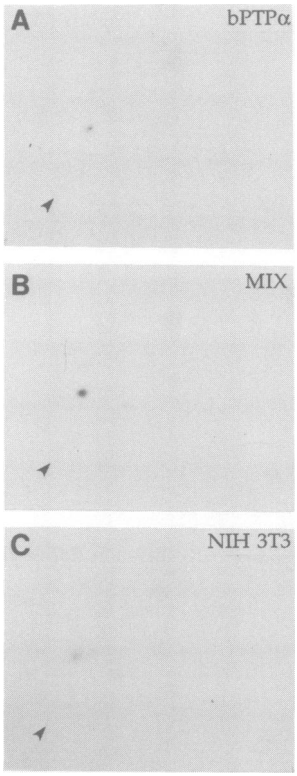

Fig. 6. Co-migration of thermolysin-digested tryptic peptides from *in vitro* and *in vivo* labeled RPTPα. The Y_A peptide from *in vivo* ^{32}P-labeled RPTPα from NIH 3T3 cells (Figure 1A) and the phosphopeptide containing Tyr789 from *in vitro* phosphorylated bPTPα (Figure 4A, thick arrow) were isolated from the TLC plates and digested with thermolysin. Subsequently these samples were subjected to two-dimensional phosphopeptide mapping. (**A**) Peptide from *in vitro* labeled bPTPα; (**B**) mix of samples in (A) and (C); (**C**) Y_A peptide from *in vivo* labeled NIH 3T3 cells.

phosphopeptides was very similar to the pattern observed with RPTPα from NIH 3T3 cells (cf. Figures 5A and 1A). Co-expression of wild type c-Src enhanced phosphorylation of the Y_A peptide (cf. Figure 5A and B). The Y_A peptide co-migrated exactly with the synthetic peptide when a mix of RPTPα from transiently transfected 293 cells with *in vitro* phosphorylated synthetic peptide was analyzed (Figure 5B–D). The enhanced phosphorylation of the Y_A peptide observed in response to co-expression of wild type c-Src indicates that c-Src may phosphorylate Tyr789 *in vivo*.

Co-migration of thermolysin-digested peptides from in vitro and in vivo labeled bPTPα

In order to obtain further biochemical evidence that the *in vitro* and *in vivo* labeled peptides that co-migrate in phosphopeptide maps (Figure 5) are identical, the Y_A peptide from *in vivo* labeled RPTPα from NIH 3T3 cells (Figure 1A) and the major tryptic phosphopeptide from *in vitro* labeled bPTPα (Figure 4A, thick arrow) were isolated from the TLC plates and digested with thermolysin. Several putative thermolysin cleavage sites (small hydrophobic residues) are present in the C-terminal tryptic peptide. However, it is hard to predict thermolysin cleavage sites, because thermolysin will recognize most nonpolar residues to some extent (Boyle *et al.*, 1991). Two-dimensional peptide mapping of the major phosphopeptide from

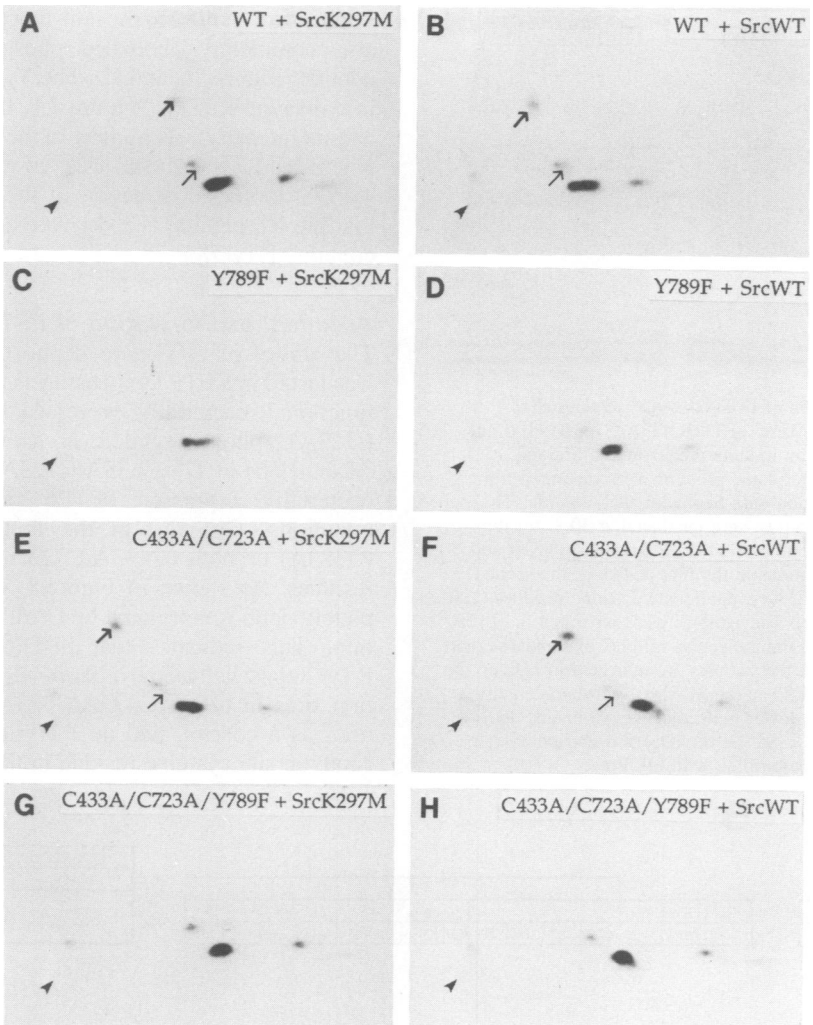

Fig. 7. Tyr789 is phosphorylated *in vivo*. Human embryonic kidney (293) cells were transiently co-transfected with CMV promoter driven expression vectors for SrcK297M (A, C, E and G), or wild type c-Src (B, D, F and H) and SV40 promoter driven expression vectors for wild type RPTPα (**A** and **B**), and the mutants RPTPαY789F (**C** and **D**), RPTPαC433A/C723A (**E** and **F**) and RPTPαC433A/C723A/Y789F (**G** and **H**). The transiently transfected cells were labeled overnight with [^{32}P]orthophosphate and following isolation of RPTPα by immunoprecipitation and SDS–PAGE, RPTPα was subjected to tryptic phosphopeptide mapping. The Y_A and Y_B peptides are indicated in (A), (B), (D) and (F) with a thick and a thin arrow, respectively.

in vitro labeled bPTPα, digested with thermolysin, resulted in two phosphopeptides that ran relatively close to the origin (Figure 6A). The thermolysin-digested Y_A peptide from *in vivo* labeled NIH 3T3 cells and from *in vitro* labeled bPTPα showed identical patterns of phosphopeptides following two-dimensional peptide mapping (Figure 6C), and a mix of the *in vitro* and *in vivo* labeled thermolysin-digested tryptic peptides confirmed that these peptides co-migrate (Figure 6B). These results show that Tyr789 is phosphorylated both in *in vivo* labeled RPTPα from NIH 3T3 cells and in *in vitro* labeled, bacterially expressed bPTPα.

Tyr789 of RPTPα is phosphorylated in vivo

Tyrosine phosphorylation of RPTPα *in vivo* was further investigated by transient co-transfection of 293 cells with SV40 promoter driven expression vectors for RPTPα or various mutants of RPTPα and CMV promoter driven expression vectors for wild type c-Src or kinase inactive c-Src (SrcK297M). The mutants of RPTPα that were used for these studies contain a Tyr→Phe mutation at position 789 (RPTPαY789F), Cys→Ala mutations in both catalytic site cysteines (positions 433 and 723, RPTPαC433A/C723A) or all three mutations (RPTPαC433A/C723A/Y789F). The c-Src and RPTPα expression vectors were co-transfected into 293 cells and subsequently these cells were labeled with [^{32}P]orthophosphate. RPTPα was then immunoprecipitated and tryptic phosphopeptide mapping was performed (Figure 7). Co-expression of wild type c-Src led to an increase in phosphorylation of the Y_A peptide (Figure 7A and B, thick arrow) of ~2-fold relative to total RPTPα phosphate content, as determined with a PhosphorImager. Mutation of Tyr789 completely abolished phosphorylation of the Y_A peptide, demonstrating that Tyr789 is the major tyrosine phosphorylation site *in vivo* (Figure 7C and D). Phosphorylation of the Y_A peptide in non-functional RPTPαC433A/C723A was 2- to 3-fold higher than in wild type RPTPα (cf. Figure 7A and E), and co-expression of wild type c-Src enhanced phosphorylation of the Y_A peptide 1.2- to 1.5-fold (Figure 7F).

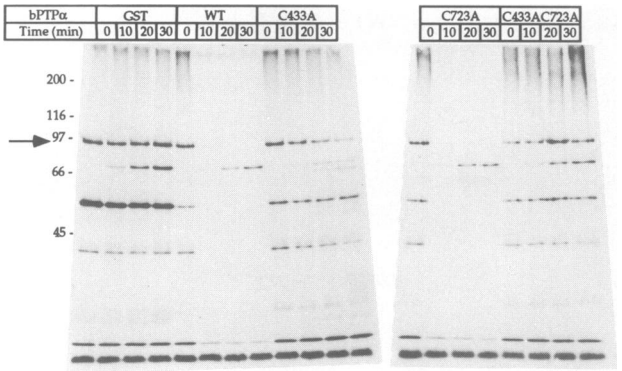

Fig. 8. Autodephosphorylation of bPTPα *in vitro*. Bacterially expressed, non-functional RPTPα (bPTPαC433A/C723A) (100 ng) was phosphorylated *in vitro* by immunoprecipitated c-Src and [γ-^{32}P]ATP (50 μCi). Phosphorylated proteins were separated from free [γ-^{32}P]ATP by gel filtration on a Sephadex G50 column. The phosphorylated proteins (~ 15 μg) were incubated at 30°C for the times indicated with GST alone or GST–PTPα fusion proteins and mutants with Cys→Ala mutations in the first (C433A), the second (C723A) or both (C433AC723A) catalytic site cysteine residues (100 ng fusion protein per sample). The samples were separated on a 10% SDS–polyacrylamide gel and the molecular weights of standards that were co-electrophoresed with the samples are indicated in kDa on the left. The position of GST–PTPα in the SDS–polyacrylamide gel is indicated with an arrow. The lower molecular weight bands, detected here, are autophosphorylated c-Src (at 60 kDa) and phosphorylated bacterial proteins that were co-purified with bPTPα.

Mutation of Tyr789 in the non-functional mutant of RPTPα also completely abolished phosphorylation of the Y_A peptide (Figure 7G and H). The Y_B peptide was detectable in wild type RPTPα (Figure 7A, B, E and F, thin arrow), and its intensity was highest in the non-functional mutant, when wild type c-Src was co-expressed. Mutation of Tyr789 abolished detection of the Y_B peptide, indicating that the Y_B peptide is a derivative of the Y_A peptide, and contains P.Tyr789.

Autodephosphorylation of RPTPα

The ability of bPTPα to dephosphorylate itself *in vitro* was tested in a PTP assay using as a substrate mutant, non-functional, bacterially expressed bPTPα (bPTPαC433A/C723A), phosphorylated *in vitro* by c-Src. *In vitro* ^{32}P-labeled bPTPαC433A/C723A was incubated with bacterially expressed bPTPα or mutants with point mutations (Cys Ala) in the first (C433A), the second (C723A) or both (C433A/C723A) catalytic site cysteine residues. As shown in Figure 8, wild type bPTPα completely dephosphorylated bPTPαC433A/C723A within 10 min. This indicates that bPTPα can dephosphorylate P.Tyr789 as well as the other *in vitro* tyrosine phosphorylation sites in bPTPαC433A/C723A (cf. Figure 2B). GST, used as a control, had no PTP activity. Mutation of the catalytic site cysteine residue in the first domain (C433A)

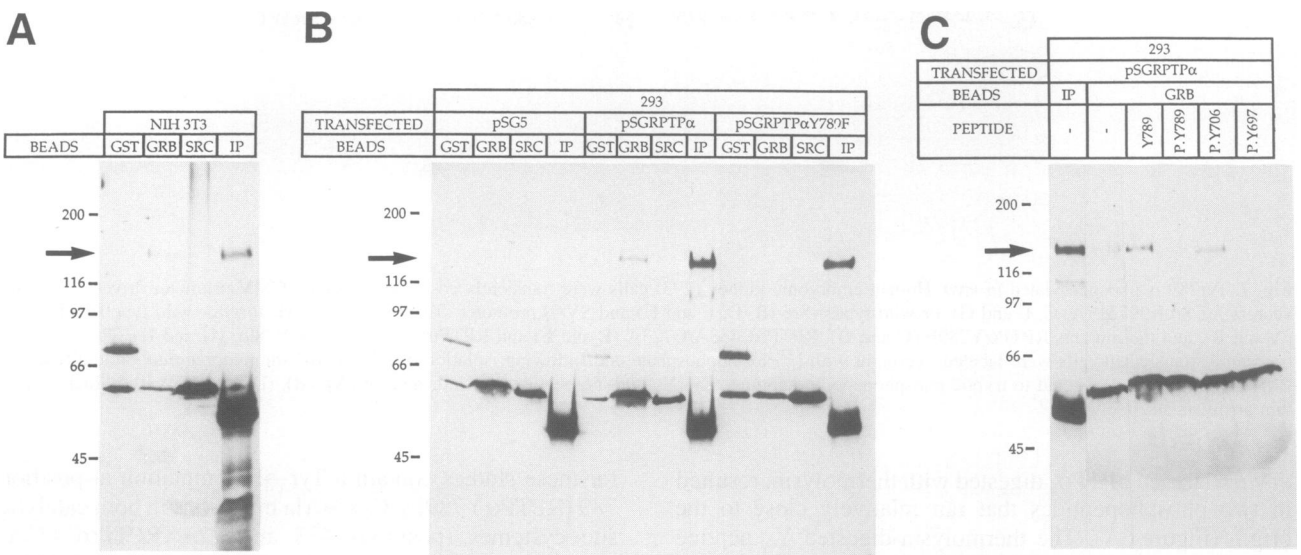

Fig. 9. The SH3-SH2-SH3 adaptor protein GRB2 associates with RPTPα through P.Tyr789 *in vitro*. (**A**) Nearly confluent NIH 3T3 cells were lysed under non-denaturing conditions (1% Triton X-100). The lysates were incubated with 5 μg GST fusion proteins, expressed in bacteria and bound to glutathione–agarose beads. The fusion proteins encoded GST alone, full-length GST–GRB2 (GRB), or the Src-SH2 domain (SRC). As a control RPTPα was immunoprecipitated using affinity purified anti-RPTPα antiserum 5478 (IP). After extensive washing the beads were boiled in reducing sample buffer and the samples were run on a 10% SDS–polyacrylamide gel. The material on the gel was transferred to Immobilon and probed with anti-RPTPα antiserum 5478. The immunoblot was developed using HRP-conjugated donkey anti-rabbit antibodies and enhanced chemiluminescence (ECL), as described in Materials and methods. Immunoblots are shown with the molecular weights of markers that were co-electrophoresed with the samples indicated in kDa on the left. The position of RPTPα is indicated with an arrow. (**B**) Immunoblotting analysis, as described under (A) of 293 human embryonic kidney cells transiently co-transfected with vector alone (pSG5), an SV40 driven expression vector for wild type RPTPα (pSGRPTPα) or mutant RPTPα with a Tyr→Phe mutation at position 789 (pSGRPTPαY789F) together with a CMV promoter driven expression vector for activated Src (SrcY527F). Lysates were incubated with GST, GST–GRB2 or GST–SRC-SH2 bound to glutathione–agarose beads, or RPTPα was immunoprecipitated. Following SDS–PAGE immunoblotting was performed using anti-RPTPα antiserum, as described under (A). (**C**) Lysates of 293 cells, transiently transfected with pSGRPTPα were incubated with GST–GRB2 bound to glutathione–agarose beads (GRB), or RPTPα was immunoprecipitated (IP). The GRB2-conjugated beads were pre-incubated with peptides or phosphopeptides (50 μM final concentration) for 5 min prior to the addition of lysate. The peptides that were used are: a C-terminal peptide of RPTPα (residues 776–793) (Y789), a phosphopeptide encompassing the C-terminus of RPTPα (residues 782–793, containing P.Tyr789) (P.Y789), and two phosphopeptides encompassing CSF-1-R autophosphorylation sites, P.Tyr706 (P.Y706) and P.Tyr697 (P.Y697). The samples were electrophoresed on an SDS–polyacrylamide gel, transferred to Immobilon and probed with anti-RPTPα antisera 5478, as described under (A). The lower molecular weight bands, detected here, were due to cross-reaction of the anti-RPTPα antiserum 5478 with bacterial proteins that were co-purified with the GST fusion proteins. In the immunoprecipitation lanes the heavy chain of anti-RPTPα antiserum was detected.

strongly reduced PTP activity of bPTPα, but did not abolish activity completely. Mutation of the catalytic site cysteine in the second PTP domain (C723A) did not affect activity significantly in this assay, in that bPTPαC433A/C723A was completely dephosphorylated within 10 min. These results confirm the observations by Wang and Pallen (1991) that the first domain of RPTPα contains the majority of the PTP activity, whereas the second domain of RPTPα contains low, but detectable PTP activity. Mutation of both catalytic site cysteines in bPTPα (C433A-C723A) completely abolished PTP activity, and no dephosphorylation of the substrate was observed (Figure 8).

When expressed transiently, RPTPαC433A/C723A had a 2- to 3-fold higher level of Tyr789 phosphorylation than wild type RPTPα (Figure 7). The enhanced phosphorylation of the Y_A peptide in the non-functional mutant of RPTPα indicates that wild type RPTPα may dephosphorylate itself, and thus that RPTPα may have autodephosphorylation activity *in vivo*.

The SH3-SH2-SH3 adaptor protein GRB2 binds to P.Tyr789 of RPTPα in vitro

P.Tyr-containing proteins provide binding sites for SH2-containing proteins. SH2 domains are P.Tyr binding moieties that bind to specific proteins through consensus recognition sequences. Comparison of sequences C-terminal to Tyr789 in RPTPα (789-YANFK-793) with consensus binding sequences of several SH2 domains indicated that Tyr789 of RPTPα is located in a consensus binding site for the SH3-SH2-SH3 adaptor protein GRB2 (consensus binding site: YXNX, Songyang *et al.*, 1993). The ability of GRB2 to bind to RPTPα was investigated using a GST fusion protein, encompassing full-length GRB2 (Lowenstein *et al.*, 1992). As controls, GST alone and GST–Src-SH2, a fusion protein containing GST fused to the v-Src SH2 domain (Liu *et al.*, 1993), were used. The fusion proteins, immobilized on glutathione–agarose beads, were incubated with NIH 3T3 cell lysates, washed, and the associated proteins were resolved on a 10% SDS–polyacrylamide gel. Immunoblotting analysis with anti-RPTPα antiserum as a probe demonstrated that RPTPα bound to GRB2, but not GST alone or the Src SH2 domain. As a positive control RPTPα was immunoprecipitated from NIH 3T3 cells (Figure 9A). The 105 kDa band that was detected in the RPTPα immunoprecipitates is either a precursor or a degradation product of RPTPα, since peptide maps of this protein, immunoprecipitated from ^{35}S-labeled cells are highly similar to peptide maps of RPTPα (data not shown).

In order to investigate whether GRB2 binding to RPTPα is dependent on phosphorylation of Tyr789, 293 cells were transiently co-transfected with vector alone (pSG5), pSGRPTPα or pSGRPTPαY789F and a CMV promoter driven expression vector for a constitutively active mutant of c-Src with a point mutation at the inhibitory tyrosine phosphorylation site (SrcY529F). Lysates of the transiently transfected 293 cells were incubated with GST, GST–GRB2 or GST–Src-SH2 bound to glutathione–agarose beads. RPTPα was immunoprecipitated from the same lysates to determine the total amount of RPTPα. Subsequent immunoblotting analysis demonstrated that GRB2, but not GST alone or Src-SH2 bound to wild type RPTPα

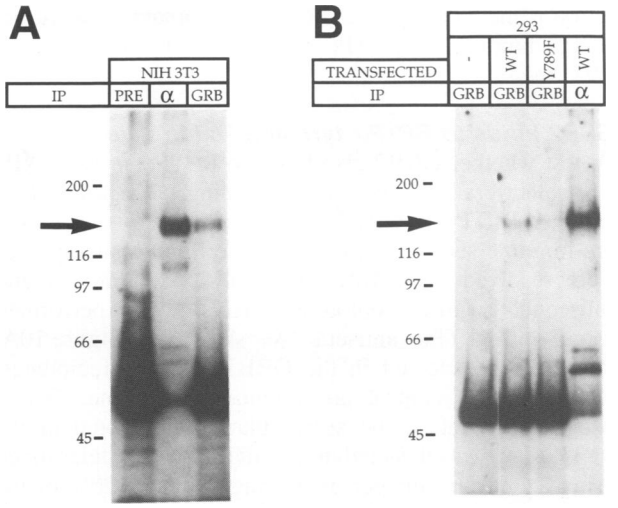

Fig. 10. GRB2 binds to RPTPα *in vivo*. (**A**) NIH 3T3 cells were lysed under non-denaturing conditions (1% Triton X-100). Immunoprecipitations were performed with 5478 pre-immune serum (pre), affinity purified anti-RPTPα antiserum 5478 (α), or anti-GRB2 antiserum (GRB). The samples were separated on a 10% SDS–polyacrylamide gel and transferred to Immobilon. The blots were probed with anti-RPTPα antiserum 5478, and detection was done using ^{125}I-labeled protein A and autoradiography. Molecular weights of marker proteins that were co-electrophoresed are shown on the left in kDa. The position of RPTPα is indicated with an arrow. (**B**) Human embryonic kidney 293 cells were transiently transfected with vector alone (–), pSGRPTPα (WT), or pSGRPTPαY789F (789). The cells were lysed and immunoprecipitations were performed with either anti-GRB2 (GRB) or anti-RPTPα (α) antiserum. Immunoblotting analysis using anti-RPTPα antiserum was performed as described in (A).

(Figure 9B). Approximately 10% of the immunoprecipitable RPTPα was found to bind to GRB2 *in vitro*. The majority of the GRB2 binding to RPTPα was dependent on Tyr789, since mutation of this site led to a 90–95% reduction in GRB2 binding, although RPTPαY789F was expressed at similar levels to wild type RPTPα (Figure 9B).

Further evidence for the dependence of GRB2–RPTPα association on tyrosine phosphorylation of RPTPα was obtained by pre-incubation of GRB2, bound to glutathione–agarose beads, with specific peptides or phosphopeptides, prior to incubation with lysates from 293 cells transiently transfected with pSGRPTPα. The peptides that were used for these studies were: non-phosphorylated C-terminal peptide of RPTPα corresponding to residues 776–793 (Y789), a C-terminal synthetic phosphopeptide of RPTPα, residues 782–793 containing P.Tyr at position 789 (P.Y789), and two synthetic phosphopeptides corresponding to colony stimulating factor-1 receptor (CSF-1-R) autophosphorylation sites, one of which (P.Y697) has been shown to be a GRB2 binding site, and the other (P.Y706) not to be a GRB2 binding site (van der Geer and Hunter, 1993). Pre-incubation of GRB2-conjugated beads with non-phosphorylated RPTPα peptide did not alter binding of GRB2 to RPTPα from transiently transfected 293 cells. However, pre-incubation with the RPTPα P.Tyr789 and CSF-1-R P.Tyr697 peptides completely abolished GRB2 binding to RPTPα, whereas the non-related phosphopeptide CSF-1-R P.Tyr706 had no effect (Figure 9C). These results demonstrate that GRB2 binding to RPTPα is dependent on P.Tyr residues, indicating that the inter-

action is mediated by the GRB2 SH2 domain. In addition, GRB2 binding to RPTPα is dependent on Tyr789 of RPTPα.

GRB2 binds to RPTPα tyrosine 789 in vivo

To test whether GRB2 also binds to RPTPα *in vivo*, NIH 3T3 cells were lysed in buffer containing 1% Triton X-100, and then RPTPα or GRB2 was immunoprecipitated, using pre-immune serum as a control. The immunoprecipitates were resolved on a 10% SDS–polyacrylamide gel and subsequently immunoblotting analysis was performed using anti-RPTPα antisera. As shown in Figure 10A, RPTPα was detected in the GRB2 immunoprecipitates, but not in the control pre-immune serum lane. Ten to twenty per cent of the signal that was detected in the RPTPα lane was detected in the GRB2 lane, as determined using a PhosphorImager, indicating that ~10–20% of the cellular RPTPα is associated with GRB2 *in vivo*. Similar experiments were performed with transiently transfected 293 cells. Cells transfected with vector alone, pSGRPTPα or pSGRPTPαY789F were lysed and GRB2 was immunoprecipitated. As shown in Figure 10B, some endogenous RPTPα was found to be associated with GRB2 following immunoblotting with anti-RPTPα antiserum. Expression of wild type RPTPα led to an increase in the signal, whereas expression of the mutant RPTPαY789F did not. These results demonstrate that GRB2 binds to RPTPα *in vivo*, and that phosphorylation of Tyr789 is essential for the process.

Sos is not detectable in RPTPα immunoprecipitates

To date, the only protein that has been identified to associate with the SH3 domains of GRB2 *in vivo* is Sos, a guanine nucleotide releasing factor for the Ras GTPase (Pawson and Schlessinger, 1993 and references therein). The GTPase dynamin binds to the SH3-domains of GRB2 *in vitro* (Gout *et al.*, 1993), but GRB2 binding to dynamin *in vivo* remains to be established. We could not detect dynamin in RPTPα immunoprecipitates by immunoblotting (data not shown), suggesting that dynamin is not associated with the RPTPα–GRB2 complex. Association of Sos with RPTPα was investigated by co-immunoprecipitation assays. Immunoblotting analysis with Sos-specific antibodies was performed on RPTPα immunoprecipitates from NIH 3T3 cells and vice versa. To be able to detect low levels of Sos in the RPTPα immunoprecipitates, four 10 cm dishes of 80–90% confluent NIH 3T3 cells were used per sample. The efficiency of the immunoprecipitation was estimated by running lysate (one-eighth of a 10 cm dish) on the same gel. As shown in Figure 11A, RPTPα was efficiently immunoprecipitated, but no RPTPα was detectable in Sos immunoprecipitates made with this antiserum. The anti-Sos antibodies did not efficiently immunoprecipitate Sos, since the signals in the Sos immunoprecipitation lane and the lysate lane are approximately equal, although 32 times more lysate was used for the immunoprecipitation (Figure 11B). A similar estimate of the total amount of Sos in the lysate of four 10 cm dishes was also obtained by the *in vitro* binding assay with GST–GRB2, where the Sos signal was also ~30 times higher in the GST–GRB2 lane than in the lysate lane (Figure 11B). No Sos could be detected in RPTPα immunoprecipitates from four 10 cm dishes by immunoblotting (Figure 11B), confirming the result in Figure 11A. In addition, we failed to detect Sos in RPTPα immunoprecipitates from ^{35}S-labeled NIH 3T3 lysates, or RPTPα in Sos immunoprecipitates in sequential immunoprecipitation assays. By contrast, both RPTPα and Sos were detected in GRB2 immunoprecipitates from ^{35}S-labeled NIH 3T3 cells (data not shown). These results indicate that Sos is not detectably associated with RPTPα via GRB2.

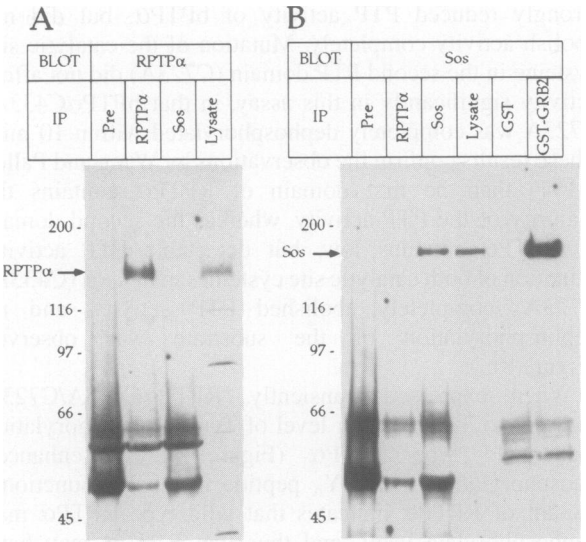

Fig. 11. Sos is not detectable in RPTPα immunoprecipitates. NIH 3T3 cells (four 10 cm dishes per sample) were lysed in buffer containing 1% Triton X-100 and immunoprecipitations with pre-immune serum (pre), affinity purified anti-RPTPα antiserum 5478 (α) or anti-Sos antiserum (Sos) were performed. Alternatively, lysate (one-eighth of a 10 cm dish) was loaded directly, or the lysate (four 10 cm dishes per sample) was incubated with fusion proteins, GST alone or GST–GRB2, bound to glutathione–agarose beads. The samples were fractionated on a 7.5% SDS–polyacrylamide gel. Immunoblotting analysis was done using affinity purified anti-RPTPα antiserum (**A**) or anti-Sos antiserum (**B**), using HRP-conjugated protein A and ECL detection. Molecular weights of marker proteins that were co-electrophoresed with the samples are indicated on the left in kDa. The positions of RPTPα and Sos are indicated with arrows.

Discussion

The PTPs have been recognized as a separate family of enzymes, distinct from the protein-serine/threonine phosphatases, with high specificity for P.Tyr residues (Fischer *et al.*, 1991; Charbonneau and Tonks, 1992; Pot and Dixon, 1992). Relatively little is known about regulation of PTP activity, and about the role of PTPs in signal transduction. Phosphorylation may play an important role in regulation of PTPs, and we have investigated tyrosine phosphorylation of the transmembrane PTP RPTPα. Here we demonstrate that RPTPα is constitutively phosphorylated on Tyr789 *in vivo*. Phosphorylation of this tyrosine residue, located very close to the C-terminus of RPTPα, creates a binding site for the SH3-SH2-SH3 adaptor protein GRB2, a key component in the receptor PTK-Ras signal transduction pathway.

Both cytoplasmically localized as well as transmembrane PTPs have been found to be phosphorylated on serine and tyrosine residues (Yamada *et al.*, 1990; Ostergaard and Trowbridge, 1991; Stover *et al.*, 1991; Feng *et al.*, 1993;

Flint *et al.*, 1993; Vogel *et al.*, 1993). Tyrosine phosphorylation of PTPs is complex, since PTPs conceivably have autodephosphorylation activity. In this respect it is noteworthy that tyrosine phosphorylation of CD45 was only observed following treatment of cells with the PTP inhibitor phenylarsene oxide (Stover *et al.*, 1991). Here we demonstrate that RPTPα is phosphorylated constitutively on a tyrosine at its extreme C-terminus *in vivo*. Treatment of NIH 3T3 cells with the PTP inhibitor orthovanadate, or with growth factors, including EGF and PDGF, did not significantly modulate tyrosine phosphorylation of RPTPα (data not shown). Tyrosine phosphorylation of RPTPα *in vivo* may be mediated by c-Src, since c-Src was able to phosphorylate Tyr789 *in vitro* and transient overexpression of c-Src together with RPTPα led to an increase of ~2-fold in Tyr789 phosphorylation in 293 cells. However, we cannot exclude the possibility that other related PTKs, e.g. Src family members, or non-related PTKs phosphorylate RPTPα *in vivo* as well.

Analysis of the effects of tyrosine phosphorylation on PTP activity is complicated. Syp (variously known as PTP1D, SH-PTP2 and PTP2C) has been demonstrated to be phosphorylated on tyrosine in response to PDGF, EGF and in v-Src-transformed cells (Feng *et al.*, 1993). In addition, co-expression of PTP1D (Syp) with a chimeric receptor PTK (EGFR/βPDGFR) in 293 cells illustrated that PTP1D is phosphorylated on tyrosine upon co-transfection of the receptor PTK (Vogel *et al.*, 1993). Immune complex PTP assays with these immunoprecipitates indicated that co-expression of the chimeric receptor PTK leads to an ~2-fold increase in PTP1D activity (Vogel *et al.*, 1993). However, since Syp (PTP1D) has autodephosphorylation activity (Feng *et al.*, 1993), these experiments are technically difficult. Another factor that further complicates these experiments is the recent finding that binding of phosphopeptides to the N-terminal SH2 domain of SH-PTP2 (Syp, PTP1D) leads to a 5- to 10-fold increase in SH-PTP2 activity (Lechleider *et al.*, 1993a). Therefore, tyrosine phosphorylation of SH-PTP2 (PTP1D) may actually inhibit its activity, since the increase in activity of tyrosine phosphorylated PTP1D in the stimulated EGFR/PDGFR 293 cells (Vogel *et al.*, 1993) was less than predicted for PTP1D bound via its SH2 domains to an activated receptor PTK.

Like PTP1D, we found that RPTPα has autodephosphorylation activity *in vitro*. In addition, since the transient transfection experiments in 293 cells showed that Tyr789 phosphorylation is 2- to 3-fold higher in non-functional mutants of RPTPα than in wild type RPTPα, it is tempting to speculate that RPTPα has autodephosphorylation activity *in vivo* as well. Since tyrosine phosphorylated non-functional mutant (bPTPαC433A/C723A) was used as a substrate in the *in vitro* autodephosphorylation assay, the observed dephosphorylation by bPTPα and its mutants was intermolecular. Potentially, RPTPα has intramolecular autodephosphorylation activity as well. However, technically it is hard to distinguish between inter- and intramolecular dephosphorylation *in vivo*.

The influence of tyrosine phosphorylation on RPTPα activity was investigated by *in vitro* PTP assays using bacterially expressed bPTPα and bPTPαY789F phosphorylated by c-Src *in vitro* with ATP-γ-S to prevent rapid autodephosphorylation. bPTPα and bPTPαY789F were equally potent in *in vitro* PTP assays, indicating that mutation of Tyr789 does not in itself affect PTP activity. Tyrosine phosphorylation of wild type bPTPα, but not bPTPαY789F, reduced PTP activity to ~50%, indicating that phosphorylation of Tyr789 specifically reduces bPTPα activity *in vitro* (data not shown). However, proper interpretation of these experiments is complex, since it is technically difficult to determine stoichiometries of thiophosphorylation of bPTPα under the conditions used (1 mM ATP-γ-S, final concentration), and sites other than Tyr789 are phosphorylated *in vitro* in bPTPα by c-Src.

Tyrosine phosphorylation of PTPs may influence their activity directly. In addition, tyrosine phosphorylation of PTPs may provide binding sites for SH2-containing proteins, by analogy with autophosphorylated PTKs. The consensus recognition sequences of a number of SH2 domains have been determined, and comparison of these consensus binding sites with sequences surrounding Tyr789 in RPTPα indicated that P.Tyr789 of RPTPα could be a binding site for the SH3-SH2-SH3 adaptor protein GRB2. The consensus recognition sequence of GRB2 is pY[V/I/L/M]NX (Songyang *et al.*, 1993). It appears that specificity is predominantly determined by the asparagine at position +2. At the +1 position a small hydrophobic residue is preferred, but recently a GRB2 binding site was identified in the CSF-1-R with a lysine residue at position +1 (pYKNI) (van der Geer and Hunter, 1993). Tyr789 of RPTPα has an alanine residue at the +1 position (pYANF), and we show that GRB2 can bind to RPTPα *in vitro* and *in vivo*. Quantitative analysis of RPTPα tyrosine phosphorylation and GRB2 binding *in vivo* indicates that almost all tyrosine-phosphorylated RPTPα is bound to GRB2. Approximately 20% of the cellular RPTPα in NIH 3T3 cells is phosphorylated on Tyr789, and co-immunoprecipitation experiments indicated that ~20% of the cellular RPTPα is associated with GRB2. In this respect it is noteworthy that co-transfection of RPTPα with an expression vector for GRB2 led to an increase in Tyr789 phosphorylation (data not shown). Co-transfection of GRB2R86K, a mutant with an Arg→Lys mutation in the highly conserved 'FLVRES' sequence of the GRB2 SH2 domain, which disrupts binding to P.Tyr-containing proteins (Skolnik *et al.*, 1993), did not increase RPTPα tyrosine phosphorylation (data not shown). These results indicate that GRB2 binding to P.Tyr789 may protect this site from (auto)dephosphorylation. Immunoprecipitation of RPTPα and GRB2 in parallel from ^{35}S-labeled NIH 3T3 cells indicated that GRB2 is in large molar excess over (P.Tyr-containing) RPTPα (data not shown). Apparently, since only 20% of the cellular RPTPα is phosphorylated on Tyr789, the PTK activity that phosphorylates Tyr789 in RPTPα is limiting in NIH 3T3 cells, since GRB2 is present in molar excess over RPTPα and protects P.Tyr789 from dephosphorylation.

Mutation of Tyr789 in RPTPα almost completely abolished GRB2 binding (typically GRB2 binding to RPTPαY789F was <5–10% of that to wild type RPTPα). Two P.Tyr-containing peptides were detected in RPTPα from NIH 3T3 cells (Y_A and Y_B). However, since neither Y_A nor Y_B was detectable in tryptic phosphopeptide maps of mutant RPTPαY789F, we deduced that both peptides contain P.Tyr789. The residual GRB2 binding to RPTPαY789F may result from GRB2 binding to other

tyrosine phosphorylation sites, which are not readily detected *in vivo*. It is noteworthy that RPTPα has five potential GRB2 binding sites in its cytoplasmic domain, two of which are located in highly conserved regions of the PTP domains, and are present in all PTPs (consensus: YINA, located at positions 286 and 579 in RPTPα). It has been speculated that these tyrosine residues are potential binding sites for GRB2 in CD45 (Songyang *et al.*, 1993). However, in RPTPα from NIH 3T3 cells the only tyrosine residue that can be detected to be phosphorylated is Tyr789, located to the C-terminal side of the second PTP-domain. Sequence comparison of the extreme C-termini of the transmembrane PTPs that have been cloned to date shows that RPTPα Tyr789 is conserved between mouse and human (Krueger *et al.*, 1990; Sap *et al.*, 1990). In addition, an identical sequence is present at a similar position in the C-terminus of the highly related RPTPε (YANF) (Krueger *et al.*, 1990). Furthermore, RPTPμ, but not the highly related RPTPκ, contains a potential GRB2 binding site five residues from its C-terminus (YLNS) (Gebbink *et al.*, 1991; Jiang *et al.*, 1993). Finally, RPTPβ contains a potential GRB2 binding site 17 residues from the C-terminus (YENV) (Krueger *et al.*, 1990). It will be interesting to see whether these PTPs are phosphorylated on their C-terminal tyrosine residues and bind GRB2 *in vivo*.

Certain non-transmembrane PTPs can bind GRB2 as well. Recent work by Li *et al.* (1994) indicates that tyrosine phosphorylation of Syp (PTP1D, SH-PTP2) may play a role in coupling GRB2 to the PDGFR. Whereas GRB2 can bind to some activated growth factor receptors directly, e.g. EGFR and CSF-1-R (Lowenstein *et al.*, 1992; Li *et al.*, 1993; van der Geer and Hunter, 1993), GRB2 could not be detected to bind directly to autophosphorylated PDGFR. Syp has been shown to bind to activated growth factor receptors, including PDGFR and EGFR, through its SH2 domains (Feng *et al.*, 1993; Kazlauskas *et al.*, 1993; Lechleider *et al.*, 1993b; Vogel *et al.*, 1993). GRB2 can be detected in immunoprecipitates of Syp, under conditions where Syp is phosphorylated on tyrosine. Binding of Syp to activated PDGFR leads to tyrosine phosphorylation of Syp, thereby enabling GRB2 to bind to Syp and thus to bring GRB2 with its associated protein, Sos, in the vicinity of the PDGFR, close to the cell membrane (Li *et al.*, 1994). The site(s) of tyrosine phosphorylation and GRB2 binding have not yet been identified in Syp or PTP1D. Syp contains three potential binding sites for GRB2 that are conserved in PTP1D, and PTP1D has an additional potential GRB2 binding site at position 580, 14 residues from the C-terminus (YENV). Recently, two tyrosine phosphorylation sites have been mapped in the SH2-containing PTP SH-PTP1 (also known as PTP1C). Both tyrosines are located to the C-terminal side of the PTP domain, and the surrounding sequences indicate that these tyrosine residues are potential GRB2 binding sites (536-YGNI and 564-YENL). *In vitro* tyrosine phosphorylated SH-PTP1 binds to GRB2, and SH-PTP1 is stably associated with GRB2 in LSTRA cells (Lorenz *et al.*, 1994).

The transmembrane PTP CD45 is phosphorylated on tyrosine following treatment of cells with phenylarsene oxide (Stover *et al.*, 1991). Autero *et al.* (1994) identified Csk and Lck as two PTKs that phosphorylate CD45 *in vitro* on peptides that run in positions similar to two peptides from *in vivo* labeled CD45 in tryptic phosphopeptide maps. One of the *in vitro* phosphorylation sites of Csk was mapped to CD45 Tyr1193, in a highly conserved region of the second PTP domain, which is not a consensus GRB2 binding site. *In vitro* phosphorylation of this site by Csk leads to an increase in CD45 activity, and enables binding of the Src-family member Lck through its SH2 domain. Following treatment with phenylarsene oxide CD45 can be detected in Lck immunoprecipitates, indicating that CD45 and Lck may be associated under those conditions *in vivo*. Tyr789 in RPTPα is distinct from the tyrosine residue that is homologous to Tyr1193 in CD45 (RPTPα Tyr772). In addition, Tyr789 is the only detectable P.Tyr residue in RPTPα immunoprecipitated from NIH 3T3 cells, even following treatment with the PTP inhibitor sodium orthovanadate, indicating that the site equivalent to Tyr1193 of CD45 is not phosphorylated in RPTPα.

Association of RPTPα and GRB2 *in vivo* may imply a role for RPTPα in regulating GRB2-mediated signaling. To date, the only protein that has been found to associate with the SH3 domains of GRB2 *in vivo* is Sos, a guanine nucleotide releasing factor for the Ras GTPase (Pawson and Schlessinger, 1993 and references therein). GRB2 binds constitutively to Sos and this complex is translocated to receptor PTKs upon ligand-mediated activation. Sos is not phosphorylated on tyrosine upon receptor PTK activation. Serine/threonine phosphorylation of Sos is enhanced in response to receptor PTK activation, but so far these phosphorylation events have not been shown to modulate Sos activity. It has been speculated that translocation of the GRB2–Sos complex to activated receptor PTKs at the cell membrane is sufficient for activation of Ras (Pawson and Schlessinger, 1993). In contrast to Syp (Li *et al.*, 1994), we failed to detect Sos associated with tyrosine phosphorylated RPTPα. It is not clear why Sos is not associated with GRB2 bound to RPTPα, but this may be due to the fact that access to the SH3 domains is sterically hindered. Alternatively, the GRB2 SH3 domains may bind to RPTPα itself, or to other proteins, thereby stabilizing the complex. The absence of Sos means that GRB2 associated with RPTPα cannot activate the Ras pathway, and RPTPα might even play a negative role in Ras signaling by sequestering GRB2. However, other proteins present in the GRB2–RPTPα complex might have signaling functions. Several tyrosine phosphorylated proteins could be detected to associate with GRB2 *in vitro* in an SH2-independent manner, i.e. following pre-incubation with specific P.Tyr-containing phosphopeptides, or using mutant GRB2 (GRB2R86K) that is defective in binding to P.Tyr-containing proteins through its SH2 domain (J.den Hertog and T.Hunter, unpublished observation). Future work will lead to identification of additional proteins that associate with the GRB2 SH3 domains, and analysis of the tyrosine phosphorylation state of these proteins in cells expressing wild type and mutant, non-functional RPTPα, may reveal a role for RPTPα in attenuation of GRB2-mediated signaling.

Materials and methods

Cells, metabolic labeling and immunoprecipitation

NIH 3T3 cells and 293 human embryonic kidney cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%

bovine calf serum (Armour Pharmaceutical Corporation, Kankakee, IL). Metabolic labeling was done overnight either in phosphate-free medium supplemented with [^{32}P]orthophosphate (2.0 mCi/ml, ICN, Irvine, CA), or in methionine-free medium supplemented with [^{35}S]methionine/[^{35}S]cysteine (100 μCi/ml, Expre^{35}S^{35}S, NEN, Boston, MA).

Eighty to ninety per cent confluent cells were lysed in boiling lysis buffer (0.5% SDS, 10 mM sodium phosphate pH 7.2, 1 mM EDTA, 1 mM DTT, 10 U/ml aprotinin, 200 μM sodium orthovanadate), boiled for 5 min and diluted with RIPA buffer without SDS. Alternatively, the cells were lysed in non-denaturing lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 μM PMSF, 10 U/ml aprotinin, 200 μM sodium orthovanadate). The lysates were pre-cleared with protein A–Sepharose beads (Repligen, Cambridge, MA), and incubated with antisera [1–2 μg affinity purified anti-RPTPα antiserum 5478 per sample, 5 μl polyclonal anti-GRB2 rabbit antiserum, raised against a GST–GRB2 fusion protein containing the C-terminal SH3 domain (Ab 50, kind gift of Yossi Schlessinger), or 5 μl polyclonal anti-Sos rabbit antiserum, raised against a synthetic peptide spanning residues 1241–1260 in the C-terminal tail of mSos1 (kind gift of Tony Pawson)] for 30 min on ice, prior to incubation with protein A–Sepharose beads for 1.5 h at 4°C. The beads were washed four times, either with RIPA buffer or with HNTG (20 mM HEPES pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol), prior to resuspension in Laemmli sample buffer, boiling for 5 min and loading on SDS–polyacrylamide gels.

Rabbits were immunized with bacterially expressed cytoplasmic domain of RPTPα, expressed as a GST fusion protein, purified on glutathione–agarose beads and released from the GST moiety by thrombin cleavage. Analysis of thus obtained bPTPα by SDS–PAGE and Coomassie staining indicated that bPTPα was ~95% pure. Polyclonal antiserum (#5478) was purified on a GST–PTPα affinity column that was generated by cross-linking GST–PTPα to glutathione–agarose beads with dimethylpimelimidate. Binding of the pre-cleared antiserum to the column, and elution of the affinity-purified antiserum with 5 M NaI, 1 mM sodium thiosulfate was done as described by Koff *et al.* (1992).

GST fusion proteins

Expression vectors for bacterial expression of RPTPα GST fusion proteins were derived by insertion of PCR-generated *Nco*I–*Hin*dIII fragments into pGEX-KG opened with *Nco*I and *Hin*dIII (Guan and Dixon, 1991b). The oligonucleotides that were used for the generation of the deletion mutants of RPTPα are as follows. NI: 5′-CCATGGGGTT-TAAGAAATACAAGCAAGCT-3′; NII: 5′-GGGCCATGGCTGCCTC-CAAGGAAGAAAAC-3′; NIII: 5′-GGGCCATGGGAAACCTTCCAG-CCAACATG-3′; CI: 5′-CCCTCAAGCTTCCAGTTCTGTGTCCCCA-TA-3′; CII: 5′-AAGCTTAATTCTCCTGTCTGTGGGCCTTGTCA-3′.

bPTPα, as depicted in Figure 2A, was generated using oligonucleotides NI and CII, I+II using NII and CII, domain I using NII and CI, and domain II using NIII and CII.

GST–GRB2 contains full-length GRB2 (amino acids 2–217) fused to GST (Lowenstein *et al.*, 1992), and was a kind gift of Yossi Schlessinger. GST–Src-SH2 contains the SH2 domain of v-Src (amino acids 148–251), which is identical in sequence to the c-Src SH2 domain, fused to GST (Liu *et al.*, 1993), and was a kind gift of Tony Pawson.

The GST fusion protein constructs were transformed into BL21 bacteria for high level GST fusion protein expression. Overnight cultures were diluted 1:10 in fresh medium and after shaking for 1 h, isopropyl β-D-thiogalactopyranoside (IPTG, 100 μM final concentration) was added. After another 4 h shaking at 37°C the cells were lysed in PBS, 1% Triton X-100, 1 mg/ml lysozyme, 10 U/ml aprotinin by sonication and the supernatants were incubated with glutathione–agarose beads (Sigma, St Louis, MO) for 10 min at room temperature. After extensive washing in PBS with 1% Triton X-100, the GST fusion proteins were eluted with 5 mM reduced glutathione for 10 min at room temperature.

In vitro kinase assays and phosphopeptide mapping analysis

Phosphorylation of the fusion proteins (100 ng) and the synthetic peptide (1 μg) was done in kinase buffer (20 mM HEPES pH 7.4, 5 mM $MnCl_2$, 1 mM DTT) using immunoprecipitated, baculovirus-expressed c-Src (40–60 ng per sample, kind gift of Martin Broome) and 25–50 μCi [γ-^{32}P]ATP (Amersham, Arlington Heights, IL) at 30°C for 15 min.

Phosphopeptide mapping and phosphoamino acid analysis were done exactly as described (Boyle *et al.*, 1991). ^{32}P-labeled proteins were eluted from the gel, precipitated with trichloroacetic acid, oxidized with performic acid and digested with TPCK-trypsin. The samples were loaded onto TLC plates and electrophoresed in pH 1.9 buffer (1.5 kV, 30 min, first dimension), and separated in the second dimension by ascending chromatography in P.Chromo buffer (Boyle *et al.*, 1991). Phosphopeptides were detected by autoradiography. Individual phosphopeptides were isolated from the TLC plates for phosphoamino acid analysis and secondary digestion by scraping the spots off the TLC plates, and elution of the phosphopeptides in 20% acetonitrile, 0.08% trifluoroacetic acid. These samples were lyophilized and subjected to acid hydrolysis in 6 M HCl at 110°C for 1 h, or digestion by thermolysin (2× 1 μg of thermolysin per sample for 1 h at 55°C in the presence of 1 mM $CaCl_2$). Phosphoamino acids were separated by two-dimensional TLC, electrophoresis in pH 1.9 buffer (1.5 kV, 25 min) and pH 3.5 buffer (1.3 kV, 16 min), for the first and second dimension, respectively. The thermolysin-digested samples were fractionated on TLC plates using conditions as described for the tryptic phosphopeptide maps.

Plasmids, site-directed mutagenesis and transfections

An SV40 driven expression vector, pSG5 (Green *et al.*, 1988), for transient expression of RPTPα in 293 cells has been described (den Hertog *et al.*, 1993). CMV promoter driven expression vectors for c-Src and the mutants c-SrcK297M and c-SrcY529F were derived by insertion of a *Bam*HI fragment, containing the full-length cDNA of murine c-*src*, or its mutants, into pSLX (kind gift of Martin Broome). Site-directed mutagenesis was done on the complete cDNA of RPTPα in pBluescript SK–. Mutations were verified by sequencing, and subsequently a 1.1 kb *Bam*HI fragment containing the mutation was inserted in pSGRPTPα that had been digested with *Bam*HI. Alternatively, for mutation of the GST fusion proteins, PCR was performed using mutant RPTPα as template, followed by insertion of the *Nco*I–*Hin*dIII PCR products into pGEX-KG. The oligonucleotides that were used for site-directed mutagenesis are as follows. C433A: 5′-CTACACCTGCACTGGCG-TGGACCACGATAG; C723A: 5′-CTGCCCCGGCACTGGCGTGC-ACAGTGATGG; Y782F: 5′-GGCGTCAATGAATTCCTGTACCAC; Y789F: 5′-CTTGAAGTTGGCAAAATCTGAAAAGGC.

Transient transfection of 293 cells was done using a calcium phosphate precipitation method, as described (den Hertog *et al.*, 1993). Briefly, 10 μg plasmid DNA was mixed with 200 μl 2 × HBSP (42 mM HEPES, 275 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 10 mM dextrose, pH 7.05), and 200 μl 250 mM $CaCl_2$, and left for 25 min at 25°C. The precipitate was resuspended and added directly to the medium on the cells. After incubation overnight, the medium was changed and another 24 h later the cells were harvested.

Autodephosphorylation of bPTPα in vitro

Bacterially expressed non-functional mutant of RPTPα (bPTPαC433A/C723A) (100 ng) was phosphorylated by immunoprecipitated, baculovirus expressed c-Src with [γ-^{32}P]ATP, as described above. Phosphorylated bPTPαC433A/C723A was separated from free [γ-^{32}P]ATP by gel filtration on a Sephadex G-50 spin column, equilibrated in 20 mM MES pH 6.0. ^{32}P-labeled bPTPαC433A/C723A (10 000 c.p.m./sample) was incubated with 100 ng bacterially expressed GST, wild type bPTPα, bPTPαC433A, bPTPαC723A or bPTPαC433A/C723A at 30°C in 20 mM MES containing 0.2% β-mercaptoethanol (100 μl final volume). Aliquots (20 μl) were taken after 0–30 min and resolved on a 10% SDS–polyacrylamide gel.

In vitro association and immunoblotting analysis

GST fusion proteins (GST alone, GST–GRB2 or GST–Src-SH2) were expressed in bacteria and purified using glutathione–agarose beads as described above. Nearly confluent cells were lysed in non-denaturing cell lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 10 U/ml aprotinin, 1 μM PMSF, 200 μM sodium orthovanadate). The lysates were pre-cleared by rotation with glutathione–agarose beads, and incubated for 1.5–2 h at 4°C with GST fusion proteins (2–5 μg per sample) that were immobilized on glutathione–agarose beads. The beads were washed four times with HNTG, resuspended in Laemmli sample buffer and boiled for 5 min, and the supernatants were loaded onto SDS–polyacrylamide gels. For some experiments the GST–GRB2 beads were pre-incubated with a peptide or phosphopeptides (50 μM, final concentration). The (phospho)-peptides that were used are: RPTPα residues 776–793, CYKVVQEYID-AFSDYANFK, with an additional cysteine at its N-terminus, RPTPα 782–793 containing P.Tyr789, YIDAFSDpYANFK (where pY denotes P.Tyr), CSF-1-R residues 691–703 containing P.Tyr697, YSEGDSSpYK-NIHLE and CSF-1-R residues 700–712 containing P.Tyr706, YIH-LEKKpYVRRDSG, both with an additional tyrosine residue at their N-terminus.

For immunoblotting analysis, polyacrylamide gels were transferred to

Immobilon (Millipore, Bedford, MA) by semi-dry blotting for 45 min at 200 mA, exactly as described (Kyhse-Anderson, 1984). Following transfer the filters were incubated in blocking solution (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.2% Tween-20, 5% non-fat milk, for 1 h at 25°C), and in blocking solution containing affinity purified anti-RPTPα antiserum 5478 (0.1 μg/ml, 1 h 25°C, or overnight at 4°C). The filters were washed four times in TBS-T (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20), and incubated for 1 h at 25°C either with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibodies or HRP-conjugated protein A in TBS-T for enhanced chemiluminescence (ECL) detection, or with ^{125}I-labeled protein A (0.4 μCi/ml) in blocking solution. Finally, the blots were washed four times in TBS-T. ECL was done exactly as described by the manufacturer (Amersham, Arlington Heights, IL).

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Recently, Sap and co-workers have found that RPTPα is phosphorylated on tyrosine and can bind to GRB2 [J.Su, A.Batzer and J.Sap (1994) *J. Biol. Chem.*, in press].