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# Tight association of GRB2 with receptor protein-tyrosine phosphatase α is mediated by the SH2 and C-terminal SH3 domains

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**Receptor protein-tyrosine phosphatase α (RPTPα), a transmembrane member of the extensive family of protein-tyrosine phosphatases (PTPs), is constitutively phosphorylated on Tyr789, a consensus binding site for the SH2 domain of the SH3–SH2–SH3 adaptor protein GRB2. We have previously shown that GRB2 binds to P.Tyr789 *in vivo* and *in vitro* via its SH2 domain. Here, we report that not only the GRB2 SH2 domain, but also the C-terminal SH3 domain is involved in binding to RPTPα *in vitro* and *in vivo*. Although the N-terminal SH3 domain of GRB2 is essential for binding to the Ras guanine nucleotide exchange factor Son of Sevenless (Sos), an RPTPα–GRB2–Sos complex could not be detected. The inclusion of peptides encompassing an hSos1 proline-rich motif in cell lysates resulted in enhanced binding of RPTPα to GRB2 *in vitro*, suggesting that steric hindrance prohibits formation of the RPTPα–GRB2–Sos complex. *In vitro* binding experiments indicated that the binding of GRB2 to Sos/dynamin and RPTPα was mutually exclusive. Analysis of *in vitro* binding kinetics coupled with results from transient co-transfections demonstrated that RPTPα is tightly bound to GRB2. The site of interaction of the C-terminal SH3 domain of GRB2 with RPTPα was mapped using deletion mutants to an 18-residue region in the N-terminal PTP domain. Arg469, within this region, was identified as one of the residues that is involved in the interaction with the C-terminal SH3 domain of GRB2. RPTPα residues 469–486 are localized close to the catalytic site cleft in the structure of the N-terminal PTP-domain, suggesting that interaction with the C-terminal SH3 domain may block access to the catalytic site, thus inhibiting RPTPα activity.**

*Keywords*: GRB2/protein-tyrosine phosphatase/RPTPα/SH2/SH3

## Introduction

Tyrosine phosphorylation, one of the main signalling mechanisms of eukaryotic cells, is regulated by the antagonistic activities of protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). Much emphasis has been placed on elucidation of signalling by (receptor) PTKs leading to identification of signal transduction cascades from the plasma membrane to the nucleus (Schlessinger and Ullrich, 1992; Pawson and Schlessinger, 1993; Pawson, 1995). One of the key players in such a signalling cascade is an adaptor protein, consisting of a central Src-homology 2 (SH2) domain flanked by two Src-homology 3 (SH3) domains, known as GRB2, Drk or Sem-5. Mammalian GRB2, *Drosophila* Drk and *Caenorhabditis elegans* Sem-5 are homologous adaptor proteins that bind to phosphotyrosine (P.Tyr) in activated autophosphorylated PTKs through their SH2 domain and to the Ras guanine nucleotide exchange factor, Son of Sevenless (Sos), through their SH3 domains (Clark *et al.*, 1992; Lowenstein *et al.*, 1992; Olivier *et al.*, 1993). Formation of an activated PTK–GRB2–Sos complex at the plasma membrane leads to activation of Ras into its GTP-bound state, thereby triggering downstream signal transduction pathways (Lowenstein *et al.*, 1992; Buday and Downward, 1993; Gale *et al.*, 1993).

Relatively little is known about the role of the enzymatic counterpart of PTKs, PTPs, in eukaryotic cell signalling. Like the PTKs, the PTPs constitute a large family of cytoplasmic and transmembrane PTPs (for reviews see Fischer *et al.*, 1991; Charbonneau and Tonks, 1992; Walton and Dixon, 1993). The transmembrane PTPs are tentatively called receptor PTPs (RPTPs), since they have the potential to transfer signals across the plasma membrane due to their topology. The extracellular domains of RPTPs are diverse, ranging from very large with multiple fibronectin type III-like and immunoglobulin repeats [e.g. LAR (Streuli *et al.*, 1989)] to very short [e.g. PTPε (Krueger *et al.*, 1990)]. The extracellular domains of RPTPμ and the highly homologous RPTPκ engage in homophilic interactions when expressed at high levels on adjacent cells (Brady-Kalnay *et al.*, 1993; Gebbink *et al.*, 1993; Sap *et al.*, 1994). Recently, it has been demonstrated that the carbonic anhydrase domain in the extracellular domain of RPTPβ is a functional ligand of contactin (Peles *et al.*, 1995). Whether binding of ligands to the extracellular domain of these PTPs modulates intracellular PTP activity remains to be determined.

Receptor Protein-Tyrosine Phosphatase α (RPTPα) is a transmembrane PTP with a relatively short, heavily glycosylated extracellular domain (123 aa) and a tandem repeat of two cytoplasmic PTP domains (Matthews *et al.*, 1990; Sap *et al.*, 1990; Daum *et al.*, 1994). The N-terminal PTP domain exhibits the majority of the PTP activity towards exogenous P.Tyr-containing substrates, while the C-terminal PTP domain possesses detectable, but low PTP activity (Wang and Pallen, 1991; den Hertog *et al.*, 1994). Previously, we have shown that RPTPα is phosphorylated on tyrosine and serine residues (den Hertog *et al.*, 1994, 1995; Traçy *et al.*, 1995). Phosphorylation of two serine residues in the juxtamembrane domain is enhanced in response to activation of protein kinase C, leading to an increase in RPTPα activity (den Hertog *et al.*, 1995; Tracy

*et al.*, 1995). The tyrosine phosphorylation site in RPTPα is localized at Tyr789, five residues from the C-terminus. Src phosphorylates this site *in vitro* and co-transfection of Src together with RPTPα leads to enhanced RPTPα Tyr789 phosphorylation indicating that Src may also be the kinase that phosphorylates RPTPα *in vivo* (den Hertog *et al.*, 1994). The sequence to the C-terminal side of RPTPα Tyr789 indicated that this was a consensus binding site for the SH3–SH2–SH3 adaptor protein GRB2. We and others have demonstrated that RPTPα binds to GRB2 (den Hertog *et al.*, 1994; Su *et al.*, 1994). Tyrosine phosphorylation is essential for GRB2 binding to RPTPα, but the function of the GRB2–RPTPα complex remains to be elucidated, since we were unable to detect Sos in RPTPα immunoprecipitates or vice versa (den Hertog *et al.*, 1994).

Here, we report that *in vitro* and *in vivo* binding of GRB2 to RPTPα was not only dependent on the GRB2 SH2 domain, but also on the C-terminal SH3 domain. *In vitro* RPTPα and Sos/dynamin binding to GRB2 was mutually exclusive and it appeared that Sos bound with higher affinity than RPTPα. On the other hand, *in vivo* RPTPα was tightly bound to endogenous GRB2; the tight binding is likely due to the fact that both the SH2 and C-terminal SH3 domains of GRB2 are involved in binding to RPTPα. A region necessary for the interaction with the C-terminal SH3 domain of GRB2 was mapped to RPTPα residues 469–486 using deletion mutants. We identified Arg469 as one of the residues that is involved in the interaction with the C-terminal SH3 domain. RPTPα residues 469–486 do not contain proline residues, and the location of this region in the structure of the N-terminal PTP domain suggests that binding of the C-terminal SH3 domain may block access to the catalytic site, thus inhibiting RPTPα activity.

## Results

### *GRB2 binding to RPTPα is dependent on the SH2 and C-terminal SH3 domains*

RPTPα is phosphorylated on Tyr789, five residues from its C-terminus, a consensus binding site for the SH2 domain of GRB2. GRB2 binds to RPTPα *in vivo* and *in vitro*, and tyrosine phosphorylation is essential for this interaction (den Hertog *et al.*, 1994; Su *et al.*, 1994). Using mutants of GRB2, we investigated the involvement of the SH3 domains of GRB2 in RPTPα binding. The SH3 domains were expressed individually as glutathione-*S*-transferase (GST) fusion proteins in bacteria. In addition, loss-of-function point mutations in the SH3 domains (proline to leucine) and in the SH2 domain (arginine to lysine) were made in full-length GRB2. The GRB2 mutants (P49L, P206L, P49LP206L and R86K), were expressed in bacteria as GST fusion proteins and immobilized on glutathione–agarose beads (as shown in Figure 1C, equal amounts of all the fusion proteins were used). These beads were incubated with lysates of NIH 3T3 cells and following extensive washing of the beads binding of RPTPα and Sos was analysed by immunoblotting. As shown in Figure 1A, the individual SH3 domains of GRB2 did not bind RPTPα. Full-length GRB2 bound RPTPα, and mutation of the N-terminal SH3 domain (P49L) did not affect RPTPα binding; however, mutation of the C-terminal SH3 domain (P206L) almost completely abolished RPTPα binding. Mutation of both SH3 domains (P49LP206L) resulted in slightly enhanced binding of RPTPα as compared with mutation of the C-terminal SH3 domain alone. Mutation of the GRB2 SH2 domain (R86K) greatly reduced RPTPα binding (Figure 1A). The individual SH3 domains both bound Sos (Figure 1B). However, Sos binding by the N-terminal SH3 mutant (P49L) GRB2 was hardly detectable. Mutation of the C-terminal SH3 domain or the SH2 domain did not affect Sos binding significantly. These results demonstrate that the SH2 and C-terminal SH3 domains of GRB2 are involved in RPTPα binding *in vitro*. The N-terminal SH3 domain is essential for Sos binding, while the C-terminal SH3 domain can bind Sos weakly.

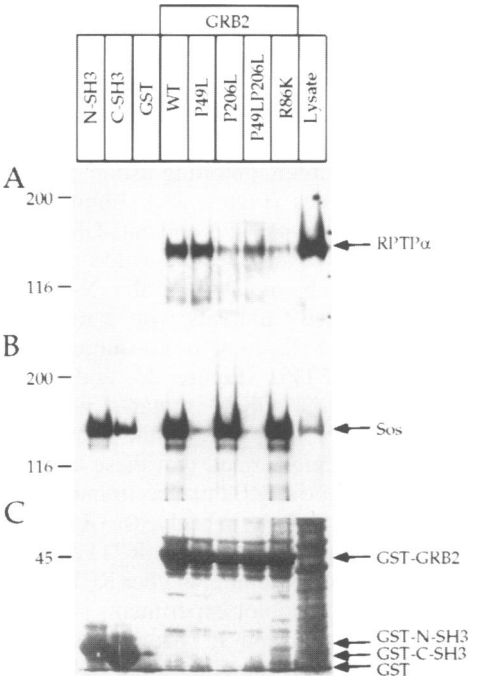


**Fig. 1.** Binding of GRB2 to RPTPα is dependent on the SH2 and C-terminal SH3 domain. Nearly confluent NIH 3T3 cells were lysed in 1% Triton X-100 buffer. The lysates were incubated with immobilized GST fusion proteins for 3 h at 4°C, or an aliquot of the lysate was run on the gel directly (Lysate). The GST fusion proteins encoded: the N-terminal SH3 domain alone (N-SH3), the C-terminal SH3 domain alone (C-SH3), GST, full-length wild-type GRB2 (WT), and mutants of full-length GRB2 with mutations in the N-terminal SH3 domain (P49L), the C-terminal SH3 domain (P206L), both SH3 domains (P49LP206L) or the SH2 domain (R86K). After extensive washing the samples were boiled in reducing sample buffer and the samples were run on a 10% SDS–polyacrylamide gel. Transfer of the gel and immunoblotting using HRP-conjugated protein A and enhanced chemiluminescence (ECL) was performed as described in Materials and methods. The blots were probed with anti-RPTPα antiserum 5478 (**A**). Subsequently, the filters were stripped and reprobed with anti-Sos antiserum (**B**). Immunoblots are shown with the molecular weights of markers that were co-electrophoresed with the samples indicated in kDa on the left. The positions of RPTPα and Sos are indicated. Before probing with anti-RPTPα antibodies the blot was stained using Coomassie blue to monitor equal loading of the fusion proteins. The stained blot is depicted with the positions of the fusion proteins indicated (**C**).

The involvement of the C-terminal SH3 domain in *in vivo* binding to RPTPα was investigated by co-immunoprecipitation assays using 293 cells transiently co-transfected with epitope-tagged RPTPα, activated Src

(SrcY529F) and wild-type or mutant GRB2. The haemagglutinin (HA) epitope tag was inserted in the extracellular domain of RPTPα immediately following the signal peptide. The HA-tagged RPTPα construct was tested by immunoprecipitation using anti-HA epitope tag antibodies (MAb 12CA5) and immunoblotting using affinity-purified anti-RPTPα antibodies (Figure 2A). Immunoblotting of parallel blots using anti-RPTPα and anti-GRB2 antibodies demonstrated that co-transfected wild-type GRB2 or GRB2P49L, which is mutated in the N-terminal SH3 domain, but not GRB2 mutants with a mutation in the C-terminal SH3 or SH2 domain, co-immunoprecipitated with HA-tagged RPTPα (Figure 2A and B), although equal amounts of the GRB2 mutants were expressed (Figure 2C). Longer exposure of the blots depicted in Figure 2B and C demonstrated that these cells expressed endogenous (wild-type) GRB2 that co-immunoprecipitated with epitope-tagged RPTPα in each case (data not shown). As a control, when epitope-tagged RPTPα was omitted from the transfection procedure, neither RPTPα nor GRB2 was detected in immunoblot experiments similar to those depicted in Figure 2A and B (data not shown). These results demonstrate that the C-terminal SH3 domain and the SH2 domain are both required for efficient interaction of GRB2 with RPTPα *in vivo*.

Previously, we have demonstrated that RPTPα has autodephosphorylation activity. In addition, phosphorylation of Tyr789, a consensus GRB2 SH2 binding site, is essential for GRB2 binding (den Hertog *et al.*, 1994), suggesting that binding of the GRB2 SH2 domain to P.Tyr789 is direct. RPTPα Tyr789 phosphorylation is a measure for GRB2 binding *in vivo*, since binding of the GRB2 SH2 domain to RPTPα P.Tyr789 protects this site from (auto)dephosphorylation. Therefore, as an independent means of determining the requirements for GRB2 binding to RPTPα *in vivo*, we investigated the extent of Tyr789 phosphorylation of RPTPα in transiently transfected 293 cells, expressing RPTPα and activated c-Src together with wild-type or mutant GRB2. Co-transfection of wild-type GRB2 resulted in a 2.5-fold increase in RPTPα Tyr789 phosphorylation, as determined by quantitative analysis of the tryptic phosphopeptide maps (Figure 3A, B and G). Co-transfection of the N-terminal SH3 mutant also led to an increase in Tyr789 phosphorylation (Figure 3C and G), while co-transfection of the C-terminal SH3 mutant did not change RPTPα Tyr789 phosphorylation significantly (Figure 3D and G). A slight increase in Tyr789 phosphorylation was observed upon co-transfection of the double SH3 mutant (Figure 3E and G). The SH2 mutant, which cannot bind P.Tyr, did not affect Tyr789 phosphorylation significantly (Figure 3F and G). In conclusion, the *in vitro* binding assays (Figure 1) and *in vivo* co-immunoprecipitation experiments (Figure 2), as well as the *in vivo* Tyr789 phosphorylation assays (Figure 3), demonstrate that the SH2 and C-terminal SH3 domains of GRB2 are involved in binding to RPTPα.

### *RPTPα is not present in the Sos–GRB2 complex*

As shown above, the C-terminal SH3 domain and the SH2 domain of GRB2 are involved in binding to RPTPα, but the N-terminal SH3 domain is not. Therefore, it is conceivable that the N-terminal SH3 domain is available for binding to Sos. However, previously we have shown

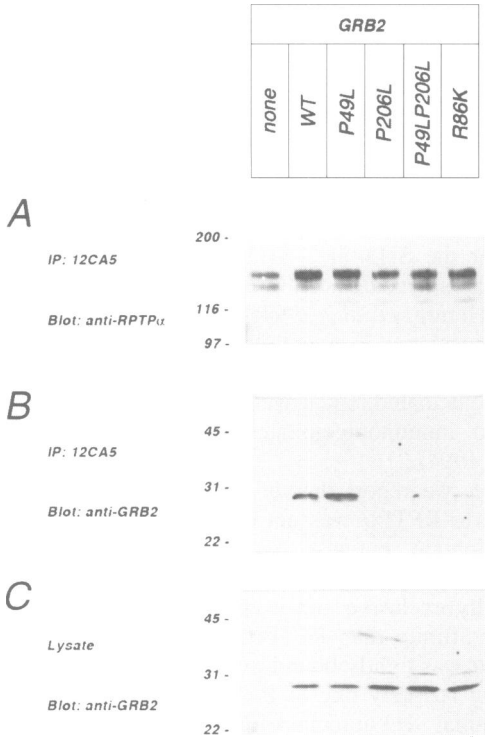


**Fig. 2.** The GRB2 C-terminal SH3 domain is involved in RPTPα binding *in vivo*. Human embryonic kidney cells (293 cells) were transiently transfected with activated murine c-Src (SrcY529F) and epitope-tagged RPTPα. Wild-type GRB2 and mutants thereof were co-transfected as indicated. The cells were lysed using cell lysis buffer containing 1% Triton X-100 and immunoprecipitations were performed using an anti-haemagglutinin epitope tag antibody (MAb 12CA5). Transfer of the gels and immunoblotting using HRP-conjugated protein A and ECL were as described in Materials and methods. Immunoblots are depicted of parallel immunoprecipitations using the same cell lysates of a 10% polyacrylamide gel: (**A**) probed with anti-RPTPα antibodies; and (**B**) on a 15% polyacrylamide gel, probed with anti-GRB2 antibodies. (**C**) An aliquot of the lysate was run directly on a 15% polyacrylamide gel to monitor expression of the GRB2 mutants. The molecular weights of marker proteins that were co-electrophoresed with the samples are indicated in kDa on the left.

that RPTPα did not co-immunoprecipitate with Sos or vice versa (den Hertog *et al.*, 1994). To investigate this further, we used a GST fusion protein encoding the region of hSos1 that contains the proline-rich GRB2 SH3 binding sites (residues 1135–1245), and tested whether RPTPα bound to GRB2–Sos complexes formed in lysates of NIH 3T3 cells or transiently transfected 293 cells. As shown in Figure 4A, RPTPα was not detectable in the GST–Sos sample, even though large amounts of GST–Sos (up to 10 μg) were used, which resulted in the binding of almost all the GRB2 from the lysate (Figure 4B). Co-transfection of RPTPα together with GRB2 into 293 cells and subsequent assay of binding of the GRB2–RPTPα complex to GST–Sos still did not result in detectable levels of bound RPTPα (data not shown). These results indicate that the RPTPα–GRB2 and GRB2–Sos complexes are mutually exclusive.

### *Enhanced RPTPα binding to GST–GRB2 in response to proline-rich peptides*

Since the GRB2 C-terminal SH3 domain is involved in RPTPα binding, we investigated the ability of proline-

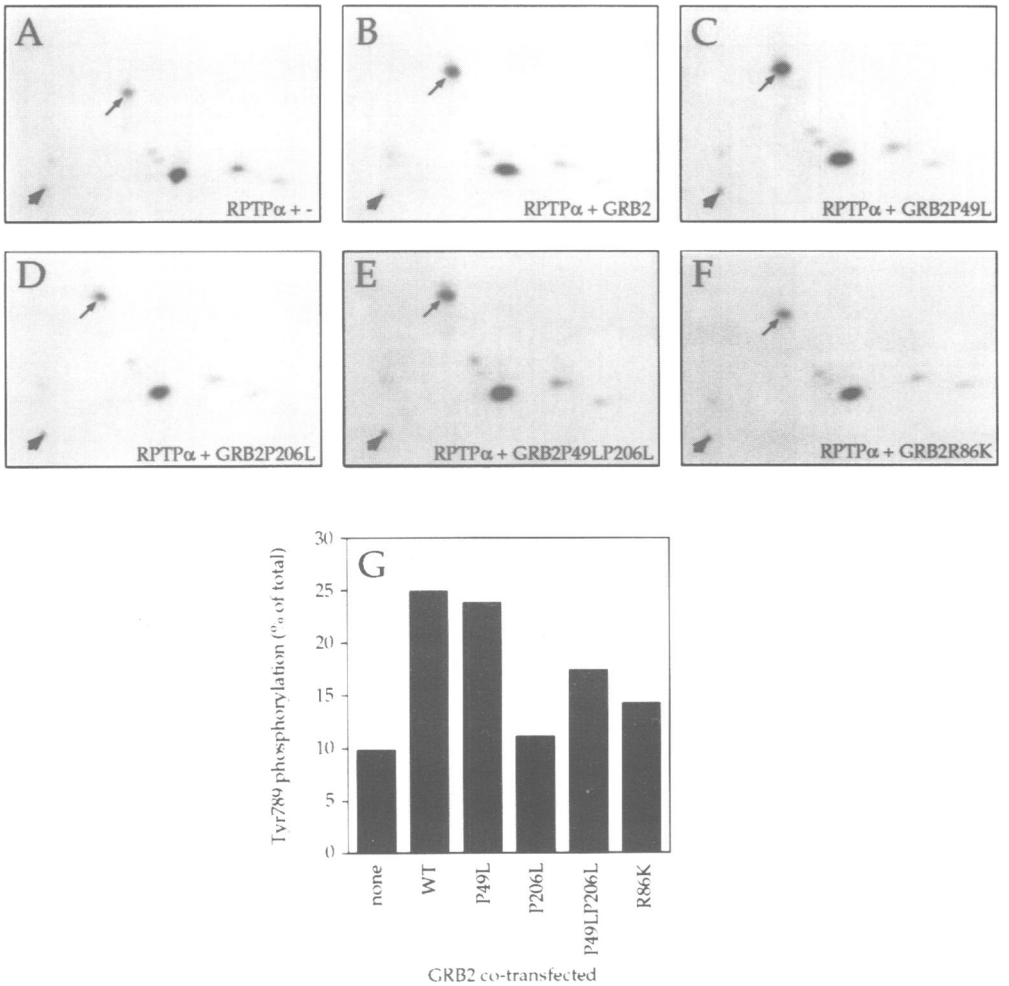


**Fig. 3.** The GRB2 SH2 and C-terminal SH3 domain are required for efficient binding to RPTPα *in vivo*. Transiently transfected 293 human embryonic kidney cells, expressing RPTPα, activated c-Src and (mutant) GRB2 were labelled overnight with [$^{32}$P]orthophosphate (2 mCi/ml). RPTPα was immunoprecipitated using affinity-purified anti-RPTPα antiserum 5478. The immunoprecipitates were fractionated on a 7.5% SDS–polyacrylamide gel and the 130 kDa RPTPα was visualized by autoradiography. $^{32}$P-labelled RPTPα was eluted from the dried gel, digested with trypsin, and the tryptic phosphopeptides were separated in two dimensions on cellulose TLC plates by electrophoresis at pH 1.9 and ascending chromatography. Autoradiographs are depicted with the anode at the left, chromatography in the vertical direction and with a short arrow marking the origin. The cells were co-transfected with RPTPα and activated murine Src (Src-Y529F) alone (**A**) or with RPTPα and Src-Y529F together with wild-type GRB2 (**B**), or with mutants of GRB2, GRB2P49L (**C**), GRB2P206L (**D**), GRB2P49LP206L (**E**) or GRB2R86K (**F**). A long arrow indicates the position of the phosphopeptide that contains RPTPα P.Tyr789. The amount of radioactivity in the different phosphopeptides was quantified using a PhosphorImager. (**G**) Relative Tyr789 phosphorylation depicted as a percentage of total phosphorylation.

rich peptides that bind to the SH3 domain(s) of GRB2 to block binding of RPTPα to GRB2. The peptides that were used were: hSos1: PVPPPVPPRRRP (hSos1 1148–1159) and, as a control, an RPTPα peptide: CYKVVQEYIDAFS-DYANFK (RPTPα 775–793). Increasing concentrations of peptide (0–0.5 mM) were added to NIH 3T3 lysates immediately before incubation with immobilized GST–GRB2. Subsequently, the binding of RPTPα or Sos to GST–GRB2 agarose beads was analysed by immunoblotting. Surprisingly, the hSos1 peptide caused an increase in RPTPα binding to GST–GRB2 in a concentration-dependent manner with detectable effects at 0.05 mM (Figure 5A). The proline-rich hSos1 peptide competed for binding of Sos to GST–GRB2, and at the highest peptide concentration (0.5 mM) no Sos binding was detectable (Figure 5B). The control RPTPα peptide had no effect on RPTPα or Sos binding to GST–GRB2. Coomassie blue staining of the blots revealed an ~100 kDa protein that apparently bound to GST–GRB2 and was competed by the hSos1 peptide (Figure 5C). Affinity purification and protein sequencing of this 100 kDa band demonstrated that it was dynamin, a protein that has previously been identified to bind to the SH3 domains of GRB2 (Gout *et al.*, 1993; Ando *et al.*, 1994). *In vitro* binding experiments using mutants of GRB2 indicated that the 100 kDa dynamin bound specifically to the SH3 domains of GRB2 (data not shown). Binding of dynamin to GST–GRB2 was efficiently blocked by high concentrations of the proline-rich peptides, but not by the control RPTPα peptide (Figure 5C). Experiments, similar to that depicted in Figure 5, using bacterially expressed Sos (residues 1135–1245) or other proline-rich peptides (PPPLPPPRRR and RHYRPLPPLP) also resulted in enhanced RPTPα binding and reduced Sos binding to GST–GRB2 (data not shown).

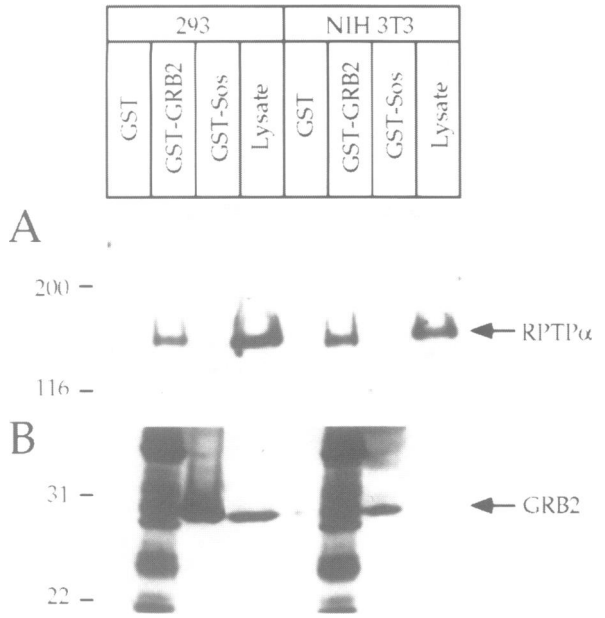


**Fig. 4.** RPTPα does not bind to Sos. Transiently transfected 293 human embryonic kidney cells, expressing RPTPα, or NIH 3T3 cells were lysed in 1% Triton X-100 lysis buffer. The lysates were incubated with GST fusion proteins immobilized on glutathione–agarose beads. An aliquot of the lysate was loaded directly on the gel. The GST fusion proteins encoded GST alone, GST–GRB2 (full-length) or GST–Sos (hSos1 residues 1135–1245, containing the proline-rich SH3 binding sites). Following extensive washing the samples were boiled in denaturing sample buffer and run on a 15% SDS–polyacrylamide gel. The gel was blotted onto Immobilon and the top of the filter probed with anti-RPTPα antiserum 5478 (**A**), while the bottom of the blot was probed with anti-GRB2 antiserum (**B**). Immunoblots are depicted with the molecular weights (in kDa) of marker proteins that were co-electrophoresed with the samples on the left. The positions of RPTPα and GRB2 are indicated.

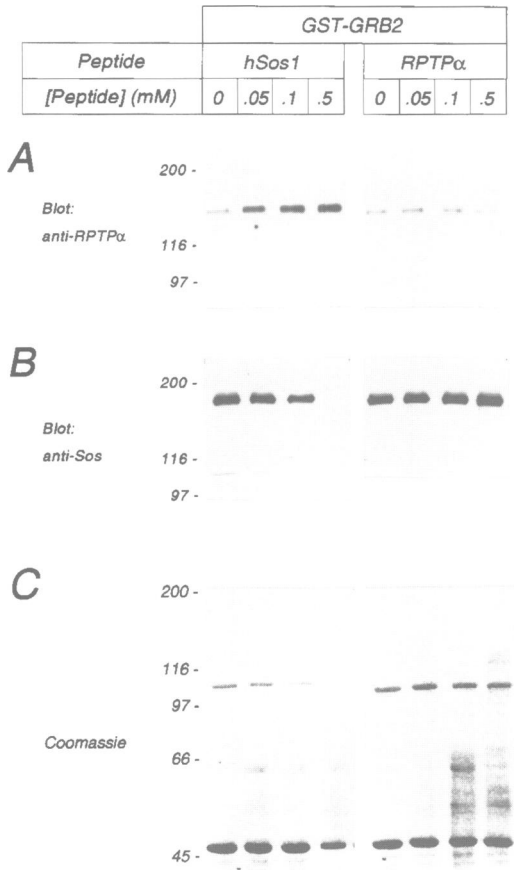


**Fig. 5.** A proline-rich peptide that contains an SH3 binding site enhances RPTPα binding to GRB2. Nearly confluent NIH 3T3 cells were lysed in 1% Triton X-100 lysis buffer. The lysates were incubated with GST–GRB2 immobilized on glutathione–agarose beads for 3 h at 4°C in the presence of varying amounts of peptide as indicated. The peptides that were used are: hSos1: PVPPPVPPRRRP (hSos1 1148–1159) and, as a control, an RPTPα peptide: CYKVVQEYIDAFSDYANFK (RPTPα 775–793). Following extensive washing the samples were boiled in denaturing sample buffer and run on a 10% SDS–polyacrylamide gel. Transfer of the material on the gel, staining of the blots with Coomassie blue, immunoblotting and stripping of the blots was as described in Materials and methods. Depicted are immunoblots using anti-RPTPα antiserum (**A**), anti-Sos antiserum (**B**) and Coomassie blue staining (**C**). Molecular weights (in kDa) of marker proteins that were co-electrophoresed with the samples are indicated on the left. The 100 kDa Coomassie blue-stained protein in (C) binds specifically to the SH3 domains of GRB2 and was identified as dynamin.

These results demonstrate that proline-rich peptides block binding of Sos and dynamin and enhance binding of RPTPα to GST–GRB2.

### *Differential binding of RPTPα and Sos to GRB2*

Since both the C-terminal SH3 domain as well as the SH2 domain of GRB2 are involved in RPTPα binding, we were interested to determine whether these interactions would be reflected in the relative binding affinity. Increasing amounts of GST–GRB2 were incubated with lysates of NIH 3T3 cells and binding of P.Tyr-containing proteins, RPTPα and Sos was determined by immunoblotting. P.Tyr-containing proteins were already detectable with the lowest amount of GST–GRB2 used (1 μg) (Figure 6A). In general, the levels of the different P.Tyr-containing protein bands increased with the amount of GST–GRB2, but even at the highest amount (18 μg) the binding of certain P.Tyr-containing proteins was not maximal. RPTPα binding was not detectable with 1–4 μg of GST–GRB2 (Figure 6B), but was observed with 6 μg and was maximal with 8 μg, indicating that all P.Tyr789-containing RPTPα was bound to GST–GRB2. In contrast, Sos binding was detectable with 1 μg GST–GRB2, and maximal with only 4 μg (Figure 6C), reflecting depletion of Sos from the lysate. Analysis of the lysates following incubation with GST–GRB2 confirmed that all Sos had bound to GST–GRB2 (data not shown). Coomassie blue staining of the blots revealed that the ~100 kDa dynamin band was detected with 2 μg GST–GRB2, and its binding was maximal with 4 μg (Figure 6D). The fact that RPTPα binding was observed only using higher amounts of GST–GRB2 might suggest that its binding affinity is relatively low as compared with Sos and dynamin binding, but this is not the case (see below). Since Sos and dynamin were depleted from the lysate before RPTPα binding was detectable (using 6 μg GST–GRB2), and since Sos and RPTPα binding are mutually exclusive (Figure 4), apparently RPTPα can only bind to GST–GRB2 when there is no free Sos or dynamin left.

### *Tight binding of RPTPα by GRB2*

The time-dependence of RPTPα, Sos and dynamin binding to GST–GRB2 was determined by incubation of NIH 3T3

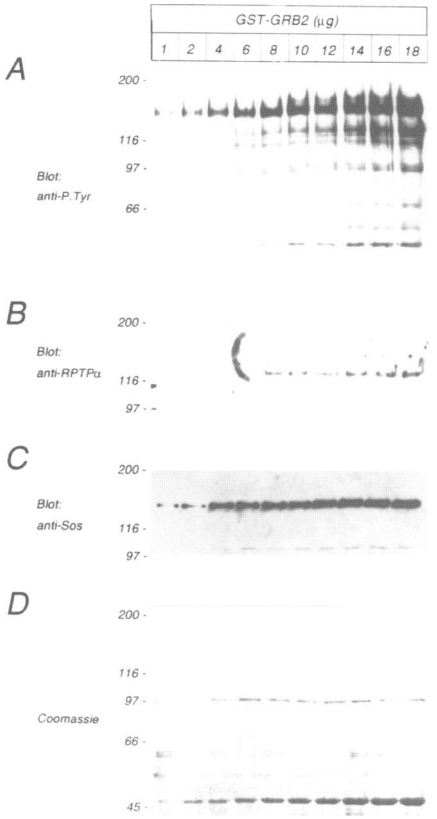


**Fig. 6.** Differential binding of P.Tyr-containing proteins, RPTPα, Sos and dynamin to GST–GRB2. NIH 3T3 cells were lysed in 1% Triton X-100 lysis buffer. The lysates were incubated with varying amounts of immobilized GST–GRB2 as indicated for 3 h at 4°C. Following extensive washing of the beads the samples were boiled in denaturing sample buffer and run on a 10% SDS–polyacrylamide gel. After transfer of the gel, the blot was stained using Coomassie blue and the blot subsequently probed sequentially with antibodies as indicated. Immunoblots are shown using anti-P.Tyr antibody PY-20 (**A**), anti-RPTPα antiserum 5478 (**B**) and anti-Sos antiserum (**C**). The Coomassie blue-stained blots are depicted in (**D**). The molecular weights (in kDa) of marker proteins are indicated on the left.

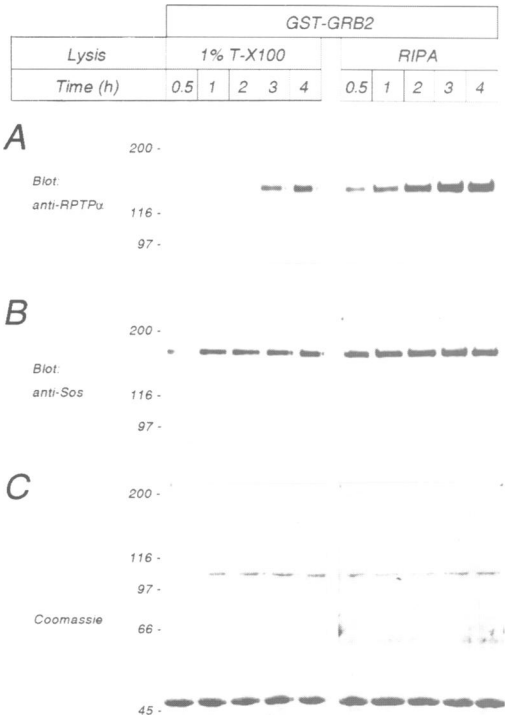


**Fig. 7.** Time-course of RPTPα binding to GST–GRB2. NIH 3T3 cells were lysed in 1% Triton X-100 buffer or in RIPA buffer as indicated. The lysates were incubated with immobilized GST–GRB2 for the time indicated at 4°C. Following extensive washing the samples were boiled in denaturing sample buffer and run on a 10% SDS–polyacrylamide gel. The material on the gel was transferred to Immobilon and the blot stained with Coomassie blue to monitor equal loading of GST–GRB2. Immunoblots are shown, probed sequentially with anti-RPTPα antiserum 5478 (**A**) and anti-Sos antiserum (**B**). The Coomassie blue-stained blot is depicted in (**C**). Molecular weights (in kDa) of marker proteins that were co-electrophoresed with the samples are indicated on the left.

lysates with GST–GRB2, immobilized on glutathione–agarose beads, for increasing periods of time. Under mild lysis conditions (1% Triton X-100) RPTPα binding to GST–GRB2 was detectable after 2 h incubation with GST–GRB2 beads (Figure 7A), while Sos and dynamin binding were detectable after 0.5 h, and reached maximal levels after 1 h (Figure 7B and C). More stringent lysis conditions (RIPA buffer) resulted in more rapid binding of RPTPα to GST–GRB2 with detectable levels after 0.5 h and maximal levels after 2 h (Figure 7A). In addition, more rapid Sos and dynamin binding was observed using RIPA lysates, as compared with 1% Triton X-100 lysates (Figure 7B and C). It is noteworthy that the maximal levels of RPTPα, Sos and dynamin binding to GST–GRB2 were not dependent on the lysis conditions, indicating that recovery of transmembrane and cytoplasmic proteins is equally efficient in 1% Triton X-100 buffer and RIPA buffer. That Sos binds more rapidly than RPTPα suggests that the on-rate of binding to GST–GRB2 is higher for Sos than for RPTPα. The more rapid binding of RPTPα from RIPA lysates than from 1% Triton X-100 lysates indicates that the availability of RPTPα for binding to GST–GRB2 is higher under more stringent conditions. This may suggest that RPTPα is sequestered by endogenous GRB2 in the lysates and is only slowly released, thereby becoming available for binding to GST–GRB2.

The hypothesis that RPTPα is tightly bound to endogenous GRB2 in lysates was tested by transient co-transfection of RPTPα, activated c-Src and GRB2 into 293 cells. Co-transfection of GRB2 dramatically reduced the amount of RPTPα that bound to GST–GRB2 (Figure 8A), even though similar amounts of RPTPα were expressed by the cells (Figure 8B). Similar effects were observed under different lysis conditions, although the effect is more pronounced using 1% Triton X-100 lysis buffer than using RIPA buffer. Co-transfection of GRB2 mutants (R86K and P206L, but not P49L) did not affect binding of RPTPα to GST–GRB2 (data not shown), indicating that the SH2 and C-terminal SH3 domain of GRB2 are essential for this effect. It is noteworthy that co-transfection of GRB2 led to a 2.5-fold increase in RPTPα Tyr789 phosphorylation (Figure 3). Therefore, even though there was 2.5-fold more tyrosine phosphorylated RPTPα that could potentially bind to GST–GRB2, a reduction in RPTPα binding was still observed. These results demonstrate that not all P.Tyr-containing RPTPα is free to bind GST–GRB2, suggesting that RPTPα is sequestered by endogenous (transfected) GRB2.

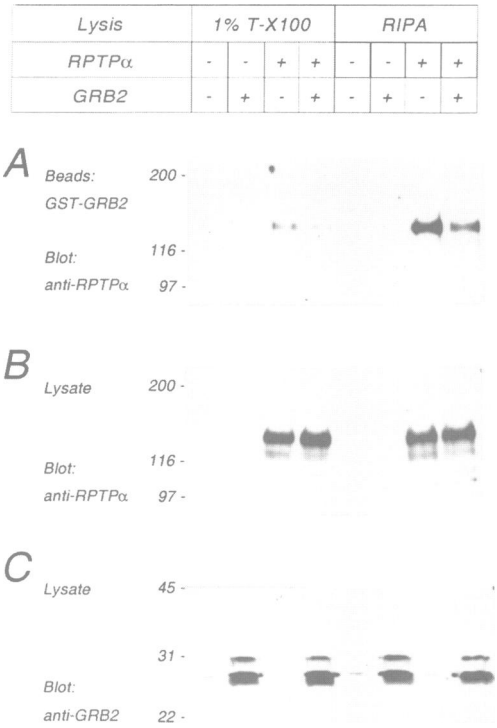


**Fig. 8.** RPTPα is sequestered by endogenous GRB2. Transiently transfected 293 cells expressing activated Src (Src-Y529F) and GRB2 alone, RPTPα alone or GRB2 and RPTPα as indicated were lysed in 1% Triton X-100 lysis buffer or in RIPA buffer. The lysates were incubated with GST–GRB2 for 3 h at 4°C, or an aliquot of the lysate was loaded directly on SDS–polyacrylamide gels. Following extensive washing the samples were boiled in denaturing sample buffer and run on 10% (**A** and **B**) or 17.5% (**C**) SDS–polyacrylamide gels. Following transfer of the material on the gels to Immobilon, the blots were probed with anti-RPTPα antiserum 5478 (A and B) or anti-GRB2 antiserum (C). Depicted are immunoblots with the molecular weights (in kDa) of marker proteins that were co-electrophoresed with the samples indicated on the left.

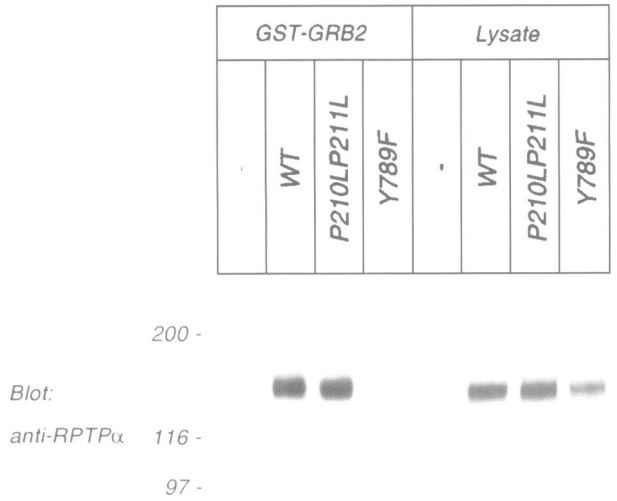


**Fig. 9.** The GRB2 C-terminal SH3 domain does not bind directly to a proline-rich region in the juxtamembrane domain of RPTPα. 293 cells were transiently co-transfected with Src-Y529F and wild-type RPTPα (WT), mutant RPTPα with mutations in the proline-rich region in the juxtamembrane domain (RPTPα–P210LP211L) or RPTPα with a mutation in Tyr789, the binding site for the SH2 domain of GRB2 (RPTPα–Y789F). The cells were lysed in 1% Triton X-100 buffer. An aliquot of the lysates was loaded directly on a 10% SDS–polyacrylamide gel (Lysate) and the rest was incubated with immobilized GST–GRB2. Following extensive washing of the beads the samples were boiled in denaturing sample buffer and run on a 10% SDS–polyacrylamide gel. The material on the gel was transferred to Immobilon and the blot probed with anti-RPTPα antiserum 5478. The immunoblot is shown with the molecular weights of marker proteins (in kDa) that were co-electrophoresed with the samples indicated on the left.

### *Mapping of the region in RPTPα that interacts with the GRB2 C-terminal SH3 domain*

The interaction of the C-terminal SH3 domain of GRB2 with RPTPα could in principle be direct. Analysis of a large number of SH3 binding sites has led to the identification of an absolutely conserved motif in proline-rich SH3 binding sites, PXXP (Feng *et al.*, 1994; Lim *et al.*, 1994; Yu *et al.*, 1994). There is one region in RPTPα that looks remotely like an SH3 binding site. This tentative SH3 binding site (residues 207–215: RKYPPLPVD) is localized in the juxtamembrane region. The prolines at positions 210 and 211 were mutated to leucine, and binding of this RPTPα mutant to GST–GRB2 was analysed. Figure 9 shows that RPTPα–P210LP211L, expressed in 293 cells, bound to GST–GRB2 as well as wild-type RPTPα. As a control, RPTPα with a tyrosine to phenylalanine mutation at position 789 was expressed in 293 cells, and this mutant did not bind to GST–GRB2 (Figure 9). A peptide encompassing the proline-rich region in RPTPα (residues 206–217, NRKYPPLPVDKL) also did not affect binding of RPTPα to GST–GRB2 (data not shown). In conclusion, the putative proline-rich SH3 binding site in the juxtamembrane region of RPTPα, residues 207–215, is not a binding site for the C-terminal SH3 domain of GRB2.

The region in RPTPα that is involved in binding to the C-terminal SH3 domain of GRB2 was mapped by analysis of the ability of RPTPα deletion mutants to bind to GST–GRB2 mutants. RPTPα mutants with large deletions (residues 330–669, 469–669 and 469–607) bound equally well to wild-type GRB2 and GRB2–P206L, but not to GRB2–R86K (data not shown), indicating that binding was mediated solely by the SH2 domain. Therefore, two mutants with relatively small deletions between residues 469 and 551 were made, as well as a single mutation within this region (RPTPα–R469L) (Figure 10A). These mutants were expressed in 293 cells (Figure 10B) and *in vitro* binding assays using wild-type GST–GRB2 indicated that these mutants contained phosphorylated Tyr789 (Figure 10C). Comparison of the expression levels of the RPTPα deletion mutants and RPTPα–R469L with the extent of binding to GST–GRB2 indicated that a higher proportion of the mutants bound to GST–GRB2 (compare Figure 10B and C). Analysis of P.Tyr content by immunoprecipitation and anti-P.Tyr blotting demonstrated that tyrosine phosphorylation was enhanced 5- to 20-fold in the deletion mutants and RPTPα–R469L compared with wild-type RPTPα (data not shown), thus explaining the relative increase in binding to GRB2. Enhanced tyrosine phosphorylation of these mutants suggests that autodephosphorylation is impaired by deletion of residues 469–486 or mutation of Arg469. Binding of wild-type RPTPα to GRB2 was dramatically reduced by mutation of the C-terminal SH3 domain, but not the N-terminal SH3 domain (Figure 10D). In contrast, the two deletion mutants bound equally well to wild-type GRB2 and GRB2–P206L (Figure 10E and F). The deletion mutants

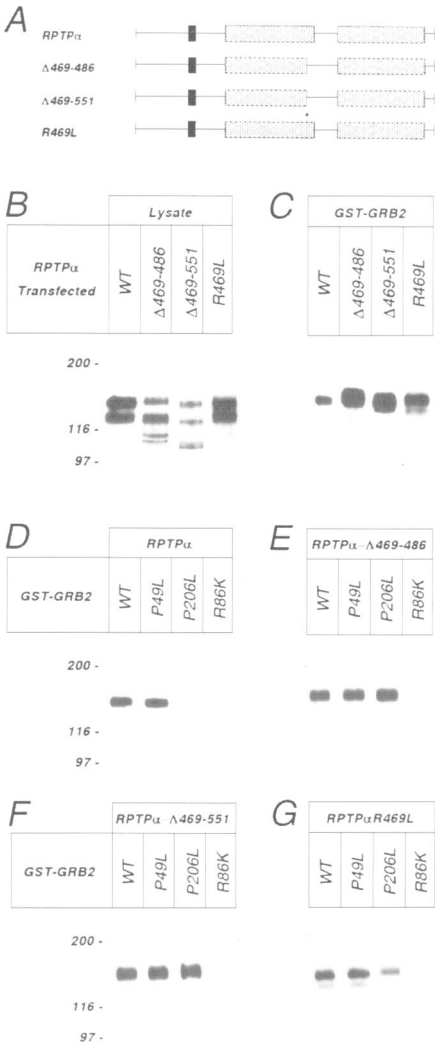


**Fig. 10.** Mapping of the GRB2 C-terminal SH3-interaction site in RPTPα. Deletion mutants of RPTPα and RPTPα–R469L were derived as described in Materials and methods and are depicted in (**A**). The RPTPα deletion mutants were co-transfected together with activated Src, Src-Y529F, into 293 cells. The cells were lysed in 1% Triton X-100 lysis buffer and an aliquot of the lysate was boiled in denaturing sample buffer and loaded directly onto a 10% SDS–polyacrylamide gel (**B**). Lysates were incubated with full-length wild-type GST–GRB2, immobilized on glutathione–agarose beads (**C**), or with a panel of GRB2 mutants (WT, P49L, P206L or R86K). Following extensive washing the samples were boiled in denaturing sample buffer and run on 10% SDS–polyacrylamide gels. The material on the gels was transferred to Immobilon and the filter incubated with anti-RPTPα antiserum 5478. Immunoblots are depicted with molecular weights (in kDa) of marker proteins that were co-electrophoresed with the samples indicated on the left. Wild-type RPTPα is depicted in (**D**), the RPTPα deletion mutants in (**E**) and (**F**) as indicated, and RPTPα–R469L in (**G**). Since relative binding of the deletion mutants to wild-type GRB2 is different (C), the exposure times of the blots shown here with wild-type RPTPα (D) and RPTPα–R469L (G) were 10-fold longer than of the blots with RPTPα–Δ469-486 (E) and RPTPα–Δ469-551 (F) to obtain comparable signals for binding of the deletion mutants to wild-type GST–GRB2.

did not bind to GRB2–R86K, indicating that GRB2 binding of these deletion mutants was mediated solely by the SH2 domain. Apparently, residues 469–486 encompassed the region in RPTPα that was required for interaction with the C-terminal SH3 domain of GRB2. Binding of mutant RPTPα with a single mutation in this region, RPTPα–R469L, to GRB2–P206L was readily detectable, but reduced as compared with binding to wild-type GRB2 (Figure 10G), indicating that Arg469 is one of the residues that is involved in the interaction with GRB2. In conclusion, RPTPα residues 469–486, and in particular Arg469, in the N-terminal PTP domain are involved in the interaction of RPTPα with the C-terminal SH3 domain of GRB2.

## Discussion

The role of PTPs in eukaryotic cell signalling is not well understood. Phosphorylation of PTPs plays an important role in regulation of PTP activity and function. Serine phosphorylation has been shown to modulate activity of several PTPs, including CD45, PTP-PEST and RPTPα (Ostergaard and Trowbridge, 1991; den Hertog *et al.*, 1995; Garton and Tonks, 1994). On the other hand, tyrosine phosphorylation of PTPs, including Syp (also known as PTP1D, SH-PTP2), PTP1C (also known as SH-PTP1, HCP), CD45 and RPTPα creates binding sites for SH2-containing proteins, thus potentially regulating PTP function (Autero *et al.*, 1994; Bouchard *et al.*, 1994; den Hertog *et al.*, 1994; Li *et al.*, 1994; Lorenz *et al.*, 1994; Su *et al.*, 1994). Here, we demonstrate that two domains of the adaptor protein GRB2 are involved in binding to RPTPα. The SH2 domain binds to RPTPα P.Tyr789 and Arg469 in the N-terminal PTP domain of RPTPα is involved in the interaction with the C-terminal SH3 domain. From the fact that RPTPα is sequestered by endogenous GRB2, we deduce that GRB2 binds tightly to RPTPα, presumably because both the SH2 and an SH3 domain are involved. Although the biological function of the RPTPα–GRB2 complex remains to be determined, the location of the region in RPTPα that is involved in the interaction with the C-terminal SH3 domain suggests that binding of GRB2 may inhibit RPTPα activity.

Mutation of either the SH2 domain or the C-terminal SH3 domain of GRB2 reduced binding to RPTPα *in vitro* and *in vivo*. In contrast, mutation of the N-terminal SH3 domain, which is essential for binding to the guanine nucleotide exchange factor Sos, did not affect binding to RPTPα. Sap and co-workers have also found that the SH2 and C-terminal SH3 domain, but not the N-terminal SH3 domain are involved in binding to RPTPα (J.Sap, personal communication). Although RPTPα and Sos bind to different GRB2 domains, an RPTPα–GRB2–Sos complex could not be detected (Figure 4; den Hertog *et al.*, 1994), suggesting that RPTPα can block binding of Sos to GRB2 and vice versa. That binding of proteins to the GRB2 SH2 domain affects binding of protein(s) to the SH3 domain(s) is not unprecedented, since Ravichandran *et al.* (1995) have shown that binding of GRB2 to Shc via its SH2 domain enhances binding of Sos to its SH3 domain(s).

In contrast to protein binding to GRB2, it has been demonstrated that (phospho)peptides bind independently to GRB2 SH2 and SH3 domains (Cussac *et al.*, 1994; Lemmon *et al.*, 1994), indicating that binding of the SH2 domain to its target sequence does not induce conformational changes in the SH3 domains. Despite this, proline-rich peptides can still have effects on binding of target proteins. Binding of a peptide encompassing the first proline-rich motif in hSos1 to the SH3 domain(s) of

GST–GRB2 blocked Sos and dynamin binding and led to an increase in RPTPα binding (Figure 5). Cussac *et al.* (1994) have shown that the affinities of similar proline-rich peptides are higher for the N-terminal SH3 domain than for the C-terminal SH3 domain, indicating that the observed effects on RPTPα binding are mostly due to blocking the N-terminal SH3 domain. Since binding of Sos or dynamin on the one hand and RPTPα on the other was apparently mutually exclusive (Figures 4 and 6), a peptide-mediated block of Sos and dynamin binding to GRB2 may actually cause enhanced binding of RPTPα. Since no Sos is detected by Coomassie blue staining it is probably mostly dynamin that saturates GST–GRB2, thereby blocking RPTPα binding in the absence of proline-rich peptides, which themselves are too small to have steric effects when bound to the N-terminal SH3 domain. Finally, binding of RPTPα to mutant GRB2 that lacks functional SH3 domains and cannot bind Sos or dynamin is enhanced compared with binding to the single C-terminal SH3 mutant (Figures 1 and 3), suggesting that the accessibility to the SH2 domain is enhanced in the double mutant. In conclusion, our data suggest that steric hindrance prohibits formation of the RPTPα–GRB2–Sos complex. This is consistent with the reported crystal structure of GRB2, in which the two SH3 domains are on the same face of the molecule, meaning that binding of a protein ligand to one SH3 domain would be likely to interfere with a protein ligand binding to the other (Maignan *et al.*, 1995).

As discussed above, RPTPα is not detectable in the Sos–GRB2 complex. Using an indirect functional assay, activation of ERK2 MAP kinase, we were also unable to detect an RPTPα–GRB2–Sos complex, in that transient transfection of RPTPα into 293 cells did not lead to activation of co-transfected ERK2 MAP kinase (data not shown). This indicates that binding of GRB2 to RPTPα at the plasma membrane does not lead to translocation of Sos to the membrane, an event that has been demonstrated to be sufficient for activation of Ras and ERK MAP kinases (Aronheim *et al.*, 1994; Quilliam *et al.*, 1994). Moreover, we have been unable to detect any effect of transfected RPTPα on growth factor- or serum-induced activation of ERK MAP kinases (data not shown), suggesting that RPTPα does not play a role in attenuation of GRB2-mediated signalling by sequestering GRB2 away from Sos.

The function of the GRB2–RPTPα complex is not clear at present. However, it is evident from our binding studies that binding of RPTPα to GRB2 is tight. Although the apparent affinity of GST–GRB2 for RPTPα is relatively low, since RPTPα binding is only detected using greater amounts of GST–GRB2, time-course studies demonstrated that this is actually caused by slow release of RPTPα from endogenous GRB2. Large amounts of GST–GRB2 displace RPTPα from the tight complex with endogenous GRB2 by shifting the equilibrium towards binding to exogenous GST–GRB2. Co-transfection of GRB2 together with RPTPα and subsequent assay of binding to GST–GRB2 led to a reduction in RPTPα binding, even though there was 2.5-fold more P.Tyr789-containing RPTPα in the lysate that could potentially bind to GRB2. The C-terminal SH3 and SH2 domain are essential for this effect (data not shown), indicating that tight binding of RPTPα to GRB2 is dependent on both these GRB2 domains.

SH3 domains bind to proline-rich sequences in their target proteins (Pawson, 1995). Comparison of a large number of SH3 binding sites has led to identification of an invariable core binding sequence within these proline-rich sequences, PXXP (Feng *et al.*, 1994; Lim *et al.*, 1994; Yu *et al.*, 1994). RPTPα contains this PXXP motif three times. Only the PXXP motif in the juxtamembrane region contains an additional proline residue (207-RKYPPLPVD-215), making it the most likely candidate SH3 binding site in RPTPα. However, mutation of the two proline residues at positions 210 and 211 did not affect binding to GST–GRB2, indicating that these residues are not involved in binding to the C-terminal SH3 domain of GRB2 (Figure 9). Using deletion mutants we mapped the region of interaction in RPTPα to residues 469–486. The fact that binding of the RPTPα–R469L mutant to GRB2–P206L was readily detectable, but reduced compared with binding of this mutant to wild-type GRB2, indicates that Arg469 is involved in, but not sufficient for the interaction with the C-terminal SH3 domain. Residues 469–486 do not contain any proline residues, which raises the question whether the prolyl peptide binding groove in the C-terminal SH3 domain is involved in binding to RPTPα. The fact that mutation of Pro206, within the prolyl peptide binding groove, almost completely abolished RPTPα binding may indicate that contacts with the prolyl peptide binding groove are involved in binding to RPTPα. Recently, it was demonstrated that GRB2 binds Vav independently of proline-rich sequences through dimerization of the C-terminal SH3 domain of GRB2 and the N-terminal SH3 domain of Vav (Ye and Baltimore, 1994), indicating that proline-rich sequences are not a prerequisite for binding to SH3 domains. The interaction between the C-terminal SH3 domain and RPTPα may not be direct. We investigated whether the C-terminal GRB2 SH3 domain binds directly to RPTPα using purified, bacterially expressed GST fusion proteins, encoding (parts of) the cytoplasmic region of RPTPα, but found that RPTPα did not bind to purified, immobilized GST–GRB2, nor to GRB2 from NIH 3T3 lysates (data not shown), indicating that the interaction between the C-terminal SH3 domain and RPTPα is not sufficiently strong to detect binding in this fashion. This has precluded us from determining whether the C-terminal SH3 domain binds directly to residues 469–486 and to Arg469 in particular, and at present we can not exclude the possibility that there is an intermediary protein between the C-terminal SH3 domain of GRB2 and RPTPα. Since GRB2 does not bind to unphosphorylated bacterially expressed RPTPα and since binding to unphosphorylated mutant RPTPα–Y789F is almost completely abolished, we conclude that although the C-terminal SH3 domain of GRB2 is required for optimal binding to RPTPα, binding of the GRB2 SH2 domain to tyrosine-phosphorylated RPTPα is essential.

RPTPα residues 469–486 (469-RAQRCQMVQTDMQ-YVFIY-486) are located in the C-terminal region of the N-terminal PTP domain, and deletion of these residues renders binding independent of the C-terminal SH3 domain of GRB2 (Figure 10). The arginine residues at positions 469 and 472 and the glutamine residues at positions 477 and 481 are highly conserved among PTPs. These residues

are also conserved in PTP1B, of which the crystal structure has recently been solved (Barford *et al.*, 1994; Jia *et al.*, 1995). The stretch of amino acids corresponding to RPTPα 469–486 in PTP1B (residues 254–271) is located in α-helices 5 and 6 and loop 17. PTP1B Arg251 (RPTPα Arg469, which is involved in binding to the C-terminal SH3 domain of GRB2) is located on the surface of PTP1B close to the catalytic site cleft (Barford *et al.*, 1994). The crystal structure of the N-terminal PTP domain of RPTPα has recently been solved (J.Noel and A.Bilwes, personal communication). The overall structure of the N-terminal PTP domain is very similar to PTP1B, and RPTPα Arg469, along with several other residues in RPTPα 469–486, including the two conserved glutamine residues, Gln477 and Gln481, are located on the surface, where they could engage in protein–protein interactions with the C-terminal SH3 domain of GRB2, or with intermediary proteins. The crystal structure of PTP1B complexed with a P.Tyr-containing peptide demonstrated that PTP1B Gln262 forms van der Waals contacts and a hydrogen bond with the peptide substrate, indicating that Gln262 is involved in substrate binding or recognition (Jia *et al.*, 1995). The observation that the region in RPTPα close to Gln477 (PTP1B Gln262) is involved in binding to the C-terminal SH3 domain of GRB2 would predict that GRB2 binding to RPTPα would block access to the catalytic site, leading to inactivation of the N-terminal PTP domain. Since RPTPα needs to be tyrosine-phosphorylated in order to be able to bind GRB2, and since the stoichiometry of phosphorylation *in vivo* in transfected cells or *in vitro* using purified c-Src is low (0.1 or less), we have not yet been able to test the hypothesis that GRB2 binding modulates RPTPα activity.

The biological function of GRB2 binding to RPTPα remains to be determined. In NIH 3T3 cells a significant proportion of all endogenous RPTPα (20%) is constitutively bound to GRB2 (den Hertog *et al.*, 1994), suggesting that formation of the GRB2–RPTPα complex is not trivial. Previously, we have demonstrated that overexpression of RPTPα-Y789F, a mutant that can no longer bind GRB2, still altered the differentiation fate of pluripotent P19 EC cells (data not shown), indicating that formation of the RPTPα–GRB2 complex is not required for neuronal differentiation of transfected P19 EC cells. As discussed above, there is no evidence to suggest that the RPTPα–GRB2 complex is involved in signalling cascades involving ERK MAP kinases. However, GRB2 may modulate RPTPα activity directly by blocking access to the catalytic site cysteine. This remains to be tested. The function of GRB2 binding to other PTPs is also not yet clear. It has been demonstrated that the cytoplasmic PTPs Syp and PTP1C act as adaptor proteins, since they bind to activated receptor PTKs through their SH2 domain(s) and become tyrosine-phosphorylated (Feng *et al.*, 1993; Kazlauskas *et al.*, 1993; Lechleider *et al.*, 1993; Vogel *et al.*, 1993; Yi and Ihle, 1993), which creates a binding site(s) for GRB2 (Bouchard *et al.*, 1994; Li *et al.*, 1994; Lorenz *et al.*, 1994). In contrast to the RPTPα–GRB2 complex, Sos is present in the Syp–GRB2 complex. Therefore, binding of the GRB2–Sos complex to Syp, already bound to activated receptor PTKs at the membrane, leads to activation of Ras (Li *et al.*, 1994). The function of the PTP activity of Syp and PTP1C in PTK–Syp/PTP1C–GRB2 complexes remains to be determined. The conserved residues in RPTPα that are involved in binding to the GRB2 C-terminal SH3 domain are also conserved in Syp and PTP1C. It will be interesting to see whether the C-terminal SH3 domain binds to this region in Syp and also to PTP1C. Future studies on the interaction of GRB2 with cytoplasmic and transmembrane PTPs will lead to new insights into signal transduction by PTKs and PTPs and may lead to identification of GRB2 as a modulator of PTP activity.

## Materials and methods

### *Cells and transfections*

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 (Gibco, Paisley, UK). Culturing and stable transfection of P19 EC cells have been described (den Hertog *et al.*, 1993). 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 $CaCl_2$ (250 mM), and left for 25 min at 25°C. The precipitate was resuspended and added directly to the medium on the cells (6 cm dish). After incubation overnight, the medium was changed and the cells were harvested 24 h later.

### *Plasmids and site-directed mutagenesis*

Stable transfection of P19 cells was done by co-transfection using a plasmid encoding the neomycin resistance gene under control of the SV40 promoter, pSV2neo (Southern and Berg, 1982). SV40-driven expression vectors, based on pSG5 (Green *et al.*, 1988), for expression of RPTPα or mutants have been described (den Hertog *et al.*, 1994). A CMV promoter-driven expression vector for activated murine Src, Src-Y529F, has also been described (den Hertog *et al.*, 1994). CMV-driven expression vectors for GRB2 and mutants of GRB2 were derived by insertion of a *Bam*HI–*Xho*I fragment, containing the complete coding sequence of GRB2 into pSLX. Prokaryotic expression vectors for GST–GRB2 fusion proteins were derived by insertion of *Nco*I–*Xho*I fragments containing full-length GRB2 in-frame into pGEX-KG (Guan and Dixon, 1991).

The expression vector for haemagglutinin epitope-tagged RPTPα was derived by insertion of a *Nco*I fragment encoding the epitope tag into a *Nco*I site in RPTPα that was generated by PCR. The construct thus obtained was checked by sequencing and contained the epitope tag immediately following the signal sequence, to the N-terminal side of Asn20 in RPTPα (numbering according to Sap *et al.*, 1990).

The expression vectors encoding deletion mutants of RPTPα were derived from pSG-RPTPα as follows: pSG-RPTPα–Δ330–669 was made by digestion of pSG-RPTPα with *Bst*EII and ligation, resulting in deletion of a 1017 bp fragment. pSG-RPTPα–Δ469–669 was derived by ligation of a blunt 0.5 kbp *Bst*EII–*Bam*HI fragment encoding RPTPα 669–793 and ~100 bp 3′ non-coding into *Bam*HI-opened blunt pSG-RPTPα. The other three deletion mutants pSG-RPTPα–Δ469–486, pSG-RPTPα–Δ469–551 and pSG-RPTPα–Δ469–607 were derived by insertion of *Bam*HI fragments that were generated by PCR into *Bam*HI-opened pSG-RPTPα. The oligonucleotides that were used to generate these *Bam*HI fragments were:

RPTPαNB486s: 5′-GCGCCATGGGGATCCAGGCCCTTCTGGAG-3′,
RPTPαNB551s: 5′-GCGCCATGGGGATCCAGAAGAACCGGGTT-3′,
RPTPαNB607s: 5′-GCGCCATGGGGATCCAGGACTTCTGGCGA-3′

and

RPTPα-BH793as: 5′-GCGAAGCTTGGATCCTCACTTGAAGTTGGC-3′.

Site-directed mutagenesis was done on pSG-RPTPα or on the complete cDNA of GRB2 in pBluescript $SK^-$. Mutations were verified by sequencing and subsequently the GRB2 cDNA was inserted in pSLX for eukaryotic expression or in pGEX–KG for prokaryotic expression as described above. The oligonucleotides that were used for site-directed mutagenesis were:

RPTPα-P210LP211L: 5′-AGGAAGTACCTACTACTGCCTGTG-3′,
RPTPα-R469L: 5′-AGCCGGATCCTGGCCCAGCGC-3′
GRB2-P49L: 5′-GGCTTCATTCTCAAGAACTAC-3′,
GRB2-P206L: 5′-GGCATGTTTCTCCGCAATTAT-3′

and

GRB2-R86K: 5′-GCCTTTCTTATCAAAGAGAGTGAGAGC-3′.

### *GST fusion proteins*

Expression vectors for bacterial expression of GST fusion proteins were transformed into BL21 bacteria for high-level expression. Overnight cultures were diluted 1:10 into fresh medium and after shaking for 1 h, isopropyl-β-D-thiogalactopyranoside (IPTG, 100 μM final concentration) was added. After another 4–6 h shaking at 37°C the cells were lysed in TBS (50 mM Tris pH 8.0, 150 mM NaCl) with 1% Triton X-100 and 1 mg/ml lysozyme by sonication and the supernatants were incubated with glutathione–agarose beads (Sigma, St Louis, MO, USA) for 10 min at room temperature. After extensive washing with TBS, the fusion proteins were eluted with 10 mM reduced glutathione for 10 min at room temperature. The eluted fusion proteins were dialysed against TBS and stored at −20°C. Just before use an aliquot was thawed and immobilized on glutathione–agarose beads.

### *In vitro binding, immunoprecipitation and immunoblotting analysis*

Nearly confluent cells were lysed in 1% Triton X-100 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) or in RIPA buffer (20 mM Tris pH 8.0, 150 mM NaCl, 10 mM $Na_2HPO_4$, 5 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 10% glycerol, 10 U/ml aprotinin, 1 μM pMSF, 200 μM sodium orthovanadate). For *in vitro* binding assays the lysates were incubated with GST fusion proteins (5–10 μg per sample, unless stated otherwise) immobilized on glutathione–agarose beads for 3 h (or as indicated) at 4°C. Immunoprecipitation was done by incubation with anti-haemagglutinin epitope tag antibody (MAb 12CA5) and protein A–Sepharose (Pharmacia, Uppsala, Sweden) for 3 h at 4°C. The beads were washed four times with HNTG (20 mM HEPES pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) or with RIPA, resuspended in Laemmli sample buffer and boiled for 5 min, and the samples were loaded onto SDS–polyacrylamide gels. For some experiments, peptide (0.05–0.5 mM, final concentration) was added to the lysates. The peptides that were used are: hSos1: PVPPPVPPRRRP (hSos1 1148–1159), two proline-rich peptides: Pro-2: PPPLPPPRRR and Pro-3: RHYRPLPPLP and, as a control, an RPTPα peptide: CYKVVQEYIDAFSDYANFK (RPTPα 775–793).

For immunoblotting analysis the material on the polyacrylamide gels was transferred to Immobilon (Millipore, Bedford, MA, USA) by semi-dry blotting for 1 h at 0.8 mA/$cm^2$ gel in transfer buffer (50 mM Tris, 40 mM glycine, 0.0375% SDS, 20% methanol). Following transfer the blots were incubated in blocking buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20, 5% non-fat milk) overnight at 4°C and in blocking buffer containing affinity-purified anti-RPTPα antiserum 5478, anti-GRB2 antiserum C-23 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-Sos antiserum C-20 (Santa Cruz Biotechnology) for 1.5 h at 25°C. For immunoblotting with anti-P.Tyr antibody PY-20 (Transduction Laboratories, Lexington, KY, USA) blocking buffer contained 5% bovine serum albumin instead of milk. The filters were washed four times in TBS-T (50 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween-20), and incubated for 1 h at 25°C with horseradish peroxidase (HRP)-conjugated protein A or HRP-conjugated anti-mouse antibody in TBS-T for enhanced chemiluminescence (ECL) detection which was performed exactly as indicated by the manufacturer (Amersham, Arlington Heights, IL, USA). Before reprobing with different primary antibodies, the blots were stripped by incubation in stripping buffer (62.5 mM Tris pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) for 1 h at 70°C.

### *$^{32}$P-labelling and tryptic phosphopeptide mapping analysis*

Transiently transfected 293 cells were metabolically labelled overnight in phosphate-free medium supplemented with [$^{32}$P]orthophosphate (2.0 mCi/ml, ICN, Irvine, CA). 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 four times with RIPA buffer without SDS. The lysates were precleared using protein A–Sepharose (Pharmacia, Uppsala, Sweden) for 1 h at 4°C, and incubated with affinity-purified anti-RPTPα antiserum 5478, together with protein A–Sepharose for 2 h at 4°C. The beads were washed four times with RIPA buffer and the samples resuspended in Laemmli sample buffer, boiled for 5 min and loaded onto SDS–polyacrylamide gels. Following electrophoresis the gel was dried and exposed to film.

Phosphopeptide mapping was done as described by Boyle *et al.* (1991). $^{32}$P-labelled RPTPα was eluted from the gel, precipitated with trichloroacetic acid, oxidized with performic acid and digested with TPCK–trypsin. The samples were loaded onto cellulose TLC plates, 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 and radioactivity quantified using a PhosphorImager (Molecular Dynamics).

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