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Multiple Interactions between Receptor Protein-tyrosine Phosphatase (RPTP) α and Membrane-distal Protein-tyrosine Phosphatase Domains of Various RPTPs*

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Receptor protein-tyrosine phosphatase (RPTP) α belongs to the large family of receptor protein-tyrosine phosphatases containing two tandem phosphatase domains. Most of the catalytic activity is retained in the first, membrane-proximal domain (RPTPα-D1), and little is known about the function of the second, membrane-distal domain (RPTPα-D2). We investigated whether proteins bound to RPTPα using the two-hybrid system and found that the second domain of RPTPσ interacted with the juxtamembrane domain of RPTPα. We confirmed this interaction by co-immunoprecipitation experiments. Furthermore, RPTPα not only interacted with RPTPσ-D2 but also with RPTPα-D2, LAR-D2, RPTPδ-D2, and RPTPμ-D2, members of various RPTP subfamilies, although with different affinities. In the yeast two-hybrid system and in glutathione *S*-transferase pull-down assays, we show that the RPTP-D2s interacted directly with the wedge structure of RPTPα-D1 that has been demonstrated to be involved in inactivation of the RPTPα-D1/RPTPα-D1 homodimer. The interaction was specific because the equivalent wedge structure in LAR was unable to interact with RPTPα-D2 or LAR-D2. *In vivo,* we show that other interaction sites exist as well, including the C terminus of RPTPα-D2. The observation that RPTPα, but not LAR, bound to multiple RPTP-D2s with varying affinities suggests a specific mechanism of cross-talk between RPTPs that may regulate their biological function.

Protein-tyrosine phosphorylation is a major mechanism of intracellular signaling within superior eukaryotic organisms that has been demonstrated to be involved in a large set of cellular events like migration, proliferation, differentiation, and transformation. Protein-tyrosine phosphatases (PTPs)[1] regulate the level of phosphotyrosine in cellular proteins issued from the action of protein-tyrosine kinase. The PTP family is composed of more than 100 different known members, but this number is constantly growing, and the last estimation is that there are 500 PTPs (1). The PTP group is subdivided in two between the cytosolic and the receptor protein-tyrosine phosphatases (RPTP). The RPTPs are distinguished by their extracellular domains, which can be very large, containing domains such as fibronectin type III-like domains and Immunoglobulin-like repeats in the case of for instance LAR, or very short (*e.g.* RPTPε) and heavily glycosylated such as RPTPα (2, 3).

RPTPα is a widely but dynamically expressed RPTP that dephosphorylates Tyr^{527} of c-Src *in vitro* and that increases c-Src kinase activity when overexpressed *in vivo* (4, 5). Furthermore, cells derived from RPTPα knock-out mice have reduced Src and Fyn activity, indicating that RPTPα activates Fyn as well as Src *in vivo* (6, 7). RPTPα is also involved in m1 muscarinic acetylcholine receptor-dependent regulation of Kv1.2 channel activity. Presumably, RPTPα directly dephosphorylates Kv1.2, leading to desuppression of its K^+ channel activity (8).

RPTPα is itself phosphorylated on Tyr^{789}, a GRB2-SH2 consensus binding site. Both the SH2 and the C-terminal SH3 domain of GRB2 cooperate for tight binding to RPTPα. Although it is clear that RPTPα interacts with GRB2 *in vivo*, the function of the binding remains elusive because it is exclusive of Sos, another partner for the SH3 domain of GRB2 (9–12).

Although it is now established that receptor protein-tyrosine kinases are activated by ligand induced dimerization, little is known about the regulation of RPTPs. One of the few examples of modulation of RPTP activity is activation of RPTPα by serine phosphorylation in response to phorbol ester-mediated activation of protein kinase C (13, 14). Although ligands have been found for some RPTPs that induced cellular changes, neither changes in activity of the RPTPs nor changes in phosphorylation state of a direct substrate were reported (15–17). This might suggest that the extracellular domain has another function in addition to regulating RPTP activity. Increasing structural (18) and functional (19–21) evidence suggests that dimerization negatively regulates RPTP activity, at least for RPTPα and CD45. In the RPTPα dimer, a helix-loop-helix so-called "wedge" structure interacted directly with and occluded the catalytic site of the opposing monomer. The inhibition is reciprocal in that the wedge of the second monomer interacts with the catalytic site of the first monomer (18). Mutations that destabilized the wedge structure abolished the inactivation (20, 21). Furthermore, we have used fluorescence resonance energy transfer between RPTPα fusion proteins, fused to two derivatives of green fluorescent protein mutants, cyan and yellow fluorescent protein to show that RPTPα formed homodimers in living cells.[2] All these results indicate that RPTPs, as opposed to the receptor protein-tyrosine kinases, would be inactivated by dimerization in which a key component of the inactivation is the interaction between the wedge of one monomer and the catalytic site of the other monomer in RPTP-D1 dimers. How-

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[1] The abbreviations used are: PTP, protein-tyrosine phosphatase; RPTP, receptor PTP; D1, membrane-proximal PTP domain; D2, membrane-distal PTP domain; GST, glutathione *S*-transferase; aa, amino acid(s); PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; HA, hemagglutinin.

[2] L. G. J. Tertoolen, C. Blanchetot, G. Jiang, J. Overvoorde, T. W. J. Gadella, Jr., T. Hunter, and J. den Hertog, submitted.

ever, dimerization may not be a general regulatory mechanism because the crystal structures of RPTPμ-D1 (22) and of the tandem phosphatase domains of RPTP LAR (23) did not show dimers despite the presence of wedges in both structures.

A striking feature of the RPTPs is the high conservation of a second PTP domain, distal to the membrane (D2), that contains no or very low catalytic activity. The second domains of LAR (23) and RPTPα[3] have the conserved PTP structure. For both RPTPs, the lack of activity in D2 resides in two amino acids that are highly conserved in active PTPs but not in RPTP-D2s: the Tyr in the KNRY motif and the Asp in the WPD loop. PTP activity was restored in D2 by mutating these key residues into their wild type D1 counterpart (23–25). The conservation of an inactive PTP domain (D2) in the RPTP family raised questions about the biological function of D2.

We used the yeast two-hybrid system to screen for proteins that interacted with RPTPα, in search of possible effector proteins of RPTPα. We found that the D2 of RPTPσ interacted with RPTPα. The observation was extended to other D2s, including RPTPα-D2, LAR-D2, RPTPδ-D2, and RPTPμ-D2 *in vitro* and *in vivo*. Using the two-hybrid system as well as GST pull-downs, we show for the first time that the wedge in RPTPα-D1, but not the equivalent wedge of LAR-D1, is involved in the interaction with D2s, indicating that these interactions are specific. *In vivo* binding of RPTPα to RPTP-D2s was further confirmed by co-immunoprecipitation. Furthermore, the affinity of binding between RPTPα and the different D2s appeared to be in the following order: RPTPδ-D2 > RPTPσ-D2/LAR-D2 > RPTPα-D2/RPTPμ-D2. Our results suggest a role for RPTP-D2s in the regulation of RPTPα because the wedge is involved in the interaction between RPTPα-D1 and RPTPα-D1 as well as between RPTPα-D1 and RPTP-D2s. Furthermore, our results suggest cross-talk between different nonrelated RPTPs that might regulate their biological function.

EXPERIMENTAL PROCEDURES

Constructs—Polymerase chain reaction fragments encompassing different RPTP domains were cloned in pGBT8 or pGAD for the two-hybrid binding assay, in pCS2+MT to make Myc-tagged fusion protein, or in pGEX-KG to make GST fusion proteins. The different RPTP domains encompassed the following residues: the juxtamembrane domain of mRPTPα (aa 175–240, numbering according to Sap *et al.* (26)), or hLAR (aa 1278–1358, accession number Y00815); the first domain of mRPTPα (aa 202–501); the second domain of mRPTPα (aa 537–793), mRPTPσ (aa 1648–1904, accession number D28530), hLAR (aa 1642–1897), hRPTPμ (aa 1200–1452, accession number X58288), mRPTPδ (aa 1037–1292, accession number D13903), or the PTP domain of zfPTP1B (aa 1–302) (27). Point mutations and deletions were made by site-directed mutagenesis and verified by sequencing. pCS2+MT-RPTPα-D2ΔC-t was made by cloning of an *Nco*I-*Eco*RI fragment in pCS2+ thus deleting residues 774–792 and the endogenous stop codon, which led to replacement of the RPTPα sequence by some vector sequence, leaving the overall size of the protein the same.

Two-hybrid Screen and Assays—For the library screening, the reporter strain YGH-1 transformed with pGBT8-RPTPα-JXT was retransformed as described (28) with a reamplified mouse brain cDNA library cloned in pGAD and plated on synthetic medium lacking leucine, tryptophane, and histidine (SC^{-LTH}). The number of transformants was approximately 1×10^7. After 4 days 150 colonies were recovered after selection and replated on new SC^{-LTH} plates for analysis of β-galactosidase activity, using a filter lift assay. The plasmid DNA from the His and 32 β-galactosidase positives colonies was isolated from liquid cultures, transformed into bacteria, and retested in yeast for specificity. For interaction assays, 3 μg of relevant plasmids and 40 μg of carrier DNA were introduced in yeast using the lithium-acetate protocol and plated on medium lacking leucine and tryptophane (SC^{-LT}) or SC^{-LTH}. After 3–4 days, independent colonies from both kind of plate (SC^{-LT} and SC^{-LTH}), were replated and tested for β-galactosidase activity using a filter lift assay.

[3] A. Bilwes and J. Noel, personal communication.

Cell Cultures and Transfections—293 and COS cells were routinely grown in DF medium (a 1:1 mixture of Dulbecco's minimum essential medium and Ham's F12 medium) supplemented with 7.5% fetal calf serum. Cells were transfected using the standard calcium-phosphate method (10). Briefly, 10-cm dishes were transfected with a total of 20 μg of DNA. The next day, the medium was refreshed and left another 16 h before harvesting.

Immunoprecipitation and Western Blotting—Subconfluent transfected cells were washed twice with ice-cold phosphate-buffered saline, and lysed with cell lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, and protease inhibitors, including benzamidine, aprotinin, and leupeptin) for 20 min on ice, harvested, and centrifuged at 14,000 $\times$ *g* for 15 min to remove the insoluble fraction. The supernatant was added to 12CA5 antibodies that had been covalently linked to protein A-Sepharose beads, using dimethylpimelimidate. After 2 h of incubation, the beads were washed four times with HNTG buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol), mixed with 2$\times$ Laemmli buffer and loaded on a 10% SDS-PAGE gel. The proteins were transferred to PVDF membrane using a semidry transfer system. After Coomassie staining, the membrane was blocked for 1 h with 5% milk in TBS-Tween (50 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20) at room temperature, incubated with the first antibody for 1.5 h, washed four times with TBS-Tween, incubated with horseradish peroxidase-conjugated anti mouse antibody (Transduction Laboratories) for 1 h, washed with TBS-Tween, and developed using enhanced chemiluminescence.

Purification of GST Fusion Proteins—All the constructs in pGEX-KG were transformed into BL21 bacteria. GST fusion proteins were purified essentially as described (29). Briefly, fresh colonies were allowed to grow until they reached an optical density of 0.6–0.8 and were then diluted 10 times. After 1 h, isopropyl-1-thio-β-D-galactopyranoside was added up to 100 μM to induce synthesis of the fusion proteins overnight at 28–30 °C. The cells were lysed by addition of lysozyme and further by sonication in TBS (50 mM Tris, pH 8.0, 150 mM NaCl) containing Triton X-100 (1%) and protease inhibitors at 4 °C. After centrifugation at 14,000 $\times$ *g*, the GST fusion proteins were collected using Sepharose-GSH beads and eluted with 10 mM reduced glutatione in TBS. The supernatant containing pure GST fusion protein was further dialyzed against a solution of TBS containing 10% glycerol.

RESULTS

RPTPα Interacts with Different RPTP-D2s in the Yeast Two-hybrid System—To find proteins interacting with RPTPα we used the yeast two-hybrid system. Different fragments of RPTPα containing the full intracellular domain or the individual PTP domains fused to the GAL4 DNA-binding domain were used to screen a mouse brain cDNA library as well as an 11.5-day-old mouse embryo cDNA library without success. The presence of RPTPα-D1 appeared to be detrimental in the two-hybrid system. Possibly, the PTP activity of RPTPα was toxic to the yeast or RPTPα-D1 homodimerization inhibited further binding of proteins. The juxtamembrane domain of RPTPα (RPTPα-JXT, aa 175–240, encompassing the region just C-terminal to the transmembrane domain and a small part of RPTPα-D1) was used to screen a mouse brain cDNA library. Three clones out of 10^7 transformants were found to bind strongly and specifically to RPTPα and were sequenced. All three clones were identical and encoded the second domain of RPTPσ-D2. To investigate whether other RPTP-D2s might interact with the juxtamembrane domain of RPTPα as well, we generated a construct of the GAL4 activation domain fused to RPTPα-D2 and found that RPTPα-D2, like RPTPσ-D2, interacted with the juxtamembrane domain of RPTPα (Table I). To validate the latter interaction, the GAL4 DNA-binding domain and GAL4 activation domain were switched, and again, a strong interaction was detected suggesting a specific interaction (Table I). To further investigate the interaction of RPTPα with other RPTP-D2s, we introduced LAR-D2, RPTPδ-D2, and RPTPμ-D2 in the two-hybrid vectors and tested for specific interaction with RPTPα-JXT. As depicted in Table I, all D2s that we tested interacted with the juxtamembrane domain of RPTPα, with RPTPδ-D2 apparently binding better. The inter-

TABLE I
Binding of RPTPD2s to the juxtamembrane domain of RPTPα in the yeast two-hybrid system

Fragments of RPTPs were fused to the GAL4 activation domain in the PGAD vector and cotransfected in YGH-1 yeast with different fragments of RPTPs, fused to the GAL4 DNA-binding domain in PGBT8 as indicated. The RPTP domains used are the juxtamembrane domain of RPTPα (RPTPα-JXT) and the membrane-distal domain (D2) of RPTPσ, RPTPα, LAR, RPTPδ, and RPTPμ. The interactions were scored as positive (++), strongly positive (+++), or negative (−) based on the yeast growth on medium lacking histidine and β-galactosidase expression.

GAL4 DNA-binding domain	GAL4 activation domain			
	−	RPTPσ-D2	RPTPα-D2	RPTPα-JXT
RPTPα-JXT	−	++	++	−
RPTPα-D2	−	−	−	++
LAR-D2	−	−	−	++
RPTPδ-D2	−	−	−	+++
RPTPμ-D2	−	−	−	++

TABLE II
Binding of RPTPα-D2 to the juxtamembrane domain of RPTPα is dependent on the wedge

YGH-1 yeast were cotransfected with pGBT8 (GAL4 DNA-binding domain) RPTPα-JXT without or with a mutation in the wedge structure (RPTPα-JXT-K230A or RPTPα-JXT-E234A) and with pGAD vector (GAL4 activation domain) alone (−) or containing RPTPα-D2. The interactions were scored as in Table I: positive (++) or negative (−) based on the yeast growth on medium lacking histidine and β-galactosidase expression.

GAL4 DNA-binding domain	GAL4 activation domain	
	−	RPTPα-D2
RPTPα-JXT	−	++
RPTPα-JXT-K230A	−	−
RPTPα-JXT-E234A	−	−

TABLE III
Binding of RPTP-D2s is specific for the juxtamembrane domain of RPTPα

YGH-1 yeast were cotransfected with pGBT8 (GAL4 DNA-binding domain) vectors encoding RPTPα-D2 or LAR-D2, and with pGAD (GAL4-AD) vector alone or containing the juxtamembrane domain of RPTPα (RPTPα-JXT) or LAR (LAR-JXT). The interactions were scored as in Table I: positive (++) or negative (−) based on the yeast growth on medium lacking histidine and β-galactosidase expression.

GAL4 DNA-binding domain	GAL4 activation domain		
	−	RPTPα-JXT	LAR-JXT
RPTPα-D2	−	++	−
LAR-D2	−	++	−

actions were not nonspecific because the RPTP-D2s did not interact with the empty vectors nor with each other. In conclusion, we show that the juxtamembrane domain of RPTPα interacted specifically with many RPTP-D2s in the two-hybrid system.

RPTPα-JXT/RPTP-D2 Interactions Are Specific and Dependent on the Wedge—The juxtamembrane domain of RPTPα that was used in the two-hybrid system contains the helix-loop-helix, so-called wedge, structure that interacted with the other monomer in the RPTPα-D1 homodimers of the crystal structure (18). Based on the crystal structure, we speculated that the wedge would also be important for the interaction between RPTPα-D1 and RPTP-D2s. We assessed which residues in the juxtamembrane domain of RPTPα were responsible for the interaction with D2s and substituted residues in the wedge that form contacts in the crystal structure with the other monomer with Ala. Single amino acid substitutions in the wedge, K230A or E234A, were sufficient to abolish binding in the yeast two-hybrid system, demonstrating that the wedge is important for the interaction (Table II). Moreover, the RPTP-D2s bound specifically to RPTPα-JXT, because none of the RPTP-D2s tested bound to the equivalent juxtamembrane domain of LAR, indicating an intrinsic difference between these two RPTPs (Table III). Taken together, these results demonstrate that the juxtamembrane domain of RPTPα, but not of LAR, specifically bound to RPTP-D2s and that the interactions were dependent on the wedge.

RPTPα-D1/RPTPα-D2 Interactions Assessed by GST Pull-down Assays—The interaction of RPTPα-D1 (containing the wedge) with RPTPα-D2 was investigated in a GST pull-down assay. Bacterially expressed GST-RPTPα-D2 was incubated with lysate of cells transfected with a Myc-tagged juxtamembrane domain of RPTPα (wild type), and binding proteins were detected by immuno-blotting. GST-RPTPα-D2, but not GST, bound MtRPTPα-JXT (Fig. 1*A*). In accordance with the yeast two-hybrid experiments (Table II), mutation of Lys^{230} in the wedge (K/A) abolished binding (Fig. 1*A*). In a more relevant context, a Myc-tagged RPTPα-D1 (Mtα-D1, aa 202–501), containing part of the juxtamembrane region and the wedge as well as a complete first domain, was pulled down by bacterially purified GST-RPTPα-D2 but not by GST alone (Fig. 1*B*). Reciprocally, Myc-tagged RPTPα-D2 (Mt-αD2) was pulled down by GST-RPTPα-D1, but not by mutant GST-RPTPα-D1 containing mutations in the wedge, GST-RPTPα-D1K230A (Fig. 1*C*), or GST-RPTPα-D1E234A (data not shown). As a control, binding of Mt-αD2 to GST-RPTPα-D2 was assessed, and we found that RPTPα-D2 did not homodimerize (Fig. 1*C*). In conclusion, the GST pull-down assays reproduced the two-hybrid experiments clearly. RPTPα-D1 interacted with RPTPα-D2, and the integrity of the wedge was required for the interaction.

In Vivo Binding of RPTPα-D1 to Distinct RPTP-D2s—We further investigated the interaction detected in the two-hybrid system and in GST pull-downs between RPTPα and different RPTP-D2s *in vivo* by co-immunoprecipitation. 293 cells were cotransfected with full-length HA-RPTPα and MtRPTPα-D2. MtRPTPα-D2 only coimmunoprecipitated with HA-RPTPα, and no MtRPTPα-D2 was detected when HA-RPTPα was not cotransfected (Fig. 2, *left* and *middle lanes*). Interestingly, full-length HA-RPTPα was a less potent partner for MtRPTPα-D2 than HA-RPTPα-ΔD2, a construct lacking RPTPα-D2, although both constructs were expressed equally (Fig. 2, *middle* and *right lanes*). These results suggest that endogenous RPTPα-D2 and cytosolic MtRPTPα-D2 may compete for the same site in RPTPα-D1 *in vivo* because less MtRPTPα-D2 bound when endogenous RPTPα-D2 was present. We next investigated *in vivo* binding of RPTPα to other RPTP-D2s. All the Myc-tagged versions of the RPTP-D2s used in the two-hybrid system coimmunoprecipitated with RPTPα (Fig. 3). Furthermore, the increase in binding to RPTPα-ΔD2, as compared with full-length HA-RPTPα, was observed with Myc-tagged LAR-D2, RPTPσ-D2, and RPTPμ-D2 (Fig. 3), as well as RPTPδ-D2 (data not shown), which might indicate a similar way of interaction between RPTPα and the different RPTP-D2s. The binding was specific for RPTP-D2s because Myc-tagged PTP1B, a cytosolic protein-tyrosine phosphatase, was not able to bind to full-length HA-RPTPα nor to HA-RPTPα-ΔD2 (Fig. 3*B*). As expected from the two-hybrid experiments, although difficult to see in Fig. 3, the binding affinities were not the same for all RPTP-D2s. As depicted clearly in Fig. 4, RPTPδ-D2 bound much better to RPTPα than RPTPα-D2, whereas LAR-D2 bound intermediately. From several independent experiments, we deduced that the affinity of RPTPα for *in vivo* binding to RPTP-D2s was RPTPδ-D2 > LAR-D2/RPTPσ-D2 > RPTPα-D2/RPTPμ-D2. In conclusion, we show that RPTPα interacts with RPTP-D2s from various RPTP subfamilies in living cells with different affinities.

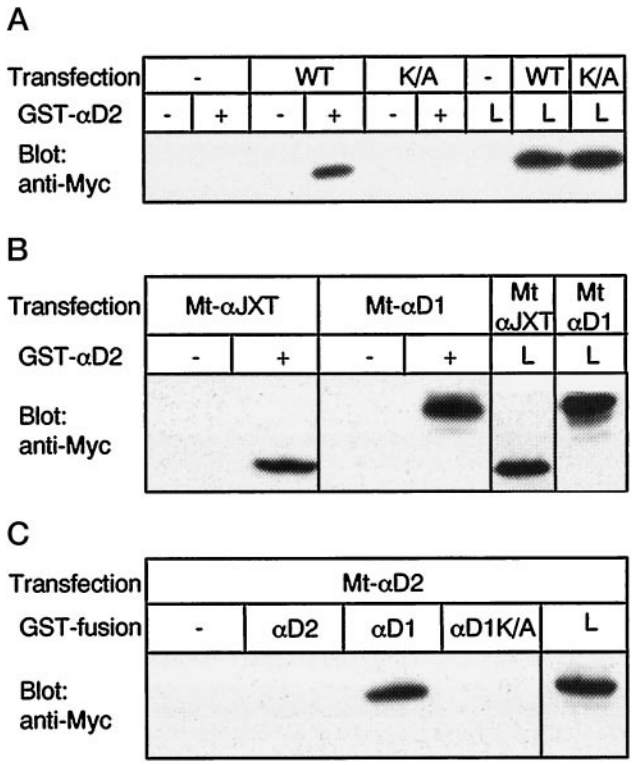

FIG. 1. **Interaction between D1 and D2 is dependent on the wedge.** *A*, bacterially expressed GST (−) or GST-RPTPα-D2 (+) were used to pull-down lysate of cells transiently transfected either with empty vector (−), Myc-tagged juxtamembrane domain of RPTPα, RPTPα-JXT (wild type (*WT*), aa 175–240), or Myc-tagged RPTPα-JXT K230A with a mutation in the wedge (*K/A*). *B*, bacterially expressed GST (−) or GST-RPTPα-D2 (+, aa 537–793) were used to pull-down lysate of cells transiently transfected either with Myc-tagged juxtamembrane of RPTPα (*Mt-αJXT*, aa 175–240) or first PTP domain, containing the wedge (*Mt-αD1*, aa 202–501). *C*, equal amounts of lysate of cells transfected with Myc-tagged second domain of RPTPα (*Mt-αD2*, aa 537–793) were used in pull-downs with 2–3 μg of bacterially expressed GST (−), GST-RPTPα-D2 (*αD2*, aa 537–793), GST-RPTPα-D1 (*αD1*, aa 202–501) or GST-RPTPα-D1K230A (*αD1K/A*). For all panels, after SDS-PAGE gel electrophoresis and blotting, the blots were probed with anti-Myc antibodies. In all panels, aliquots of the whole cell lysates were included to monitor equal expression of the different proteins (*L*).

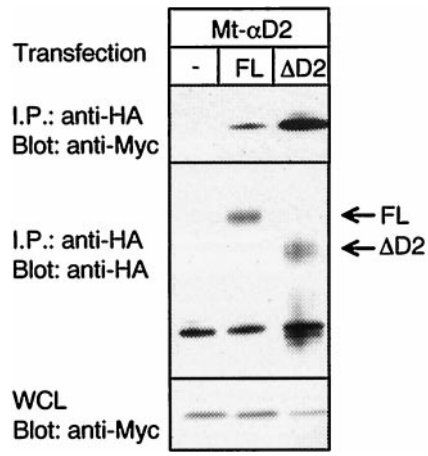

FIG. 2. **Co-immunoprecipitation of RPTPα-D2 with RPTPα *in vivo*.** 293 cells were transiently co-transfected with Myc-tagged RPTPα-D2 (*Mt-αD2*) and HA-tagged full-length RPTPα (*FL*) or a mutant, lacking D2 (Δ*D2*). The HA-tagged proteins were immunoprecipitated using 12CA5 antibody, resolved on SDS-PAGE, and transferred to PVDF membrane, and the blots were probed with anti-Myc (9E10) antibody (*top panel*) and 12CA5 antibody (*middle panel*). Equal expression of the Myc-tagged-RPTPα-D2 in the lysate was monitored (*bottom panel*). Note that deletion of the second domain (RPTPα-ΔD2) considerably increases the binding of MtRPTPα-D2 (*top panel*). *I.P.*, immunoprecipitation; WCL, whole cell lysate.

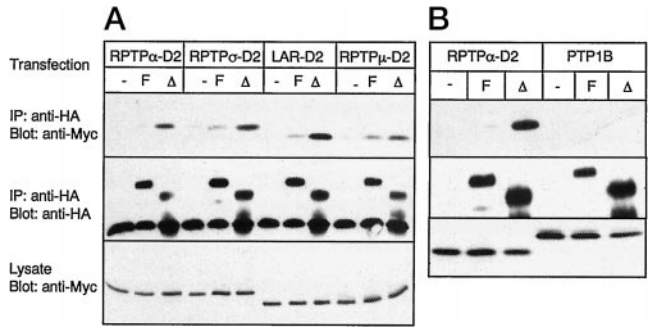

FIG. 3. **Binding of different RPTP-D2s to RPTPα *in vivo*.** HA-tagged RPTPα full-length (HA-RPTPα) or a deletion mutant (RPTPα-ΔD2) was transiently cotransfected in 293 cells with different Myc-tagged RPTP-D2s (RPTPα-D2, RPTPσ-D2, LAR-D2, or RPTPμ-D2) or as a control Myc-tagged zfPTP1B (*B*). After anti-HA immunoprecipitation (*IP*), separation on SDS-PAGE, and transfer onto PVDF membrane, the blot was probed with anti-Myc (*top panel*) and anti-HA (*middle panel*) antibodies. Equal expression of the Myc-tagged RPTP-D2s was monitored in the *bottom panel*.

In Vivo Binding between RPTPα-D1 and RPTPα-D2 Is Partially Dependent on the Wedge—To investigate the role of the wedge in the D1-D2 interaction *in vivo*, we co-expressed Myc-tagged RPTPα-D2 with HA-RPTPα wedge mutants. Relatively similar amounts of MtRPTPα-D2 coimmunoprecipitated with HA-RPTPα or with the single mutants E227A, K230A, E231A, or E234A (data not shown). Similarly, single mutations in the wedge of HA-RPTPα-ΔD2 had no effect on MtRPTP-αD2 binding (data not shown). However, single mutations might be too subtle to induce changes in *in vivo* binding. Therefore, we deleted the wedge completely from residues 207 to 238. This deletion was based on the crystal structure to minimize any possible conformational perturbation of the PTP domain. In the HA-RPTPα-ΔD2 context, complete deletion of the wedge (HA-RPTPα-ΔD2Δ207/238) led to a reproducible reduction in binding but not a complete loss (Fig. 5, *second* and *third lanes*), indicating that the *in vivo* binding was partially dependent on the wedge structure. Surprisingly, in the full-length context, the same deletion led to a reproducible increase in binding (Fig. 5, *fourth* and *fifth lanes*), suggesting that deletion of the wedge opened up a binding site for RPTPα-D2 in the full-length background. Interestingly, mutation of Glu^{228} to Ala or Arg seemed to affect the binding of RPTPα to MtRPTPα-D2 in a similar way, albeit the effects were not as pronounced (data not shown). Similar effects were found with other RPTP-D2s, including RPTP-LARD2, RPTPσ-D2, and RPTPμ-D2 (data not shown). These results indicate that the interaction between RPTPα-D1 and RPTP-D2s is partially dependent on the wedge.

Region of RPTPα-D2 Involved in Binding—To get better insight into the region in RPTPα-D2 that was important for binding to RPTPα, we made single point mutations and deletions in D2 and investigated the effect on co-immunoprecipitation. Mutations in the catalytic site of D2 (V555Y, C723S, and E690D) (24) had no effect on the binding (data not shown). Because RPTPα has autodephosphorylating activity on tyrosine 789 (Tyr^{789}), a Src phosphorylation site (9), we investigated whether the RPTPα-D1/D2 interaction was merely an enzyme-substrate interaction. Mutation of Tyr^{789} to Phe in the Myc-tagged RPTPα-D2 did not change the binding efficiency nor did the mutation of C433S in HA- RPTPα or another substrate trapping mutant, RPTPαD401A (data not shown), indicating that binding to RPTPα-D2 was not an enzyme-substrate interaction. Interestingly, deletion of the C-terminal part of RPTPα-D2 (aa 774–792) reduced the binding to RPTPα

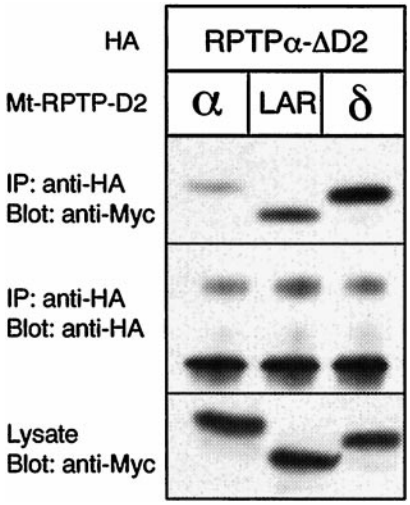

FIG. 4. **RPTP*α* has different binding affinities for different RPTP-D2s *in vivo*.** 293 cells were transfected with Myc-tagged second domain of RPTPα (α), LAR or RPTPδ (δ), and HA-RPTPα-ΔD2. After anti-HA immunoprecipitation (*IP*), separation on SDS-PAGE, and transfer onto PVDF membrane, the blot was probed with anti-Myc (*top panel*) and anti-HA (*middle panel*) antibodies. Equal expression of the Myc-tagged proteins in the lysate was monitored (*bottom panel*).

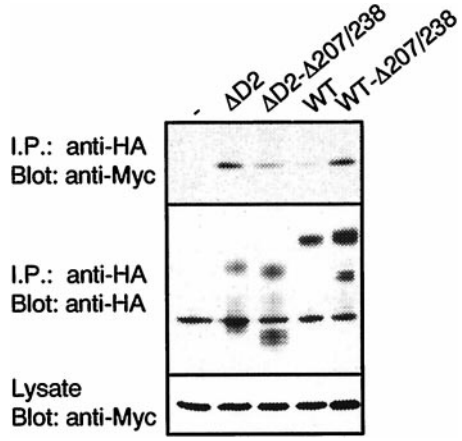

FIG. 5. **Complete deletion of the wedge affects the binding between HA-RPTP*α* and Myc-tagged RPTP*α*-D2.** 293 cells were cotransfected with Myc-tagged RPTPα-D2 and HA-RPTPα (*FL*) or HA-RPTPα-ΔD2 (Δ*D2*) with and without the wedge (Δ*207/238*). Co-immunoprecipitation (*I.P.*) was performed as described in the legend to Fig. 2. Note that deletion of the wedge in the full-length background (*FL*) leads to an increase in binding, whereas deletion of the wedge in the RPTPα-ΔD2 background leads to a decrease in binding.

and to RPTPα-ΔD2, indicating that the C terminus is involved in the interaction between RPTPαD1 and RPTPαD2 (Fig. 6). Furthermore, when HA-RPTPα-ΔD2Δ207/238 was coexpressed with RPTPα-D2ΔCt, most, if not all of the interaction was abolished, indicating independent involvement of the wedge in D1 on the one hand and the C terminus of D2 on the other (Fig. 6). These results suggest that there are at least two independent sites of binding between RPTPα and RPTPα-D2. One involves the wedge in RPTPα-D1 and the other involves the C-terminal part of RPTPα-D2.

DISCUSSION

The conservation of an inactive D2 in most RPTPs remains elusive. Here we show that RPTPα interacts directly with multiple RPTP-D2s *in vitro* and *in vivo* with varying affinities. The interactions were reproduced in three different assays: (i) in the two-hybrid system, (ii) in GST pull-downs, and (iii) by co-immunoprecipitations. Using the two first assays we showed

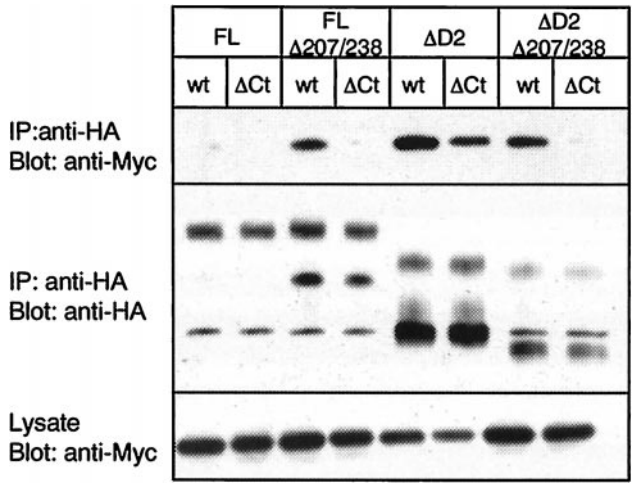

FIG. 6. **The C-terminal tail of RPTP*α*-D2 is involved in the RPTP*α*-D1/RPTP*α*-D2 interaction.** HA-RPTPα or HA-RPTPα-ΔD2 with and without the wedge (Δ*207/238*) were cotransfected in 293 cells with Myc-tagged RPTPα-D2 with (*wt*) and without the C-terminal tail (Δ*Ct*). The blot was probed with anti-Myc (*top panel*) or anti-HA (*middle panel*) antibodies. Equal expression of the Myc-tagged RPTP-D2s was monitored in the *bottom panel*.

that the integrity of the wedge in the juxtamembrane domain of RPTPα is important for efficient binding. Furthermore, we show that the binding is specific, because the equivalent wedge of LAR did not interact with any of the RPTP-D2s tested. These results are consistent with those of Wallace *et al.* (30), who showed that the juxtamembrane domain of LAR did not bind to RPTPδ-D2. Specificity was further demonstrated by variable affinities between RPTPα and different D2s. *In vivo*, multiple interaction sites were mapped between RPTPα-D1 and RPTPα-D2, in that not only the wedge in RPTPα-D1 was involved but also the C-terminal tail of RPTPα-D2.

RPTPs belong to the large PTP family, and little is known about regulation of RPTPs. Cytosolic PTPs contain specific localization sequences or protein modules that have been shown to be involved in their regulation (31). For instance, SHP-1 and SHP-2 contain a unique C-terminal PTP domain and two SH2 domains able to bind to phosphotyrosine. The crystal structure demonstrated how SHP-2 is inactivated by binding of its SH2 domain to the catalytic site of the PTP. Phosphotyrosine containing proteins that bind to the SH2 domain release the interaction leading to the opening of the structure and activation of the PTP (32), thereby confirming previous reports that Tyr(P)-containing peptides activate SHP-2 (33).

In addition to an active, membrane proximal PTP domain (D1), most RPTPs contain a second PTP domain (D2). D2 generally has no or very low activity and, at least for LAR and RPTPα, has a highly conserved three-dimensional structure in which only two residues that are absolutely conserved in active RPTP-D1s are responsible for the low D2 activity (23–25). The structural conservation of an inactive D2 in RPTPs raised the question of their biological function. Here we demonstrate that not only RPTPα-D2, but also LAR-D2, RPTPσ-D2, RPTPδ-D2, and RPTPμ-D2 interacted with RPTPα, suggesting that RPTP-D2 binding to RPTPs may play an important role in RPTP function. We were unsuccessful in detecting interactions between different full-length RPTPs in coimmunoprecipitation experiments, which was probably due to low expression levels and poor detection of some of these RPTPs.

We show here that there are multiple interaction sites between RPTPα and RPTP-D2s. For instance, the wedge to the N-terminal side of RPTPα-D1 was involved but not sufficient

for the interaction. In addition, the C terminus was involved in the interaction. Deletion of the C-terminal tail of RPTPα-D2 reduced the binding to RPTPα and, coupled to the deletion of the wedge, abolished binding completely, indicating there are two distinct sites of interaction. The presence of one of the sites apparently is sufficient to detect binding *in vivo*. It remains to be determined to which regions the wedge and the C terminus of D2 bind in RPTPα.

The role of the wedge in RPTP-D2 binding is complex (Fig. 5). Deletion of the wedge in HA-RPTPα-ΔD2 reduced RPTPα-D2 binding, suggesting the wedge directly binds RPTPα-D2, consistent with the results from our two-hybrid experiments. However, deletion of the wedge in full-length HA-RPTPα led to an increase in RPTPα-D2 binding, which suggests that a binding site for RPTPα-D2 becomes (more) available in full-length RPTPα upon deletion of the wedge. Apparently, this binding site does not become (more) available in RPTPα-ΔD2. It is noteworthy that the wedge is also involved in RPTPα-D1/RPTPα-D1 homodimerization. Deletion of the wedge will destabilize the D1/D1 interaction, which may perhaps render the second binding site more accessible to D2s. Because the wedge is involved in homodimerization of RPTPα-D1, as well as in binding to RPTPα-D2, we speculate that D2 binding to RPTPα affects RPTPα-D1 homodimerization.

Recently, the crystal structure of the tandem phosphatase domain of LAR was solved (23). The arrangement of the domains in the LAR structure indicates clearly that intramolecular interaction between the juxtamembrane domain of D1 and D2 is highly improbable, suggesting that the interaction between RPTPα-D1 and RPTPα-D2 is a reflection of an interaction *in trans*. Nevertheless, the LAR crystal structure showed an interface between LAR-D1 and LAR-D2, which mainly involves the interdomain loop, stabilized with multiple hydrogen bonds between the two PTP domains (23). Whether this interaction is strong enough to be detected in co-immunoprecipitation is not known. The residues in the interdomain loop that are involved in the LAR-D1/LAR-D2 interaction are conserved in RPTPs, including RPTPα, and they were present in all the HA-tagged constructs that we used. It is possible that, in addition to the two regions that we identified, this LAR-like interdomain interaction between RPTPα-D1 and RPTPα-D2 occurred as well. In fact, this third interface might explain the residual binding that we detected in some cases (Fig. 6). However, the presence of endogenous RPTPα (through dimerization) or a third protein might explain residual binding as well.

Our results suggest that there is competition between RPTPα-D1 and RPTP-D2s, because both RPTPα-D1 and RPTP-D2s interacted with the wedge. Such competition between D1 and D2 was already suspected for CD45 *in vitro* because homodimerization of CD45-D1 was not detected in a protein containing D1 and D2 (34). Furthermore, *in vivo*, it was shown that RPTPδ-D2 interacts with RPTPσ-D1 (30). Involvement of the wedge in the RPTPδ-D2/RPTPσ-D1 interaction was suggested on the basis of a large deletion of the juxtamembrane domain that abolished the interaction. We demonstrate, using mutagenesis of single residues, that the wedge is directly involved in binding to RPTP-D2s. The functional significance of the interaction between D2 and D1 is controversial. Wallace *et al.* (30) demonstrated that the interaction between RPTPδ-D2 and RPTPσ-D1 led to the inactivation of RPTPσ-D1. However, CD45-D1 showed an increase in catalytic activity *in vitro* when fused to CD45-D2, which was presumably due to an increase in the monomeric form (34). We were unable to detect any differences in RPTPα-D1 activity in the presence of RPTPα-D2 *in vitro* using *p*-nitrophenylphosphate as a substrate (data not shown). However, whether RPTPα-D1/D2 interactions persist during the activity assays is not known. Furthermore, interpretation of these *in vitro* PTP assays should be done with most care, because subtle differences in the experimental conditions will gravely affect the outcome of the experiments.

Because the interaction between RPTPα-D1 and RPTPα-D2 directly involved the wedge that is also involved in RPTPα-D1/RPTPα-D1 dimerization and because deletion of the wedge had striking effects on D1/D2 binding, we propose that similarly to CD45, RPTPα-D2 might regulate the dimerization state of RPTPα-D1 and thus the activity of RPTPα *in vivo*. Indeed, RPTPα dimerization, which is dependent on the wedge, negatively regulates its activity (20). Furthermore, the ability of distant RPTP-D2s (LAR, RPTPσ, RPTPδ, and RPTPμ) to bind to RPTPα raises the possibility that RPTPα is regulated by other RPTPs or regulates other RPTPs. The differences in binding efficiency that we detected are surprising, given the high homology between RPTP-D2s and suggest a precise interplay between RPTPs. It is noteworthy that the binding efficiencies appear to be low, in that only a small proportion of the RPTP-D2s bound to RPTPα in the coimmunoprecipitation experiments (Figs. 2–6). Perhaps the low binding affinities of the RPTP-D2s reflect that binding is dynamic, allowing rapid changes in the equilibrium between bound and not bound because of subtle changes in conditions.

For the interactions we describe here to occur, the different RPTPs have to be expressed in the same cell. It is noteworthy that RPTPα, LAR, and RPTPσ have different but overlapping expression patterns during mouse embryogenesis. For instance, RPTPα and LAR are both expressed in mouse dorsal root ganglia (35–37). Furthermore, both LAR and RPTPα have been shown, albeit in different studies, to localize to the growth cone of growing neurons (36, 38, 39). From *Drosophila* work, it is clear that different RPTPs cooperate in a fashion that is still unclear (40). Here, we show that directional interactions between different subfamilies of RPTPs exist with a central role for RPTPα. We further show that the interactions involve the wedge structure of RPTPα, suggesting a direct role for these interactions in regulation of catalytic activity and thus in crosstalk between RPTPs.

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Multiple Interactions between Receptor Protein-tyrosine Phosphatase (RPTP) α and Membrane-distal Protein-tyrosine Phosphatase Domains of Various RPTPs

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