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Catalytically active membrane-distal phosphatase domain of receptor protein-tyrosine phosphatase α is required for Src activation

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Receptor protein-tyrosine phosphatase α (RPTPα) is a transmembrane protein with tandem cytoplasmic phosphatase domains. Most of the catalytic activity is contained by the membrane-proximal catalytic domain (D1). We found a spontaneous Arg554 to His mutation in the pTyr recognition loop of the membrane-distal phosphatase domain (D2) of a human patient. This mutation was not linked to the disease. Here, we report that the R554H mutation abolished RPTPα-D2 catalytic activity. The R554H mutation impaired Src binding to RPTPα. RPTPα, with a catalytic site cysteine to serine mutation in D2, also displayed diminished binding to Src. Concomitant with decreased Src binding of the R554H and C723S mutants compared with wild-type RPTPα, enhanced phosphorylation of the inhibitory Src Tyr527 site was observed, as well as reduced Src activation. To confirm that catalytic activity of RPTPα-D2 was required for these effects, we analyzed a third mutant, RPTPα-R729K, which had an inactive D2. Again, Src binding was reduced and Tyr527 phosphorylation was enhanced. Our results suggest that a catalytically active D2 is required for RPTPα to bind and dephosphorylate its well-characterized substrate, Src.

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Introduction

Receptor protein-tyrosine phosphatases (RPTPs), like their cytoplasmic relatives, counteract the activity of tyrosine kinases by dephosphorylating phosphotyrosine residues. Based on the structure of their extracellular domain, the RPTPs are classified into eight subtypes [1–3]. The ectodomains may play an important role in the regulation of the RPTPs following cell–cell or cell–matrix contacts, or upon interaction with specific extracellular ligands [4,5]. Besides the extracellular domain, the majority of the RPTPs present another interesting feature: tandem catalytic domains. The membrane-proximal catalytic domain (D1) and the membrane-distal phosphatase domain (D2) of RPTPs are highly conserved, in that the D2 domains contain a protein-tyrosine phosphatase (PTP) signature motif, similarly to the D1 domains [6]. In addition, the 3D structures are conserved between D1 and D2 domains [7–9]. However, most RPTP-D2 domains have very low or no catalytic activity [10–12]. In the case of LAR and RPTPα the absence of two residues in the D2 domain– the

Abbreviations
D1, membrane-proximal catalytic domain; D2, membrane-distal phosphatase domain; GST, glutathione *S*-transferase; HA, hemagglutinin; LAR, leukocyte common antigen related; pNPP, para-nitrophenylphosphate; PTP, protein-tyrosine phosphatase; RPTP, receptor protein-tyrosine phosphatase; TCR, T-cell receptor.

tyrosine from the pTyr recognition loop (also known as the KNRY motif) and the aspartic acid from the WPD loop – was shown to be responsible for decreased catalytic activity. Upon substitution of these two residues, the catalytic activity of the D2 domains was greatly increased [7,13,14]. RPTPε-D2, but not CD45-D2, regained catalytic activity upon changing the two amino acids mentioned above [15].

The biological function of these membrane-distal domains is not completely understood. Soon after they were discovered, the RPTP-D2s were suggested to alter the substrate specificity of D1 *in vitro* [10,11]. In addition, the D2 domains were shown to be involved in intermolecular and intramolecular interactions, as well as in homodimerization and oligomerization of RPTPs [9,16–21]. Based on the crystal structures of LAR and CD45, another role of the D2 domain was proposed: stabilization of the D1 domain [7,22]. These interactions suggest that the D2 domains function as regulators of the activity of the D1 domains. LAR-D2 was shown to be important for the interaction with downstream effectors, including Trio [23], Abl kinase and Enabled [24], β-catenin [25] and Liprin-α [26]. Another study indicated that an acidic region from CD45-D2 is required for the regulation of TCR-mediated calcium-signaling pathways [27]. The involvement of D2 domains in substrate recognition was observed for CD45-D2, which seems to mediate the interaction with Lck [28].

The D2 domain of RPTPα is the only known membrane-distal domain with considerable catalytic activity [10,14,29]. It was shown that its activity is comparable to, or even higher than, the activity towards para-nitrophenylphosphate (pNPP) of dual-specificity phosphatases such as cdc25, VH1 and YPTP1 [14]. One of the main roles identified to date for RPTPα-D2 is that of a redox sensor. This function is dependent on its catalytic cysteine (Cys723). Following hydrogen peroxide-induced oxidation, this cysteine mediates the stabilization of RPTPα dimers followed by complete inactivation of the enzyme and rotational coupling of the extracellular domain [30,31]. RPTPα-D2 is essential for RPTPα homodimerization in the absence of oxidizing reagents [32] and has a role in pervanadate-induced tyrosine phosphorylation of RPTPα [33], showing that D2 is involved in protein–protein interactions. Besides the interaction with other phosphatase domains, RPTPα-D2 binds to calmodulin, leading to the inactivation of the D2 domain [34].

We discovered an R554H mutation in the KNRY motif of RPTPα-D2 in a screen for disease-related mutations in RPTPs. Whereas this mutation appears not to be linked to disease, we observed that this mutation in the pTyr recognition loop of RPTPα-D2 completely abolished catalytic activity. Furthermore, we observed decreased binding of Src, a well-known RPTPα substrate, to RPTPα-R554H and to RPTPα-C723S, another mutant with an inactive D2 domain. Src Tyr527 dephosphorylation and activation was also reduced in response to these mutations, compared with wild-type RPTPα. A third mutant, RPTPα-R729K, with impaired catalytic activity in the D2 domain, confirmed that a catalytically active D2 domain appears to be required for Src binding and Tyr527 dephosphorylation.

Results

Identification of a naturally occurring mutation in RPTPα

We hypothesized that mutations in RPTPα might be linked to Noonan syndrome, a dominantly inherited human syndrome. Several genes have been identified that are associated with Noonan syndrome, most prominently *PTPN11*, encoding the cytoplasmic PTP, Shp2. Approximately 50% of all patients with Noonan syndrome contain dominant activating mutations in *PTPN11* [35]. Other genes that are associated with Noonan syndrome encode factors in the Ras–mitogen-activated protein kinase (MAPK) pathway: SOS1, KRAS, BRAF and RAF1 [36–39]. To assess whether RPTPα is also involved in Noonan syndrome, we sequenced all 22 exons of *PTPRA* in a panel of 46 patients with Noonan syndrome who did not contain mutations in genes that are known to be associated with Noonan syndrome. We identified a heterozygous point mutation in exon 16 in a single patient, resulting in a missense mutation, R554H, in the absolutely conserved Arg residue of the KNRY motif or pTyr loop of RPTPα-D2. Subsequently, *PTPRA* was sequenced in the unaffected parents of this *de novo* patient and it was found that the mother carried the same mutation, making a causal role for the R554H mutation in RPTPα in Noonan syndrome unlikely. Subsequently, a mutation was identified in *SOS1*, resulting in the missense mutation T266K in the Sos1 protein. This mutation has also been identified in other Noonan patients and we therefore concluded that Noonan syndrome in this patient was probably caused by the missense mutation in *SOS1*, not by a mutation in *PTPRA*. Nevertheless, biochemically, RPTPα–R554H behaved differently from wild-type RPTPα.

R554H mutation abolished catalytic activity of RPTPα-D2

RPTPα Arg554 is an absolutely conserved residue in the pTyr recognition loop (the KNRY motif). This

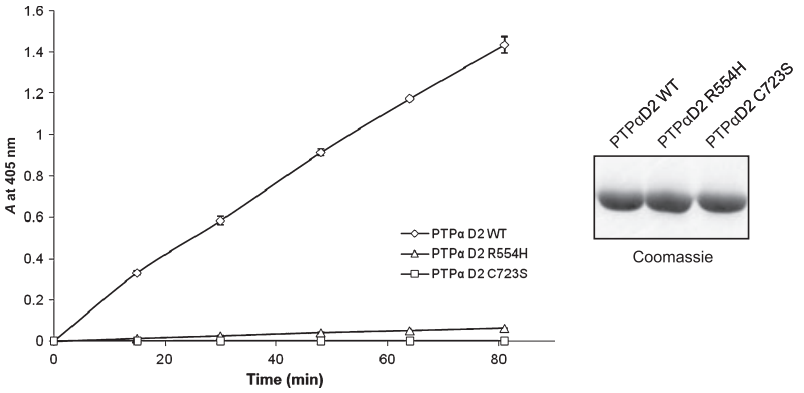

Fig. 1. The R554H mutation abolished RPTPα-D2 catalytic activity. The catalytic activity of GST-fusion proteins containing the D2 domain of RPTPα WT, R554H or C723S was tested *in vitro* using pNPP as the substrate. The error bars represent ± SD of three independent phosphatase activity determinations. Equal amounts of protein were used in the PTP experiment, as indicated in the inset, showing Coomassie Brilliant Blue-stained fusion proteins on an SDS-polyacrylamide gel.

Arg residue is important for electrostatic attraction of ligands and is a putative substrate-binding site [6]. We tested whether the R554H mutation had an influence on the catalytic activity of RPTPα-D2 *in vitro* by using glutathione *S*-transferase (GST)-fusion proteins and pNPP as a substrate. The phosphatase activity of D2-R554H was compared with that of wild-type (WT)-D2 and with that of D2-C723S, a catalytically dead mutant with a mutation in the essential catalytic site cysteine. The catalytic activity of D2-R554H was dramatically reduced compared with that of WT-D2 and only slightly higher than the activity of D2-C723S, the inactive mutant (Fig. 1). These results show that the Arg554 residue is essential for the catalytic activity of RPTPα-D2.

Catalytic activity of the RPTPα D2 domain is required for Src binding and activation

It is well known that the membrane distal domains of RPTPs are involved in protein–protein interactions [16–18,20,28]. We investigated whether the introduction of inactivating mutations in RPTPα-D2 (R554H and C723S) affected the interaction with Src, the well-established substrate of RPTPα. For this purpose, SYF cells that lack endogenous Src, Fyn and Yes were transiently cotransfected with constructs encoding Src and hemagglutinin (HA)-tagged RPTPα WT, RPTPα-R554H or RPTPα-C723S. Src was immunoprecipitated from the SYF lysates and the samples were probed for co-immunoprecipitated (mutant) RPTPα. HA-RPTPα-R554H binding to Src was substantially reduced (by approximately 50%) when compared with the binding of WT RPTPα to Src (Fig. 2A). The interaction of Src with the RPTPα-C723S mutant was decreased to a similar extent. The interaction between RPTPα and Src may be mediated by pTyr789. Therefore, we tested whether the phosphorylation of Tyr789 was affected in R554H and C723S mutants and no significant differences in the pTyr789 levels were observed. Tyr789 phosphorylation of endogenous RPTPα was not detected (Fig. 2A, bottom panel, lane 2), because expression of endogenous RPTPα is relatively low in SYF cells and only approximately 10% of the cells are transfected with Src, which induces phosphorylation of RPTPα Tyr789. These results indicate a role for RPTPα-D2 in the interaction with Src, which is separate from the phosphorylation of Tyr789.

Next, we analyzed the ability of RPTPα and mutants to activate Src. Fractions of the same lysates used for Src immunoprecipitation were tested for Src Tyr416 and Tyr527 phosphorylation, indicators of Src activation. When cotransfected with wild-type RPTPα, phosphorylation of the inhibitory pTyr527 was reduced (Fig. 2B; see also Fig. 4B, cf. lanes 2 and 3). The effects of cotransfection of mutant RPTPα-R554H on Src phosphorylation were less pronounced than of wild-type RPTPα. Src pTyr527 dephosphorylation in RPTPα-R554H transfected cells was approximately 65% of pTyr527 dephosphorylation in wild-type RPTPα cotransfected cells. Cotransfection of RPTPα-C723S with Src also led to a significant decrease in Src pTyr527 (approximately 46% of wild-type RPTPα). Src Tyr416 autophosphorylation was reduced in R554H and C723S transfected cells, to 83% and 59%, respectively, compared with wild-type cotransfected cells (Fig. 2B). These results suggest that RPTPα-mediated Src dephosphorylation was impaired in RPTPα mutants with inactive D2 domains.

To establish the ability of RPTPα-D2 mutants to activate Src, we tested the *in vitro* kinase activity of Src immunoprecipitated from SYF cells cotransfected with Src and RPTPα WT, R554H or C723S. The activity of Src in the presence of RPTPα R554H was clearly reduced compared with the activity of Src cotransfected with WT RPTPα. RPTPα-C723S activated Src only minimally under these experimental conditions (Fig. 3A). Three independent experiments

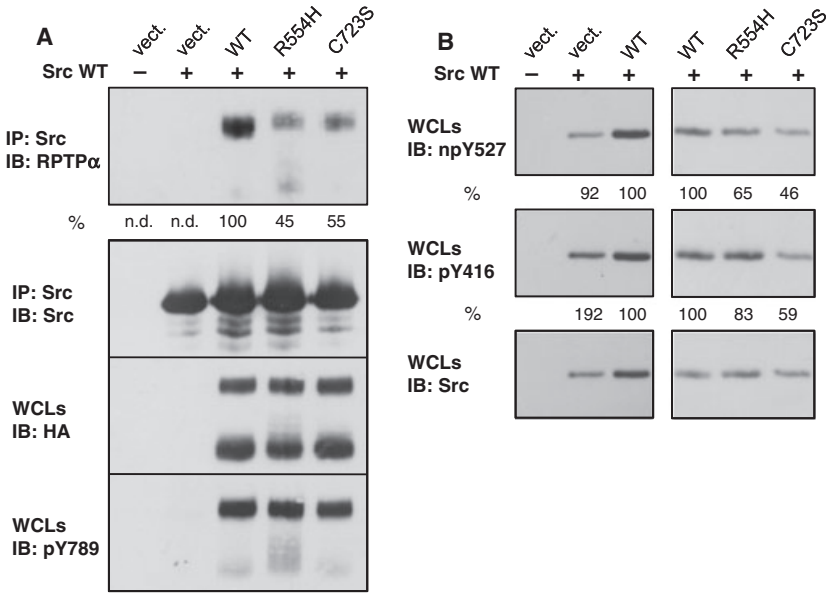

Fig. 2. Src association with RPTPα-R554H and RPTPα-C723S mutants is reduced. (A) SYF cells were cotransfected with Src and HA-RPTPα WT, R554H or C723S, lysed and Src was immunoprecipitated with cross-linked Src mAbs. The samples were fractionated on a 7.5% SDS-polyacrylamide gel, transferred to poly(vinylidene difluoride) (PVDF) membranes and immunoblotted with anti-RPTPα serum and with the Src mAb 327. Whole-cell lysates were probed with the HA mAb 12CA5 to monitor HA-RPTPα expression and with anti-pY789 to assess Tyr789 phosphorylation. The amount of co-immunoprecipitated RPTPα was quantified and normalized for total pTyr789 levels. The samples with no transfected RPTPα were not determined (n.d.). (B) A fraction of the lysates used for Src immunoprecipitation was boiled in SDS sample buffer and the samples were run on a 7.5% SDS-polyacrylamide gel. The proteins were transferred to PVDF membranes, which were then probed with anti-npY527 Ig and subsequently, after stripping, with anti-pY416 and anti-Src Igs, as indicated. Src phosphorylation was quantified and the values were normalized for the total amounts of Src. The quantification results are presented under the corresponding panels. This experiment was carried out three times and the results of one representative experiment are depicted here.

were quantified and the results indicated that WT RPTPα activated Src 2.2-fold. RPTPα-R554H activated Src 1.7-fold and RPTPα-C723S did not activate Src significantly (1.1-fold) (Fig. 3B). These results indicate that the catalytic activity of RPTPα-D2 plays an important role in Src activation.

To confirm that the catalytic activity of RPTPα-D2 is required for Src binding and activation, we used an RPTPα-D2 mutant (RPTPα-R729K) with an Arg to Lys mutation in the PTPase signature motif in the D2 domain. This Arg residue has an essential role in catalysis in PTPs, and mutation of this residue in PTPs results in the inactivation of catalytic activity, but does not result in substrate-trapping mutants. Likewise, mutant RPTPα-D2-R729K has no catalytic activity in the D2 domain [33]. Src was co-expressed in SYF cells with RPTPα-R729K and Src was immunoprecipitated. The RPTPα-R729K mutant showed a reduction in Src binding of approximately 75% compared with RPTPα WT (Fig. 4A). In the presence of RPTPα-R729K, Src pTyr527 dephosphorylation was strongly decreased to 20% of the wild-type RPTPα cotransfected response, and Tyr416 was only mildly affected (Fig. 4B), suggesting that RPTPα-R729K is not able to dephosphorylate Src pTyr527 and activate Src. Taken together, these results indicate that catalytically active RPTPα-D2 is required for binding and activation of Src.

Discussion

Here we report that inactivating mutations in the membrane-distal domain of RPTPα affected the biological function of RPTPα, impairing Src binding and its ability to activate Src. Our results indicate that a catalytically active D2 domain is required for RPTPα-mediated Src binding and activation.

We identified a heterozygous mutation in RPTPα in a patient with *de novo* Noonan syndrome and we found the same heterozygous mutation in one of the unaffected parents, indicating that this mutation was not causally linked to Noonan syndrome. We demonstrate here that RPTPα-R554H was functionally impaired. Apparently, a single wild-type allele of RPTPα is sufficient for human life and the R554H

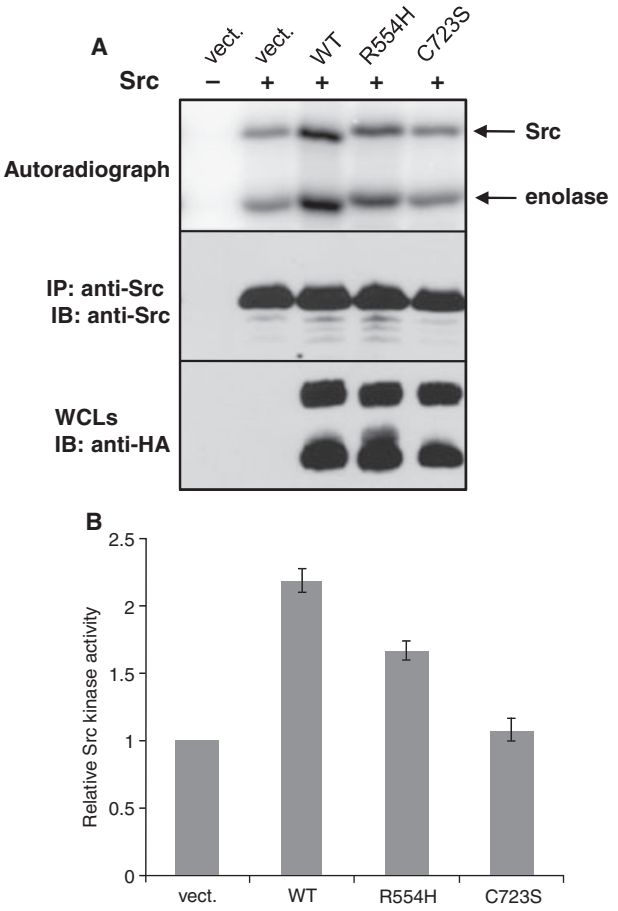

Fig. 3. Reduced activation of Src by RPTPα-R554H and RPTPα-C723S. (A) Src was immunoprecipitated from SYF cells cotransfected with Src and RPTPα constructs. Half of the immunoprecipitate was subjected to an *in vitro* kinase assay, using enolase as a substrate, and the amount of incorporated phosphate was visualized by autoradiography (top panel). The positions of enolase and Src are indicated. The other half of the immunoprecipitate was fractionated by electrophoresis on a 7.5% SDS-polyacrylamide gel, blotted, probed with anti-Src Ig and developed with enhanced chemiluminescence (ECL) (middle panel). Part of the lysate was resolved on a 7.5% SDS-polyacrylamide gel, transferred to poly(vinylidene difluoride) (PVDF) membrane and probed for total RPTPα expression (bottom panel). (B) Relative Src kinase activity. Each bar represents the average of three independent experiments ± SD, relative to Src immunoprecipitated from cells cotransfected with the empty vector, which was set to one.

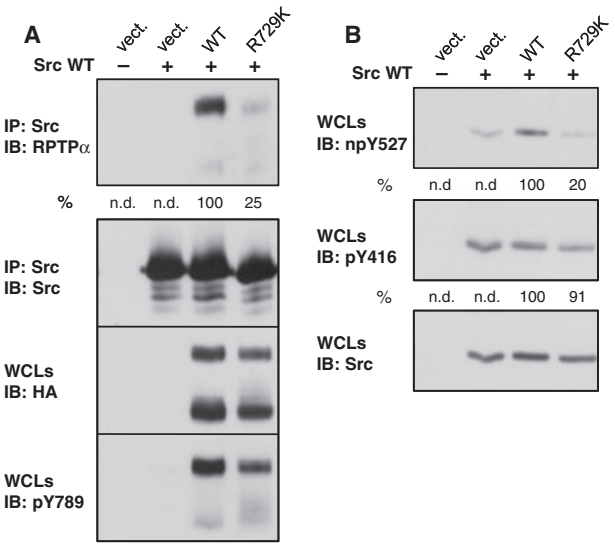

Fig. 4. Impaired Src binding to the inert RPTPα-R729K mutant. (A) SYF cells cotransfected with Src and empty vector, HA-RPTPα WT or HA-RPTPα-R729K were lysed and Src was immunoprecipitated. The samples obtained were run on 7.5% SDS-polyacrylamide gels, transferred to poly(vinylidene difluoride) (PVDF) membranes and probed for co-immunoprecipitated RPTPα and total Src. Lysates were probed for the amount of RPTPα and for pTyr789. (B) A fraction of the cell lysates used for Src immunoprecipitation was processed and tested for Src pTyr416 and npTyr527. Quantification of the blots was performed as described in Fig. 2. This experiment was carried out three times and a representative result is depicted here.

mutation does not have a dominant effect over the wild-type RPTPα allele. It is noteworthy that heterozygous RPTPα knockout mice are indistinguishable from wild-type siblings [40,41], corroborating the conclusion that a single RPTPα allele is sufficient for mammalian life.

Mutant RPTPα-D2-R554H was almost completely inactive compared with WT RPTPα-D2. Arg554 is located in the pTyr recognition loop (the so-called KNRY motif) next to Val555, one of the amino acids responsible for decreased catalytic activity of RPTPα-D2 [13]. The conserved arginine residue was proposed to be involved in the electrostatic attraction of the substrate [6], and mutation of the corresponding arginine (Arg45) to Ala in PTP1B led to very low catalytic activity, probably as a result of structural perturbation of the catalytic site [42]. As seen from the crystal structure of RPTPα-D2 [8], Arg554 is positioned very close to Cys723, and mutation of this amino acid could indeed disturb the architecture of the catalytic site. The *in vitro* catalytic activity of the entire cytoplasmic domain of RPTPα was not significantly affected by the R554H mutation (data not shown) but this is not unexpected because the D2 domain only marginally contributes to the overall catalytic activity of RPTPα [10,14,29].

Src binding to three different RPTPα-D2 inactive mutants was impaired. Moreover, the ability of these mutants to activate Src was reduced when compared with WT RPTPα. According to the current model, Src binds to phosphorylated Tyr789 of RPTPα via its SH2

domain [43]. We did not observe significant changes in Tyr789 phosphorylation of the D2 domain-inactive mutants compared with WT RPTPα (Figs 2A and 4A) that would account for reduced Src binding to these RPTPα mutants. In contrast to the current model, we have evidence that Tyr789 is not required for Src binding, in that mutation of Tyr789 in RPTPα did not abolish Src binding to RPTPα (AV, JdH, submitted), suggesting the presence of other Src-binding sites in RPTPα. Taken together, phosphorylation of Tyr789 is not the only determinant in Src binding. Instead, we provide evidence that other features of RPTPα-D2 mediate Src binding. In addition, we cannot exclude the possibility that the Src-binding site is not located in RPTPα-D2.

Functional analysis of the tandem PTP domains of RPTPα indicated that mutation of the catalytic Cys723 did not affect phosphorylation of the Src family kinase Fyn, or Fyn autophosphorylation activity [44]. The apparent difference with our results may be caused by differences in experimental conditions: they assessed total Fyn phosphorylation, whereas we detected phosphorylation on two specific sites in Src; they determined Fyn autophosphorylation, whereas we assessed Src kinase activity by measuring the *in vitro* phosphorylation of an exogenous substrate. Alternatively, the apparent difference may reflect the fact that the effects we observed are specific for Src.

Ever since their discovery, it has been speculated that RPTP D2 domains may have a role in substrate binding and substrate presentation to the catalytically active D1 domain [45]. This hypothesis was confirmed in a study showing that the catalytic site of LAR-D2 is required for binding to the insulin receptor, a known LAR substrate [46]. The interaction is decreased when the catalytic cysteine in LAR-D2 is mutated to serine. Another example is CD45 binding to the Src family kinase Lck, which is mediated by a unique acidic region in CD45-D2 [28]. CD45-D2 is also critical for substrate recruitment of TCR-zeta *in vivo*, because replacement of the membrane-distal domain of CD45 with the LAR D2 domain abolishes the binding of TCR-zeta [47].

It remains to be determined definitively how the three mutants that we analyzed affect Src binding and activation. It is unlikely that these mutations disrupt the structure of RPTPα-D2, because the mutations are subtle. Moreover, Cys to Ser mutations of other PTPs showed the expected crystal structures. In conclusion, we demonstrate here, for the first time, that a functional, catalytically active D2 is required for RPTPα to bind to its substrate, Src, and to dephosphorylate and activate it.

Materials and methods

Materials and antibodies

Anti-HA-tag (12CA5), anti-Src (327) Igs and anti-RPTPα (5478AP) serum were prepared as previously described [48,49]. Anti-Src-npY527 was from Cell Signaling and anti-Src-pY418 was from Biosource. Horseradish peroxidase-coupled anti-rabbit and anti-mouse secondary Igs were from BD Biosciences. Polyethylenimine, nocodazole, paclitaxel, glutathione–Sepharose and hydrogen peroxide were from Sigma Life Science. FuGene6 transfection reagent was from Roche.

DNA constructs

The constructs used for the expression of HA-RPTPα WT [48], Src WT [49] and HA-RPTPα R729K [33] were as previously described. HA-RPTPα R554H was obtained by site-directed mutagenesis using HA-RPTPα WT as the template and the following forward and reverse oligonucleotides: 5′- ATG AAG AAG AAC CAT GTT TTA CAG ATC -3′ and 5′ - GAT CTG TAA AAC ATG GTT CTT CTT CAT - 3′. The constructs encoding WT, R554H or C723S GST-PTPalpha D2 fusion proteins were obtained by directional cloning of PCR fragments digested with *Nco*I and *Hin*dIII into the pGEX-KG vector digested with the same restriction enzymes. The PCR fragments were obtained by amplification with 5′ - CCC ATG GCT TCT CTA GAA ACC - 3′ and 5′ - CGC AAG CTT TCA CTT GAA GTT GGC - 3′ oligonucleotides using pSG RPTPα WT, pSG RPTPα R554H or pSG RPTPα C723S as templates. The constructs were verified by sequencing.

Cell culture and transfection

For the experiments described in this study we used SYF cells. SYF cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal bovine serum. The cells were transfected with FuGene6 according to the protocol provided by the manufacturer. After transfection the cells were grown for 16 h in complete medium, then the medium was replaced with serum-free medium and the cells were grown for an additional 24 h.

Recombinant proteins

The constructs encoding GST-fusion proteins were transformed into BL21 bacteria. Expression of fusion proteins was induced by incubation with 0.1 mM isopropyl thio-β-D-galactoside (IPTG) for 5 h at 25 °C. The bacteria were harvested by centrifugation, resuspended in NaCl/Tris solution containing 1 μg·mL of leupeptin, 1 $\mu g \cdot mL^{-1}$ of aprotinin and 1 $mg \cdot mL^{-1}$ of lysozyme, and incubated for 10 min at room temperature. The suspension was sonicated on ice,

supplemented with 1% Triton X-100, kept for 10 min on ice and centrifuged to collect the soluble proteins. The supernatants were mixed with glutathione agarose to pull-down the GST-fusion proteins, and incubated for 30 min at 4 °C. The beads were washed three times with ice-cold NaCl/Tris. The GST-fusion proteins were eluted twice for 5 min at room temperature with elution buffer containing 50 mM Tris/HCl, pH 8.0, 10 mM reduced glutathione and 10% glycerol. The proteins were dialyzed against NaCl/Tris containing 10% glycerol.

Immunoprecipitation and immunoblotting

SYF cells were lysed for 20 min on ice in cell lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 1% Triton X-100, 10% glycerol, 5 mM NaF, 5 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 $\mu g \cdot mL^{-1}$ of leupeptin and 1 $\mu g \cdot mL^{-1}$ of aprotinin). The lysates were collected using a cell scraper and centrifuged for 10 min at 13000 rpm. Samples from the lysates were collected and boiled after being mixed with equal volumes of 2× SDS sample buffer (125 mM Tris/HCl, pH 6.8, 20% glycerol, 4% SDS, 2% β-mercaptoethanol and 0.04% Bromophenol Blue) and resolved on 7.5% SDS/PAGE gels. For Src immunoprecipitation the lysates were incubated for 1 h at 4 °C with the Src mAb 327 cross-linked to Protein A–Sepharose. The immunoprecipitates were washed four times with HNTG buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% Triton-X-100 and 10% glycerol).

In vitro Src kinase assay

Src was immunoprecipitated as described above. The immunoprecipitates were washed three times with HNTG buffer and once with kinase buffer, and then divided into two equal fractions, of which one was immunoblotted using anti-Src Ig and the other was subjected to the kinase assay. The reactions were performed in 40 μL of kinase reaction buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 5 mM NaF and 1 mM Na_3VO_4), containing 10 μCi of [^{32}P]dATP[γP] and 3.5 μg of acid-denatured enolase. The reactions were incubated at 30 °C for 30 min, stopped by the addition of 2× SDS sample buffer and resolved by electrophoresis on a 7.5% SDS-polyacrylamide gel. The results were visualized by autoradiography.

Phosphatase assay

The activity of the RPTPα-D2 domain was investigated *in vitro* using GST-fusion proteins and pNPP as the substrate. The reaction was conducted in 200 μL of mixture containing 20 mM Mes, pH 6.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 10 mM pNPP. The reaction was initiated by the addition of fusion protein and then incubated at 30 °C for the times indicated. One millilitre of 1 N NaOH was added to quench the reaction, and the formation of p-nitrophenol was detected at *A* 405 nm using a spectrophotometer.

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References

1 Alonso A, Sasin J, Bottini N, Friedberg I, Osterman A, Godzik A, Hunter T, Dixon J & Mustelin T (2004) Protein tyrosine phosphatases in the human genome. *Cell* **117**, 699–711.
2 Brady-Kalnay SM & Tonks NK (1995) Protein tyrosine phosphatases as adhesion receptors. *Curr Opin Cell Biol* **7**, 650–657.
3 Tonks NK (2006) Protein tyrosine phosphatases: from genes, to function, to disease. *Nat Rev Mol Cell Biol* **7**, 833–846.
4 Tabernero L, Aricescu AR, Jones EY & Szedlacsek SE (2008) Protein tyrosine phosphatases: structure-function relationships. *FEBS J* **275**, 867–882.
5 den Hertog J, Ostman A & Bohmer FD (2008) Protein tyrosine phosphatases: regulatory mechanisms. *FEBS J* **275**, 831–847.
6 Andersen JN, Mortensen OH, Peters GH, Drake PG, Iversen LF, Olsen OH, Jansen PG, Andersen HS, Tonks NK & Moller NP (2001) Structural and evolutionary relationships among protein tyrosine phosphatase domains. *Mol Cell Biol* **21**, 7117–7136.
7 Nam HJ, Poy F, Krueger NX, Saito H & Frederick CA (1999) Crystal structure of the tandem phosphatase domains of RPTP LAR. *Cell* **97**, 449–457.
8 Sonnenburg ED, Bilwes A, Hunter T & Noel JP (2003) The structure of the membrane distal phosphatase domain of RPTPalpha reveals interdomain flexibility and an SH2 domain interaction region. *Biochemistry* **42**, 7904–7914.
9 Barr AJ, Ugochukwu E, Lee WH, King ON, Filippakopoulos P, Alfano I, Savitsky P, Burgess-Brown NA, Muller S & Knapp S (2009) Large-scale structural analysis of the classical human protein tyrosine phosphatome. *Cell* **136**, 352–363.
10 Wang Y & Pallen CJ (1991) The receptor-like protein tyrosine phosphatase HPTP alpha has two active catalytic domains with distinct substrate specificities. *EMBO J* **10**, 3231–3237.
11 Streuli M, Krueger NX, Thai T, Tang M & Saito H (1990) Distinct functional roles of the two intracellular phosphatase like domains of the receptor-linked protein

tyrosine phosphatases LCA and LAR. *EMBO J* **9**, 2399–2407.
12 Gebbink MF, Verheijen MH, Zondag GC, van Etten I & Moolenaar WH (1993) Purification and characterization of the cytoplasmic domain of human receptor-like protein tyrosine phosphatase RPTP mu. *Biochemistry* **32**, 13516–13522.
13 Buist A, Zhang YL, Keng YF, Wu L, Zhang ZY & den Hertog J (1999) Restoration of potent protein-tyrosine phosphatase activity into the membrane-distal domain of receptor protein-tyrosine phosphatase alpha. *Biochemistry* **38**, 914–922.
14 Lim KL, Ng CH & Pallen CJ (1999) Catalytic activation of the membrane distal domain of protein tyrosine phosphatase epsilon, but not CD45, by two point mutations. *Biochim Biophys Acta* **1434**, 275–283.
15 Lim KL, Lai DS, Kalousek MB, Wang Y & Pallen CJ (1997) Kinetic analysis of two closely related receptor-like protein-tyrosine-phosphatases, PTP alpha and PTP epsilon. *Eur J Biochem* **245**, 693–700.
16 Blanchetot C, Tertoolen LG, Overvoorde J & den Hertog J (2002) Intra- and intermolecular interactions between intracellular domains of receptor protein-tyrosine phosphatases. *J Biol Chem* **277**, 47263–47269.
17 Wallace MJ, Fladd C, Batt J & Rotin D (1998) The second catalytic domain of protein tyrosine phosphatase delta (PTP delta) binds to and inhibits the first catalytic domain of PTP sigma. *Mol Cell Biol* **18**, 2608–2616.
18 Blanchetot C & den Hertog J (2000) Multiple interactions between receptor protein-tyrosine phosphatase (RPTP) alpha and membrane-distal protein-tyrosine phosphatase domains of various RPTPs. *J Biol Chem* **275**, 12446–12452.
19 Hayami-Noumi K, Tsuchiya T, Moriyama Y & Noumi T (2000) Intra- and intermolecular interactions of the catalytic domains of human CD45 protein tyrosine phosphatase. *FEBS Lett* **468**, 68–72.
20 Felberg J & Johnson P (1998) Characterization of recombinant CD45 cytoplasmic domain proteins. Evidence for intramolecular and intermolecular interactions. *J Biol Chem* **273**, 17839–17845.
21 Toledano-Katchalski H, Tiran Z, Sines T, Shani G, Granot-Attas S, den Hertog J & Elson A (2003) Dimerization in vivo and inhibition of the nonreceptor form of protein tyrosine phosphatase epsilon. *Mol Cell Biol* **23**, 5460–5471.
22 Nam HJ, Poy F, Saito H & Frederick CA (2005) Structural basis for the function and regulation of the receptor protein tyrosine phosphatase CD45. *J Exp Med* **201**, 441–452.
23 Debant A, Serra-Pages C, Seipel K, O'Brien S, Tang M, Park SH & Streuli M (1996) The multidomain protein Trio binds the LAR transmembrane tyrosine phosphatase, contains a protein kinase domain, and has separate rac-specific and rho-specific guanine nucleotide exchange factor domains. *Proc Natl Acad Sci U S A* **93**, 5466–5471.
24 Wills Z, Bateman J, Korey CA, Comer A & Van Vactor D (1999) The tyrosine kinase Abl and its substrate enabled collaborate with the receptor phosphatase Dlar to control motor axon guidance. *Neuron* **22**, 301–312.
25 Kypta RM, Su H & Reichardt LF (1996) Association between a transmembrane protein tyrosine phosphatase and the cadherin-catenin complex. *J Cell Biol* **134**, 1519–1529.
26 Serra-Pages C, Kedersha NL, Fazikas L, Medley Q, Debant A & Streuli M (1995) The LAR transmembrane protein tyrosine phosphatase and a coiled-coil LAR-interacting protein co-localize at focal adhesions. *EMBO J* **14**, 2827–2838.
27 Wang Y, Liang L & Esselman WJ (2000) Regulation of the calcium/NF-AT T cell activation pathway by the D2 domain of CD45. *J Immunol* **164**, 2557–2564.
28 Felberg J, Lefebvre DC, Lam M, Wang Y, Ng DH, Birkenhead D, Cross JL & Johnson P (2004) Subdomain X of the kinase domain of Lck binds CD45 and facilitates dephosphorylation. *J Biol Chem* **279**, 3455–3462.
29 Wu L, Buist A, den Hertog J & Zhang ZY (1997) Comparative kinetic analysis and substrate specificity of the tandem catalytic domains of the receptor-like protein-tyrosine phosphatase alpha. *J Biol Chem* **272**, 6994–7002.
30 Blanchetot C, Tertoolen LG & den Hertog J (2002) Regulation of receptor protein-tyrosine phosphatase alpha by oxidative stress. *EMBO J* **21**, 493–503.
31 van der Wijk T, Blanchetot C, Overvoorde J & den Hertog J (2003) Redox-regulated rotational coupling of receptor protein-tyrosine phosphatase alpha dimers. *J Biol Chem* **278**, 13968–13974.
32 Jiang G, den Hertog J & Hunter T (2000) Receptor-like protein tyrosine phosphatase alpha homodimerizes on the cell surface. *Mol Cell Biol* **20**, 5917–5929.
33 Buist A, Blanchetot C & den Hertog J (2000) Involvement of the membrane distal catalytic domain in pervanadate-induced tyrosine phosphorylation of receptor protein-tyrosine phosphatase alpha. *Biochem Biophys Res Commun* **267**, 96–102.
34 Liang L, Lim KL, Seow KT, Ng CH & Pallen CJ (2000) Calmodulin binds to and inhibits the activity of the membrane distal catalytic domain of receptor protein-tyrosine phosphatase alpha. *J Biol Chem* **275**, 30075–30081.
35 Tartaglia M, Mehler EL, Goldberg R, Zampino G, Brunner HG, Kremer H, van der Burgt I, Crosby AH, Ion A, Jeffery S *et al.* (2001) Mutations in PTPN11, encoding the protein tyrosine phosphatase SHP-2, cause Noonan syndrome. *Nat Genet* **29**, 465–468.

36 Tartaglia M, Pennacchio LA, Zhao C, Yadav KK, Fodale V, Sarkozy A, Pandit B, Oishi K, Martinelli S, Schackwitz W *et al*. (2007) Gain-of-function SOS1 mutations cause a distinctive form of Noonan syndrome. *Nat Genet* **39**, 75–79.
37 Razzaque MA, Nishizawa T, Komoike Y, Yagi H, Furutani M, Amo R, Kamisago M, Momma K, Katayama H, Nakagawa M *et al*. (2007) Germline gain-of-function mutations in RAF1 cause Noonan syndrome. *Nat Genet* **39**, 1013–1017.
38 Sarkozy A, Carta C, Moretti S, Zampino G, Digilio MC, Pantaleoni F, Scioletti AP, Esposito G, Cordeddu V, Lepri F *et al*. (2009) Germline BRAF mutations in Noonan, LEOPARD, and cardiofaciocutaneous syndromes: molecular diversity and associated phenotypic spectrum. *Hum Mutat* **30**, 695–702.
39 Bentires-Alj M, Kontaridis MI & Neel BG (2006) Stops along the RAS pathway in human genetic disease. *Nat Med* **12**, 283–285.
40 Petrone A, Battaglia F, Wang C, Dusa A, Su J, Zagzag D, Bianchi R, Casaccia-Bonnefil P, Arancio O & Sap J (2003) Receptor protein tyrosine phosphatase alpha is essential for hippocampal neuronal migration and long-term potentiation. *EMBO J* **22**, 4121–4131.
41 Ponniah S, Wang DZ, Lim KL & Pallen CJ (1999) Targeted disruption of the tyrosine phosphatase PTPalpha leads to constitutive downregulation of the kinases Src and Fyn. *Curr Biol* **9**, 535–538.
42 Guo XL, Shen K, Wang F, Lawrence DS & Zhang ZY (2002) Probing the molecular basis for potent and selective protein-tyrosine phosphatase 1B inhibition. *J Biol Chem* **277**, 41014–41022.
43 Zheng XM, Resnick RJ & Shalloway D (2000) A phosphotyrosine displacement mechanism for activation of Src by PTPalpha. *EMBO J* **19**, 964–978.
44 Lim KL, Kolatkar PR, Ng KP, Ng CH & Pallen CJ (1998) Interconversion of the kinetic identities of the tandem catalytic domains of receptor-like protein-tyrosine phosphatase PTPalpha by two point mutations is synergistic and substrate-dependent. *J Biol Chem* **273**, 28986–28993.
45 Hunter T (1998) Anti-phosphatases take the stage. *Nat Genet* **18**, 303–305.
46 Tsujikawa K, Kawakami N, Uchino Y, Ichijo T, Furukawa T, Saito H & Yamamoto H (2001) Distinct functions of the two protein tyrosine phosphatase domains of LAR (leukocyte common antigen-related) on tyrosine dephosphorylation of insulin receptor. *Mol Endocrinol* **15**, 271–280.
47 Kashio N, Matsumoto W, Parker S & Rothstein DM (1998) The second domain of the CD45 protein tyrosine phosphatase is critical for interleukin-2 secretion and substrate recruitment of TCR-zeta in vivo. *J Biol Chem* **273**, 33856–33863.
48 den Hertog J, Pals CE, Peppelenbosch MP, Tertoolen LG, de Laat SW & Kruijer W (1993) Receptor protein tyrosine phosphatase alpha activates pp60c-src and is involved in neuronal differentiation. *EMBO J* **12**, 3789–3798.
49 den Hertog J, Tracy S & Hunter T (1994) Phosphorylation of receptor protein-tyrosine phosphatase alpha on Tyr789, a binding site for the SH3-SH2-SH3 adaptor protein GRB-2 in vivo. *EMBO J* **13**, 3020–3032.