RPTPα-mediated activation of Src

RPTPα-gemediëerde activering van Src
(met een samenvatting in het Nederlands)

Proefschrift

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Andrei Mircea Vacaru
geboren op 11 februari 1979 te Boekarest, Roemenië

Promotoren: Prof. dr. H.C. Clevers
Prof. dr. J. den Hertog

Părinților mei

The research described in this thesis was performed at Hubrecht Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW), within the Graduate School of Cancer Genomics and Developmental Biology in Utrecht, the Netherlands.

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Cover: ribbon representations of RPTPα-D1 dimer (white, PDB ID 1YFO) and Src (black, PBD ID 1FMK) structures.

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Chapter 1

General introduction

Adapted from: A.M. Vacaru and J. den Hertog (2009) Reciprocal regulation of RPTPα and Src, Romanian Journal of Biochemistry, 46 (2), 187-197

Tyrosine phosphorylation is a vital mechanism in signal transduction and regulation of all eukaryotic organisms. Its importance was demonstrated for many essential cellular processes including differentiation, proliferation, cell cycle progression, metabolic homeostasis, transcriptional activation, intra- and intercellular communication and motility. Changes in the delicate tyrosine phosphorylation equilibrium may lead to immune diseases, cancer or diabetes. The cellular tyrosine phosphorylation balance is maintained by the antagonistic activities of the protein tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). Interestingly, many members of these two classes of enzymes are themselves regulated by tyrosine phosphorylation.

Protein-tyrosine phosphatases

107 genes from the human genome were discovered to encode for PTPs (Alonso et al. 2004). According to the amino acid sequence of their catalytic site they were divided into four classes (Fig.1). The first three classes are cysteine-based phosphatases containing the consensus CX_5R. The first class is further divided into classical, phosphotyrosine-specific phosphatases and the more heterogeneous group of VH1-like PTPs, also called dual specificity phosphatases (DSPs) that along with phosphotyrosine can dephoshorylate phosphoserine, phosphothreonine, mRNA and phospholipids. The best characterized PTPs of the former subclass are the MAPK phosphatases (MKPs) and PTEN. The MKPs are specific enzymes that regulate the activity of the MAPKs by dephosphorylating both phosphotyrosine and phosphothreonine from the MAPK's kinase activation loop (Owens and Keyse 2007). PTEN functions as a tumor suppressor by negatively regulating the Akt/PKB signaling pathway through dephosphorylation of the second messenger phosphatidylinositol-3,4,5-triphosphate (Leslie and Downes 2002). The low molecular weight PTP (LMW-PTP) is the only member of the second class of PTPs and it was shown to be able to dephosphorylate PDGFR, Src, and p190RhoGAP (Raugei et al. 2002). LMW-PTP is linked with a number of human diseases including allergy, obesity, Alzheimer's disease, asthma and myocardial hypertrophy (Bottini et al. 2002). The three Cdc25 proteins form the third class of PTPs and have the ability to dephosphorylate both phosphotyrosine and phosphothreonine. Their role is to activate Cdks by removing the inhibitory N-terminal phosphorylation, resulting in progression of the cell cycle at different points (Kristjansdottir and Rudolph 2004). Initially discovered as

transcription factors, the PTPs of the fourth family (Eya) use a novel mechanism of catalysis dependent on aspartic acid. The phosphatase activity of these enzymes was demonstrated to be essential in organogenesis (Jemc and Rebay 2007).

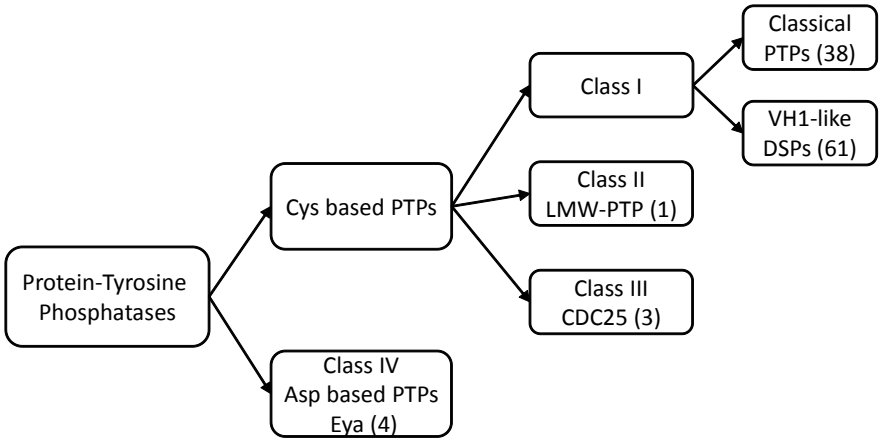

Fig.1 Classification of protein-tyrosine phosphatases

In humans, from the total number of PTPs 11 are catalytically inactive, 13 dephosphorylate phospholipids and 2 dephosphorylate mRNA, reducing the number to 81 enzymes able to dephosphorylate phosphotyrosine (Alonso et al. 2004). These proteins counteract the activity of the 85 PTKs believed to have catalytic activity (Manning et al. 2002) maintaining the phosphotyrosine equilibrium.

Classical PTPs

The hallmark of the classical PTPs is the VHCSXGXGR(T/S)G sequence within the PTP loop (Andersen et al. 2001). As mentioned above the classical PTPs are phospho-tyrosine specific. This specificity is conferred by the depth of the catalytic site where the catalytic cysteine can be reached only by phosphotyrosines (Barford et al. 1994; Yuvaniyama et al. 1996). The microenvironment within the catalytic site confers the catalytic cysteine a very low pK_a allowing it to promote a nucleophile attack on the phosphate and initiate the catalysis forming the thiol-phosphate intermediate (Denu et al. 1996). Together with the PTP-loop, nine other motifs were identified in the structure of the classical PTPs. Amongst these the WPD loop plays fundamental roles in the catalytic mechanism of the PTPs. After the catalytic cysteine binds the phosphate, the WPD loop locks over the substrate trapping it in the catalytic cleft (Stuckey et al. 1994; Pannifer et al. 1998), then the aspartic acid

functions as a general acid donating a proton for the release of the tyrosine (Zhang et al. 1994) and at the end of the catalytic process the same aspartic acid works as a general base activating a molecule of water which hydrolyses and releases the phosphate from the active site (Lohse et al. 1997). Wide understanding of the catalytic mechanism of the PTPs led to the development of the "substrate trapping" mutants, a very useful tool for PTP substrate identification (Flint et al. 1997).

Receptor protein-tyrosine phosphatases (RPTPs)

Based on their subcellular localization the classical PTPs can be divided into cytosolic PTPs and transmembrane receptor PTPs (RPTPs). The structure of the extracellular domain divides the RPTPs into 8 subtypes (Brady-Kalnay and Tonks 1995) as depicted in Fig. 2. Even though the extracellular domains are highly variable suggesting they have an important role for RPTP function, so far the only RPTPs shown to be regulated by extracellular ligands are RPTPζ and LAR. RPTPζ activity is downregulated upon Pleiotrophin binding resulting in increased phosphorylation of downstream RPTPζ targets (Meng et al. 2000). Syndecan and Dallylike have opposing effects on LAR activity influencing the axon guidance process in Drosophila (Rawson et al. 2005). For a number of RPTPs (δ, κ, μ and λ) homophilic interactions were shown to take place but besides the role in cell-cell adhesion no other function for this type of binding was discovered (reviewed in den Hertog et al. 2008). Inhibition of a chimeric protein containing the extracellular domain of EGF receptor and the intracellular domain of CD45 following EGF stimulation (Desai et al. 1993), suggested a role for ligand induced dimerization in the function of the RPTPs.

Another distinct feature of RPTPs is the presence of a second catalytic domain (D2). Despite containing the phosphate-binding loop (Andersen et al. 2001) and having a well conserved three-dimensional structure compared to the membrane-proximal domains (D1) (Nam et al. 1999; Sonnenburg et al. 2003; Barr et al. 2009), the catalytic activity of RPTP-D2s is very low or absent (Streuli et al. 1990; Wang and Pallen 1991; Gebbink et al. 1993). For some RPTPs, like LAR and RPTPα, the mutations responsible for decreased activity were found in the KNRY and WPD motifs and the reconstruction of these motifs, by replacing two amino acids, recovered the activity (Lim et al. 1997; Buist et al. 1999; Nam et al. 1999). Similar mutations in RPTPε-D2 restored the activity, however these mutations had no effect on CD45-D2 activity (Lim et al. 1999). The biological function of these catalytic impaired domains is not

entirely understood. So far it is known that the D2 domains are involved in inter- and intramolecular interactions and they play an important role in the homo- and oligomerization of the RPTPs. *In vitro* studies indicated an intramolecular interaction in CD45 that results in increased thermostability and enhanced catalytic activity (Felberg and Johnson 1998). Wallace and collaborators identified in a two-hybrid screen, and confirmed with co-immunoprecipitation experiments, an interaction between RPTPδ-D2 and RPTPσ-D1. This heterodimerization required the helix-turn-helix structure from the juxtamembrane domain of RPTPσ-D1 also known as the wedge structure and led to inhibition of the catalytic activity of RPTPσ-D1 (Wallace et al. 1998). A similar approach used in a more extensive study revealed multiple interactions between D1 and D2 domains from different RPTPs, suggesting that this interplay, based on the wedge domain of the D1 domains and the C-terminal region of the D2 domains, is a common mechanism for all the RPTPs (Blanchetot et al. 2002). LAR-D2 domain was shown to interact with a number of downstream effectors, including Trio, Abl kinase and Enabled, β-Catenin and Liprin α, part of these interactions playing an important role in synapse formation and stabilization (Stryker and Johnson 2007). D2 domains are also involved in substrate recognition as observed for CD45 where D2 mediates the interaction with Lck (Felberg et al. 2004). An acidic region from CD45-D2 seems to be required for T-cell receptor-mediated calcium signaling regulation (Wang et al. 2000) but the exact mechanism is not known. Finally, the crystal structures of LAR and CD45 suggest that the D2 domains are important for the stabilization of the D1 domain (Nam et al. 1999; Nam et al. 2005).

RPTPα

RPTPα structure and regulation

RPTPα contains a short, highly glycosylated extracellular domain and two intracellular phosphatase domains (Daum et al. 1994). The membrane proximal domain has low activity compared to the other member of the R4 class, RPTPε (Lim et al. 1997; Wu et al. 1997), whilst the membrane distal domain of RPTPα is the most active D2 domain of all the RPTPs and it was defined as a bona fide PTP with an activity higher than that of some dual specificity phosphatases (Lim et al. 1997). The function of the catalytic activity of RPTPα-D2 is not known. However, extensive studies on the function of the catalytic cysteine revealed a role of this residue in

RPTPα oxidation-induced dimerization (Blanchetot et al. 2002; van der Wijk et al. 2004).

The crystal structures of both D1 and D2 were elucidated. RPTPα-D1 domain crystallized as a dimer with a helix-turn-helix wedge structure occupying the catalytic site of the opposing monomer (Bilwes et al. 1996). From this emerged the idea that dimerization could be a general mechanism of RPTP negative regulation. The results obtained for CD45 and RPTPα strengthened the idea of a wedge-based inhibitory mechanism. Studies on a chimeric molecule containing the extracellular domain of EGF receptor and the transmembrane and cytoplasmic domain of CD45 showed that following the introduction of a mutation at the tip of the wedge, the T-cell receptor (TCR) is not inhibited anymore by EGF stimulation (Majeti et al. 1998). Furthermore, the effects of the analogous wedge mutations in a knock-in mouse were consistent with activation of CD45 (Majeti et al. 2000).

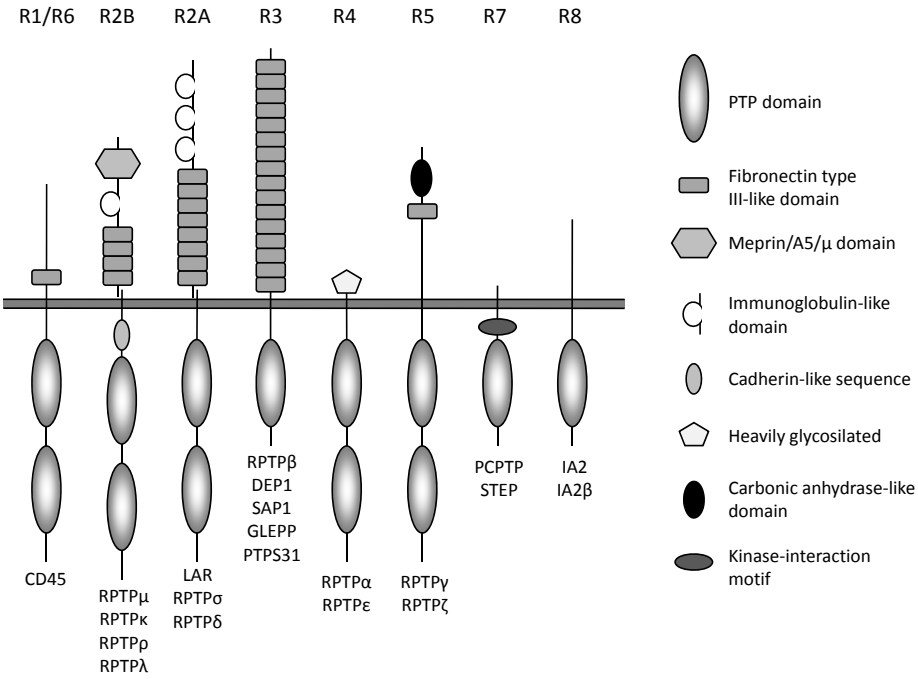

Fig.2 Schematic view of the RPTPs

The wedge structure is conserved in all the D1 domains of the RPTPs. However, until now no other RPTP crystal structure has shown a wedge mediated dimerization as seen for RPTPα. Even if the D1 domain of RPTPμ crystallizes as a dimer, the position of the dimers suggests that the wedge does not seem to be

implicated in the dimer formation (Hoffmann et al. 1997). Furthermore, the crystal structures of the D1-D2 tandems of LAR (Nam et al. 1999) and CD45 (Nam et al. 2005) show a rigid orientation of the domains which excludes the wedge-induced dimerization model for RPTPs. Recently a wedge-independent dimerization model was proposed for RPTPγ. In this model a head-to-toe arrangement of the RPTPγ phosphatase domains leads to catalytic inhibition and this type of dimerization is probably induced by ligand binding (Barr et al. 2009).

The crystal structure of RPTPα-D2 revealed that this domain alone is not sufficient for RPTPα dimerization (Sonnenburg et al. 2003). In the same study proteolysis sensitivity of RPTPα suggested that the linker region between D1 and D2 domain is highly flexible and allows the dimerization of the D1 domain and the interaction of D1 with other molecules. The crystal structure of the oxidized RPTPα-D2 domain shows a number of conformational changes supporting the idea of potential flexibility between RPTPα-D1 and D2 (Yang et al. 2007). The crystal structure of the RPTPα D1-D2 tandem is required to answer all these contradictory results concerning the wedge dimerization model.

Multiple functional studies describe that RPTPα can dimerize in cells. Two different approaches based on *in vivo* cross-linking (Jiang et al. 2000) and fluorescence-resonance-energy-transfer (Tertoolen et al. 2001) revealed that the dimer formation also implicates the ectodomain and the transmembrane domain of RPTPα. Dimerization was thought initially to be an inhibitory mechanism, but experimental data showed that this process can lead to the formation of both active and inactive dimers. In the first study promoting this idea, mutations to cysteines at certain positions in the extracellular domain resulted in molecules that formed constitutive dimers and depending on the position of the mutation these dimers were able or unable to activate Src (Jiang et al. 1999). An ectodomain-tag accessibility assay revealed that the underlying mechanism is the rotational coupling of the molecules (van der Wijk et al. 2003). The same study proposed that the rotational coupling is regulated by redox signaling. H_2O_2-induced oxidation of Cys723 results in formation of disulfide bonds with the Cys723 of a neighboring molecule. This reversible process stabilizes the RPTPα dimers resulting in complete inactivation of the enzyme and rotational coupling of the extracellular domain (Blanchetot et al. 2002; van der Wijk et al. 2003; van der Wijk et al. 2004).

RPTPα has the most active D2 domain (Wang and Pallen 1991; Lim et al.

1997; Wu et al. 1997), with a catalytic activity towards pNPP even higher than the activity of dual specificity phosphatases like cdc25, VH1 and YPTP1 (Lim et al. 1997). Mutation of the RPTPα-D2 catalytic cysteine (Cys723) abolished the pervanadate-induced tyrosine phosphorylation of RPTPα (Buist et al. 2000) suggesting that this changed the D2 domain conformation influencing the interaction with the kinase responsible for RPTPα Tyr789 phosphorylation. RPTPα-D2 activity was shown to be downregulated following Calmodulin binding (Liang et al. 2000) but the physiological role of this interaction remains to be discovered. Further studies are required to determine if D2 domain of RPTPα has a physiological catalytic function.

Along dimerization other mechanisms are involved in the regulation of RPTPα activity. Recently it was shown that two splicing variants of RPTPα had different activities (Kapp et al. 2007). RPTPα is also regulated by calpain proteolytic cleavage of the intracellular domain (Gil-Henn et al. 2001). Serine phosphorylation of two sites (Ser180 and Ser204) in the juxtamembrane region (Tracy et al. 1995) was shown to lead to increased RPTPα activity (den Hertog et al. 1995). The kinases found to be responsible for RPTPα Ser phosphorylation are PKCδ (Brandt et al. 2003) and CaMKIIα (Bodrikov et al. 2008). It was proposed that RPTPα serine phosphorylation and activity reaches its maximum in mitosis (Zheng and Shalloway 2001), but the dynamics of this process are not completely understood. Ser204 is located C-terminal to the wedge structure suggesting that phosphorylation of this site could modulate the wedge conformation, leading to further changes in activity. RPTPα is also phosphorylated on Tyr, with the C-terminal Tyr789 being the major Tyr phosphorylation site (den Hertog et al. 1994). Tyr789 was proposed to play an important role in Src activation but this aspect will be discussed later.

The highly glycosylated extracellular domain of RPTPα may function as a receptor for different ligands. One ligand shown to interact with RPTPα was the neuronal receptor Contactin (Zeng et al. 1999) which was initially identified as binding partner of RPTPβ/ζ (Peles et al. 1995). RPTPα and Contactin associate only in *cis* and independently of RPTPα glycosylation, but the effect of this interaction on RPTPα was not well defined. In addition, NB-3, a member of the F3/Contactin family and neural recognition molecule close homologue of L1 (CHL1) was shown to associate with RPTPα and regulate its activity towards Src family kinase Fyn in this way controlling the apical dendrites orientation of the pyramidal neurons in the caudal cortex (Ye et al. 2008). Integrins might also work as RPTPα ligands and

lead to activation of the enzyme by separating the inactive dimers as suggested by von Wichert and co-workers (2003). This study shows that Integrin αV/β3 upregulated RPTPα-mediated activation of the Src family kinases, a step required for the formation of force-dependent focal complexes. RPTPα was also indicated as a potential receptor for the *Helicobacter pylori* vacuolating cytotoxin, VacA (Yahiro et al. 2003). The glycosylation of RPTPα extracellular domain could have a role in dimerization similar to CD45 (Xu and Weiss 2002), in which increased glycosylation of the longer splice variant resulted in decreased dimerization and increased activity. This hypothesis is supported by the study of Kapp and collaborators who noticed changes in the activity of two RPTPα splice variants with extracellular domains of different lengths (Kapp et al. 2007).

RPTPα function

RPTPα is ubiquitously expressed in mice, with increased expression in the dorsal root ganglia, cranial ganglia, adrenal gland and cortex (Sap et al. 1990; den Hertog et al. 1996). In young rats RPTPα is highly expressed in neocortex, hippocampus and cerebellum (Sahin et al. 1995). Chicken RPTPα is also expressed in various tissues, but the highest expression was observed in the brain (Fang et al. 1996). In zebrafish RPTPα is expressed in the brain, the pharingeal arches, the pectoral fin and the spinal cord (van der Sar et al. 2001). Expression of *Xenopus* RPTPα was detected in eye, heart, skin, liver and most abundantly in brain and lungs (Yang and Friesel 1998). Taken together these expression patterns suggest an important role for RPTPα in the nervous system. This idea is confirmed by the defects observed in the knock-out or knock-down animals. Even if initially the RPTPα knock-out mice did not present any obvious phenotypes (Ponniah et al. 1999; Su et al. 1999), closer examination revealed defects in the distribution of the hippocampal pyramidal neurons (Petrone et al. 2003). Moreover, behavioral studies on the knock-out mice revealed a role of RPTPα in learning and neuroplasticity (Skelton et al. 2003). In zebrafish, morpholino-induced RPTPα knock-down caused delayed retinal differentiation and apoptosis in the brain (van der Sar et al. 2002).

RPTPα was shown to be implicated in a number of signaling processes. One of the functions indicated for RPTPα was that of down-regulator of Insulin receptor signaling by dephosphorylating the Insulin receptor β-subunit (Moller et al. 1995; Lammers et al. 1997; Lammers et al. 1998; Andersen et al. 2001; Lacasa et al. 2005). In GH4 pituitary cells RPTPα inhibits the effect of Insulin-induced prolactin

gene expression (Jacob et al. 1998; Vulin et al. 2005) while in rat adipocytes RPTPα overexpression downregulates the Insulin-stimulated translocation of the glucose transporter GLUT4 to the membrane (Cong et al. 1999). Moreover, Kapp and collaborators showed that RPTPα might be a negative regulator of Insulin secretion (Kapp et al. 2003). However, the role of RPTPα in the Insulin pathway is questionable since in RPTPα knock-out mice glucose homeostasis and Insulin signaling are not affected (Le et al. 2004). RPTPα was also shown to bind and activate the Kv1.2 potassium channel upon signaling by the m1 Muscarinic acetylcoline receptor (Tsai et al. 1999) and to activate the Hyperpolarization-activated cyclic nucleotide gated (HCN) channel (Huang et al. 2008) suggesting that RPTPα plays an important role in ion channel regulation.

Next to the functions described above, probably the most studied function of RPTPα is that of Src and Fyn kinases activator. Soon after its discovery RPTPα's ability to dephosphorylate pTyr527 and thus activate Src was revealed (Zheng et al. 1992; den Hertog et al. 1993). In brain lysates (Ponniah et al. 1999) and fibroblasts (Su et al. 1999) from RPTPα knock-out mice, Src and Src-related kinase Fyn had reduced activity and increased Tyr527 phosphorylation decisively demonstrating the physiological role of RPTPα for Src and Fyn activation. Through Src and Fyn RPTPα wields its influence in a number of important cellular processes.

Overexpression of RPTPα in mouse P19 embryonal carcinoma cells changes the differentiation fate of these cells in favor of neuronal differentiation, probably due to increased Src activity in these cells (den Hertog et al. 1993). RPTPα can also induce neuronal differentiation of mouse embryonic stem cells (van Inzen et al. 1996). Different studies demonstrated that neurite outgrowth is regulated by RPTPα-activated Src (Yang et al. 2002) or Fyn (Bodrikov et al. 2005; Bodrikov et al. 2008). RPTPα-mediated Src activation was also shown to play an important role in skeletal muscle cell differentiation (Lu et al. 2002). In rat brains N-methyl-D-aspartate (NMDA) glutamate receptors, RPTPα and Src are brought together by the scaffolding protein PSD95 forming a complex that regulates the NMDA receptors and controls the long-term potentiation of the hippocampal neurons (Lei et al. 2002). This model was confirmed by RPTPα knock-out mice which, as mentioned before, showed long term potentiation defects (Petrone et al. 2003; Skelton et al. 2003).

RPTPα-dependent Src activation was demonstrated to control cell spreading and migration processes. Integrin-mediated spreading and migration of fibroblasts

lacking RPTPα are delayed concomitantly with reduced association of Src and Fyn with the focal adhesion kinase (FAK) and reduced phosphorylation of the Src substrates FAK and p130cas (Su et al. 1999; Zeng et al. 2003). In a recent study it was proposed that RPTPα plays an early role in Integrin signaling by activating Src and Fyn SFKs. These form an active complex with activated FAK and phosphorylate downstream substrates. Later on RPTPα has a role in Integrin-induced cell spreading, focal adhesion formation and migration that requires RPTPα Tyr789 phosphorylation (Chen et al. 2006). RPTPα seems to influence focal adhesion turnover through activation of the small GTPase Rac1 but it is not known if this process is dependent on Src activation (Herrera Abreu et al. 2008). In rat kidney, the tetraspan protein CD63 seems to associate with RPTPα and Src forming a complex that further phosphorylates and downregulates the ROMK1 potassium channel (Lin et al. 2008). Studies on mast cells derived from RPTPα knock-out mice bone marrow suggested that RPTPα and c-Kit are required for stem cell factor-induced SFKs activation that further leads to mast cell polarization and migration (Samayawardhena and Pallen 2008). All these results demonstrate the fundamental role played by RPTPα in Src and Fyn activation.

Src

The gene encoding v-Src was discovered as the essential part of the Rous sarcoma virus for inducing cell transformation (Martin 1970). A normal cellular homologue was then discovered in normal avian DNA and this was the first proto-oncogene (Stehelin et al. 1976), the gene coding for Src. The protein product of the *src* gene was identified and soon after that Src was proven to be a protein tyrosine kinase and furthermore to be itself tyrosine phosphorylated (Hunter and Sefton 1980).

Src structure and conformations

As mentioned above, the human genome encodes for 90 PTKs, 58 of them being receptor PTKs and the remaining 32, non-receptor PTKs. Nine of the non-receptor protein tyrosine kinases belong to the Src family kinases (SFKs) (Erpel and Courtneidge 1995). These are divided in two subclasses based on their similarity with Lyn (Lyn, Hck, Lck and Blk) or Src (Src, Yes, Fyn, Fgr and Yrk).

Src, like all the related kinases from the family, is composed of six distinct functional domains which are, from N- to C-terminus: SH4, unique region, SH3,

SH2, catalytic domain and the C-terminal tail where the inhibitory pTyr is located (Fig. 3). The SH4 domain contains the myristoylation site (the Gly at position 2), and following lipid attachment at this site Src is targeted to the plasma membrane. The unique region is the most divergent part among the SFKs having specific roles for different family members. The SH3 domain binds to proline-rich sequences while the SH2 domain binds phosphorylated tyrosines. Both SH3 and SH2 domains regulate the catalytic activity through inter- and intramolecular interactions and direct the protein to the substrates or to the required site of action (Thomas and Brugge 1997).

Early after Src discovery it was revealed that phosphorylation of Tyr416 stimulates and phosphorylation of Tyr527 inhibits the catalytic activity of Src (Kmiecik and Shalloway 1987). The phosphorylation of these two regulatory sites was found to be controlled by two different mechanisms: Tyr416 phosphorylation was shown to be achieved by autocatalysis (Smith et al. 1993) while Tyr527 phosphorylation resulted from the action of Csk (Okada and Nakagawa 1989). Only later, the elucidation of Src crystal structure revealed the details of the modular mechanism that regulates Src activity. Inactive Src adopts a closed conformation with the SH3 and SH2 domains folded behind the catalytic domain (Fig. 3). This alignment is stabilized by the SH2 domain binding to the C-terminal pTyr527 and the SH3 domain interaction with the left-handed helix from the linker between the SH2 and the kinase domain. In this arrangement the activation loop within the catalytic domain forms an α-helix that prevents Tyr416 from phosphorylation. SH2 or SH3 ligands or dephosphorylation of pTyr527 can disassemble the inactive conformation and expose Tyr416 which gets phosphorylated (Xu et al. 1999). Autophosphorylated Tyr416 has a role in keeping the enzyme in an active state even if Tyr527 subsequently gets phosphorylated by Csk (Sun et al. 1998).

Src function

Src expression is ubiquitous, with increased abundance in platelets, osteoclasts and brain (Brown and Cooper 1996). Subcellular localization of Src is restricted to endosomes, perinuclear membranes, secretory vesicles and plasma membrane. Src is involved in a number of processes including cell differentiation, proliferation, survival, cell adhesion, morphology and motility, and bone resorption (Roskoski 2004). Src knock-out mice die few weeks after birth. The main phenotype observed is osteopetrosis, a bone accumulation resulting from impaired osteoclast

activity (Soriano et al. 1991). Src/Fyn or Src/Yes double knockouts die perinatally (Stein et al. 1994), while the Src/Fyn/Yes triple knockouts die at early embryonic stages (Klinghoffer et al. 1999).

Chackalaparampil and Shalloway (1988) discovered that Src was activated at the entry in mitosis and several years later it was suggested that Src activity was required for the entry in mitosis (Roche et al. 1995). However, high levels of Src were found in differentiated neurons which are postmitotic (Cotton and Brugge 1983) and in platelets which are anucleated cells (Golden et al. 1986), suggesting that Src has roles other than as a mitotic regulator. One of the other important functions of Src is that of transducer of the Integrin-induced signals. The fibroblasts derived from triple Src/Fyn/Yes knockouts show clear defects in Integrin signaling, confirming the important role played by SFKs in this pathway (Klinghoffer et al. 1999).

Src regulation

In cells, negative regulation of Src is acquired through catalytic and non-catalytic processes. The best described inhibitory mechanism of Src is the phosphorylation of the N-terminal Tyr527 (Cooper et al. 1986). So far, two kinases were found responsible for this action: Csk (Okada et al. 1991) and Csk-homologous kinase (Chk) (Davidson et al. 1997). Chk can also inhibit Src in a non-catalytic fashion by forming a stable complex, independent of Tyr527 phosphorylation (Chong et al. 2004). Three other proteins were shown to inactivate Src in a non-catalytic manner: WASP, Caveolin and RACK1. WASP can bind concomitantly to the SH3 and catalytic domain of Src inhibiting its activity (Schulte and Sefton 2003). Caveolin is able to form stable complexes with Src in which Src activity is suppressed (Li et al. 1996). RACK1 when overexpressed in fibroblasts inhibits Src activity (Mamidipudi et al. 2004). Inhibition of Src activity may also be mediated by dephosphorylation of Tyr416 and three PTPs were suggested to reduce Src activity in this way: RPTPα (den Hertog et al. 1993), PTP-BL (Palmer et al. 2002) and TC-PTP (van Vliet et al. 2005).

Similar to inactivation, Src activation is promoted through non-catalytic mechanisms, when the inhibitory intramolecular binding of SH3 or SH2 domain is lost in favor of interaction with neighboring molecules which harbor SH3 and SH2 binding domains. Integrin-induced Src activation is mediated by Src SH2 domain binding to FAK pTyr397 and Src SH3 domain binding to the cytoplasmic tail of Integrin β3 (Mitra and Schlaepfer 2006). Src is also activated after binding to PDGF receptor, an interaction mediated by Src SH2 domain (Twamley-Stein et al. 1993). Another

example of non-catalytic activation is the interaction of Src via its SH3 domain with β-Arrestin (Luttrell et al. 1999).

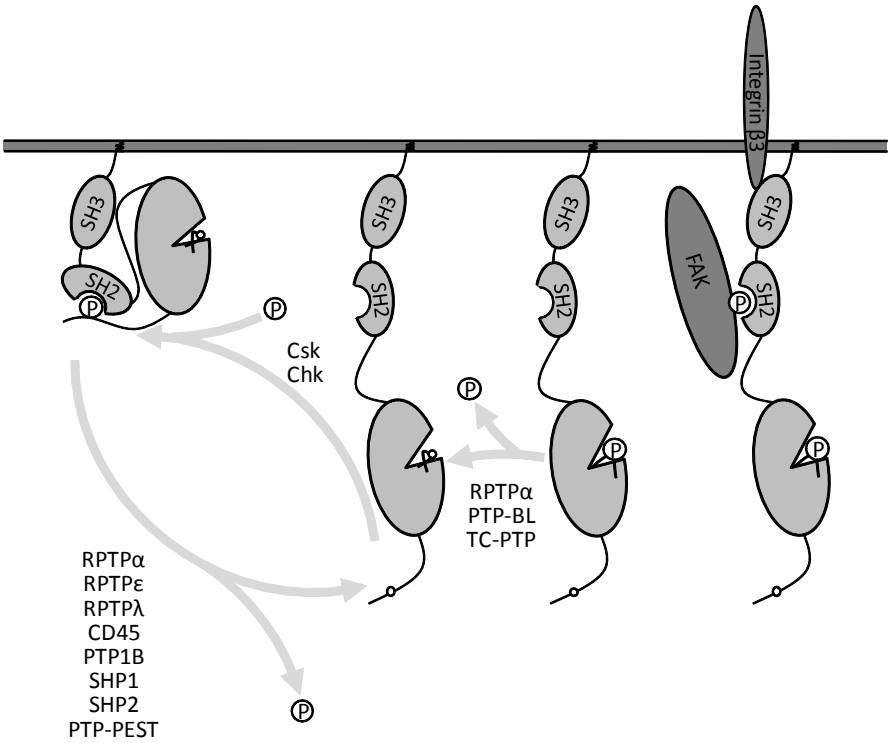

Fig.3 Various ways of Src regulation. Normally Src is in a dormant, closed conformation with the SH2 domain binding pTyr527 and the SH3 domain binding the SH2-kinase domain linker. Dephosphorylation of pTyr527 opens the molecule and Src autophosphorylates itself on Tyr416 becoming fully active. Opening of the inactive conformation can occur after binding of the SH2 or SH3 domains to proteins containing pTyr or polyproline type II helices. Src is inactivated after dephosphorylation of pTyr416 and folds into the closed conformation following Tyr527 phosphorylation.

It was suggested that Src can be positively regulated by hydrogen peroxide-induced oxidation (Rosado et al. 2004) and the activation was proposed to be the result of a disulfide bond formation between Cys245 from the SH2 domain and Cys487 located in the kinase domain (Giannoni et al. 2005). However, the effect of H_2O_2 on Src activity is still a subject of debate since a different study (Tang et al. 2005) shows that treatment with low to medium doses of H_2O_2 leads to Src inactivation which is caused by complete dephosphorylation of Src pTyr416.

Phosphorylation of Src on other sites than Tyr416 also leads to Src activation. Cdk1/Cdc2, the kinase essential for the G2/M transition in the cells, phosphorylates Src on three different sites: Thr34, Thr46 and Ser72. It was proposed

that phosphorylation of these sites reduces the intramolecular interactions with SH2 and SH3 domains promoting Src activation (Shenoy et al. 1992; Stover et al. 1994).

As discussed before, dephosphorylation of Src pTyr527 seems to be the most important step in Src activation. Both PTPs and RPTPs were found to be responsible for dephosphorylation of Tyr527: PTP1B (Bjorge et al. 2000), Shp1 (Somani et al. 1997), Shp2 (Zhang et al. 2004), PTP-PEST (Chellaiah and Schaller 2009), CD45 (Stover et al. 1994), RPTPλ (Fang et al. 1994), RPTPε (Gil-Henn and Elson 2003), and RPTPα (Zheng et al. 1992; den Hertog et al. 1993).

All these regulatory mechanisms keep the activity of Src at functional levels, avoiding the potential oncogenic effects that could result from its overactivation.

RPTPα-mediated Src regulation

As discussed above, the physiological role of RPTPα is mainly linked to its ability to activate Src and Fyn kinases by dephosphorylating their C-terminal inhibitory phosphotyrosine (Zheng et al. 1992; den Hertog et al. 1993). Zheng and collaborators proposed a displacement mechanism for RPTPα-mediated Src activation (Zheng et al. 2000). In this mechanism the scaffolding protein GRB2 which normally binds RPTPα pTyr789 through its SH2 domain and C-terminal SH3 domain (den Hertog et al. 1994; Su et al. 1994) releases pTyr789 which is bound by the SH2 domain of Src. pTyr527 after losing the interaction with the SH2 domain is dephosphorylated by RPTPα and Src is activated. The same authors suggested that in mitosis GRB2 binding to RPTPα is lost due to hyperphosphorylation of RPTPα Ser180 and Ser204. Increased serine phosphorylation would also enhance RPTPα catalytic activity. Subsequently, Src binds the GRB2-free pTyr789 and is dephosphorylated by RPTPα reaching its highest activity within the cell cycle (Zheng and Shalloway 2001; Zheng et al. 2002). It is not clear if this mechanism of RPTPα-mediated Src activation is correct since several studies showed that mutant RPTPα lacking Tyr789 is capable of activating Src. In this respect, Lammers and collaborators showed that RPTPα pTyr789 was not required for activation of Src. On the contrary, this mutant enhanced the Src-induced focus formation in NIH3T3 cells. Further it was suggested that Tyr789 phosphorylation was required only for RPTPα localization at the focal adhesion sites (Lammers et al. 2000). In another study WT RPTPα and RPTPα-Y789F mutant had the same effect on Src activation after expression in PC12 rat

phaeochromocytoma cells. Moreover, Src-induced neurite outgrowth in response to EGF was enhanced in the presence of RPTPα-Y789F mutant compared to WT RPTPα (Yang et al. 2002). Recently, Chen and colleagues demonstrated that Integrin-stimulated Src and Fyn activation does not require RPTPα pTyr789, although the phosphorylation of this site is necessary for the later processes, like focal adhesion formation and cell migration (Chen et al. 2006). In addition, it was suggested that RPTPα-induced Src activation is required for Src-mediated Tyr789 phosphorylation of RPTPα following Integrin stimulation, an event that probably takes place after FAK-Src complex formation. In a previous study it was shown that Src coexpression with RPTPα leads to an increase in pTyr789, suggesting that Src is responsible for RPTPα phosphorylation in vivo (den Hertog et al. 1994). Thus, RPTPα is not only able to dephosphorylate Src thus activating it, but Src phosphorylates RPTPα, thus regulating its role in downstream signaling cascades.

Interestingly, it was shown that RPTPα can also dephosphorylate Src pTyr416 *in vitro* and phosphorylation of Src Tyr416 is not increased in cells overexpressing RPTPα suggesting that RPTPα can also dephosphorylate Src pTyr416 *in vivo* (den Hertog et al. 1993). These results imply that RPTPα-Src interaction is a multiple step process that results in tight regulation of Src activity.

The interplay between RPTPα and Src becomes more complicated when the proteins interacting with these two enzymes are considered. More work will be required to understand the role of the external factors on this intricate interaction.

Scope of this thesis

The scope of this thesis was to investigate in more detail RPTPα regulation and function in the cellular context. Src is the best characterized substrate of RPTPα, therefore we used the ability of RPTPα to activate Src as a readout for our research. In Chapter2 we investigated mitotic RPTPα phosphorylation using phospho-specific antibodies. In contrast to what was suggested previously we discovered that in mitosis RPTPα pSer204 is dephosphorylated, concomitant with increased Src binding to RPTPα and Src activation. Further, in Chapter3, we tried to elucidate the details of the mechanism that led to increased RPTPα-Src interaction following mitotic RPTPα pSer204 dephosphorylation and we found that RPTPα wedge could be responsible for that effect. Chapter4 was dedicated to the study of a spontaneous RPTPα mutation which revealed that catalytically active of RPTPα-D2 is required for Src activation. Finally, in Chapter5 we tested different approaches for identification of new RPTPα substrates and we identified a number of interesting candidate substrates that remain to be confirmed.

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Chapter 2

Serine dephosphorylation of RPTPα in mitosis induced Src binding and activation

Andrei M. Vacaru[1] and Jeroen den Hertog[1,2]

[1]Hubrecht Institute – KNAW and University Medical Center, Utrecht, the Netherlands
[2]Institute of Biology Leiden, Leiden University, the Netherlands

Submitted

Abstract

Receptor protein-tyrosine phosphatase alpha (RPTPα) is the mitotic activator of the protein-tyrosine kinase Src. RPTPα serine hyperphosphorylation was proposed to mediate mitotic activation of Src. We raised phosphospecific antibodies to the two main serine phosphorylation sites and we discovered that RPTPα Ser204 was almost completely dephosphorylated in mitotic NIH3T3 and HeLa cells, whereas Ser180 and Tyr789 phosphorylation were only marginally reduced in mitosis. Concomitantly, Src pTyr527 and pTyr416 were dephosphorylated, resulting in 2.3-fold activation of Src in mitosis. Using inhibitors, we found that dephosphorylation of RPTPα pSer204 in mitosis is likely mediated by PP2A. Mutation of Ser204 to Ala did not activate RPTPα and intrinsic catalytic activity of RPTPα was not affected in mitosis. Interestingly, binding of endogenous Src to RPTPα was induced in mitosis. GRB2 binding to RPTPα, which was proposed to compete with Src binding to RPTPα, was only modestly reduced in mitosis, which could not account for enhanced Src binding. Moreover, we demonstrate that Src bound to mutant RPTPα-Y789F, lacking the GRB2 binding site, illustrating that Tyr789 is dispensable for Src binding. Mutation of RPTPα Ser204 to Asp, mimicking phosphorylation, significantly reduced co-immunoprecipitation with Src, suggesting that phosphorylation of Ser204 prohibits binding to Src. Based on our results, we propose a new model for mitotic activation of Src in which RPTPα pSer204 dephosphorylation facilitates Src binding, leading to RPTPα-mediated dephosphorylation of Src pTyr527 and pTyr416 and hence modest activation of Src.

Introduction

Protein tyrosine phosphatases (PTPs) are responsible for dephosphorylation of the phosphotyrosyl residues. The human genome contains approximately 100 genes that encode members of the four PTP families and most of them have mouse orthologues (Alonso et al. 2004; Tonks 2006). According to their subcellular localization, the classical PTPs, encoded by less than half of the total PTP genes, are divided in two subfamilies: cytoplasmic and receptor protein-tyrosine phosphatases (RPTPs). The majority of the RPTPs contain besides a variable extracellular domain and a transmembrane domain, two highly homologous phosphatase domains (Krueger et al. 1990), with the membrane-proximal domain comprising most of the catalytic activity (Lim et al. 1997).

RPTPα is a typical RPTP with a small, highly glycosylated extracellular domain (Daum et al. 1994). RPTPα function is regulated by many mechanisms, including proteolysis (Gil-Henn et al. 2001), oxidation (Yang et al. 2007), dimerization (Jiang et al. 1999; Jiang et al. 2000; Tertoolen et al. 2001; Blanchetot et al. 2002; van der Wijk et al. 2003) and phosphorylation of serine and tyrosine residues (den Hertog et al. 1994; den Hertog et al. 1995; Tracy et al. 1995). RPTPα is broadly expressed in many cell types and over the years, RPTPα has been shown to be involved in a number of signaling mechanisms, including neuronal (den Hertog et al. 1993) and skeletal muscle cell differentiation (Lu et al. 2002), neurite elongation (Yang et al. 2002; Bodrikov et al. 2005; Bodrikov et al. 2008), insulin receptor signaling down-regulation (Moller et al. 1995; Lammers et al. 1997; Lammers et al. 1998; Andersen et al. 2001; Lacasa et al. 2005), insulin secretion (Kapp et al. 2003), activation of voltage-gated potassium channel Kv1.2 (Tsai et al. 1999), long-term potentiation in hippocampal neurons (Lei et al. 2002; Petrone et al. 2003), matrix-dependent force transduction (von Wichert et al. 2003), and cell spreading and migration (Su et al. 1999; Zeng et al. 2003; Herrera Abreu et al. 2008).

The majority of the roles played in these cellular processes involve RPTPα's ability to activate the proto-oncogenes Src and Fyn by dephosphorylating their C-terminal inhibitory phosphotyrosine (Zheng et al. 1992; den Hertog et al. 1993; Bhandari et al. 1998; Ponniah et al. 1999; Su et al. 1999). Normally, this phosphotyrosine (pTyr527 in chicken Src) binds to the Src SH2 domain, keeping the protein in an inactive closed conformation. A displacement mechanism was proposed for RPTPα mediated Src activation in which pTyr789 of RPTPα is required

to bind the SH2 domain of Src before RPTPα dephosphorylates pTyr527 (Zheng et al. 2000). This model is subject of debate since other studies show that RPTPα lacking Tyr789 is still able to dephosphorylate and activate Src (Lammers et al. 2000; Yang et al. 2002; Chen et al. 2006; Kapp et al. 2007). In normal cells, Src reaches its activation peak during mitosis (Chackalaparampil and Shalloway 1988; Shenoy et al. 1989; Bagrodia et al. 1991; Roche et al. 1995) and with the help of overexpressing cells it was shown that this activation is triggered mainly by RPTPα. The model that emerged is that RPTPα is activated in mitosis due to serine hyperphosphorylation and detaches from the GRB2 scaffolding protein (Zheng and Shalloway 2001; Zheng et al. 2002) that normally binds most of the pTyr789 of RPTPα via its SH2 domain (den Hertog et al. 1994; den Hertog and Hunter 1996; Su et al. 1996). Two serine phosphorylation sites were mapped in the juxtamembrane domain of RPTPα, Ser180 and Ser204 (Tracy et al. 1995). The kinases that were found responsible for their phosphorylation were PKCδ (Brandt et al. 2003) and CaMKIIα (Bodrikov et al. 2008) but there is no clear evidence that these kinases are activated in mitosis. We set out to investigate the role of serine phosphorylation of RPTPα in mitotic activation of Src.

We generated phospho-specific antibodies and show that RPTPα pSer204, but not pSer180, is dephosphorylated in mitotic NIH3T3 and HeLa cells, concomitant with activation of Src. Using inhibitors, we identified PP2A as the phosphatase that dephosphorylates pSer204. It is noteworthy that PP2A is known to be activated in mitosis. Intrinsic PTP activity of RPTPα was similar in unsynchronized and mitotic cells and mutation of Ser204 did not activate RPTPα in *in vitro* PTP assays. Yet, Src binding to RPTPα was induced in mitotic NIH3T3 cells and RPTPα-S204D with a phosphomimicking mutation at Ser204 co-immunoprecipitated less efficiently with Src. Based on our results, we propose a mechanism for mitotic activation of Src that is triggered by dephosphorylation of RPTPα pSer204, resulting in enhanced affinity for Src and subsequent dephosphorylation and activation of Src.

Results

Specificity of anti-pSer180 and anti-pSer204 antibodies.

To study RPTPα phosphorylation in mitosis, we generated antibodies against phosphorylated peptides derived from the mouse RPTPα protein sequence corresponding to Ser180 and Ser204. To test the specificity of these antibodies, HEK 293 cells were transfected with vectors coding for HA-tagged wild type RPTPα and mutants containing Ser to Ala mutations. Both antibodies recognized wild type RPTPα and mutations of individual phosphorylation sites of RPTPα abolished binding. Further, the antibodies did not bind to RPTPα after blocking with the phosphopeptides that were used for immunization. The non-phosphorylated peptides had no effect (Fig. 1A, B). The pSer180 and pSer204 antibodies were specific for their respective phosphorylation sites in RPTPα.

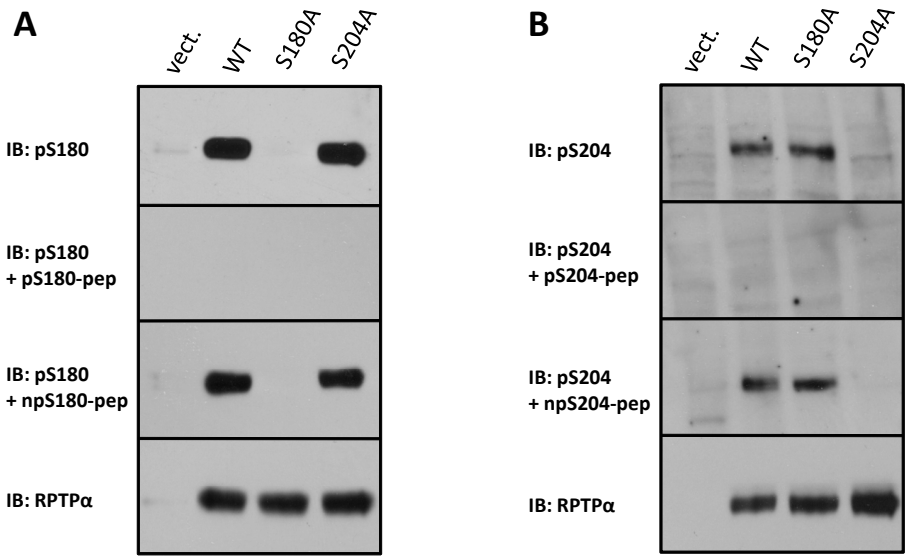


Fig.1 Specificity of anti-pSer180 and anti-pSer204 antibodies. HEK293 cells were transiently transfected with empty vector, WT HA-RPTPα, HA-RPTPα-S180A or HA-RPTPα-S204A. The cells were lysed and the lysates were fractionated on 7.5% SDS-polyacrylamide gels, transferred to PVDF membranes and immunoblotted with anti-pS180 (A) or anti-pS204 (B) antibodies. As indicated, the antibodies were used alone or together with the phospho- or nonphospho-peptides against which the antibodies were raised. The total levels of HA-RPTPα in the lysates were probed with anti-RPTPα antibody and shown in the bottom panels (A and B).

Mitotic dephosphorylation of RPTPα.

Previously it was suggested that RPTPα serine phosphorylation is increased after mitotic arrest (Zheng and Shalloway 2001). Using the pSer180 and pSer204

antibodies and the commercially available pTyr789 antibody we followed the phosphorylation state of RPTPα in the cell cycle. NIH3T3 cells were arrested with nocodazole and released from mitotic block. The cells were lysed and RPTPα was immunoprecipitated with anti-RPTPα 5478 antibody. In control, unsynchronized cells all three RPTPα phosphorylation sites were phosphorylated (Fig. 2A). After mitotic arrest pSer204 was almost completely dephosphorylated whereas pSer180 and pTyr789 were less affected. RPTPα Ser204 phosphorylation increased rapidly upon release from the mitotic block and reached the levels from unsynchronized cells 3 h after release. Phosphorylation of Ser180 presented a slower recovery after the cells were released and Tyr789 phosphorylation recovered 1 - 3 h after release (Fig. 2A). FACS analysis was used to monitor cell cycle distribution. The efficiency of the nocodazole induced mitotic block was high since more than 90% of the cells were mitotic. The cells were viable and after removal of nocodazole and washing with PBS the cells reentered the cell cycle (Fig. 2A).

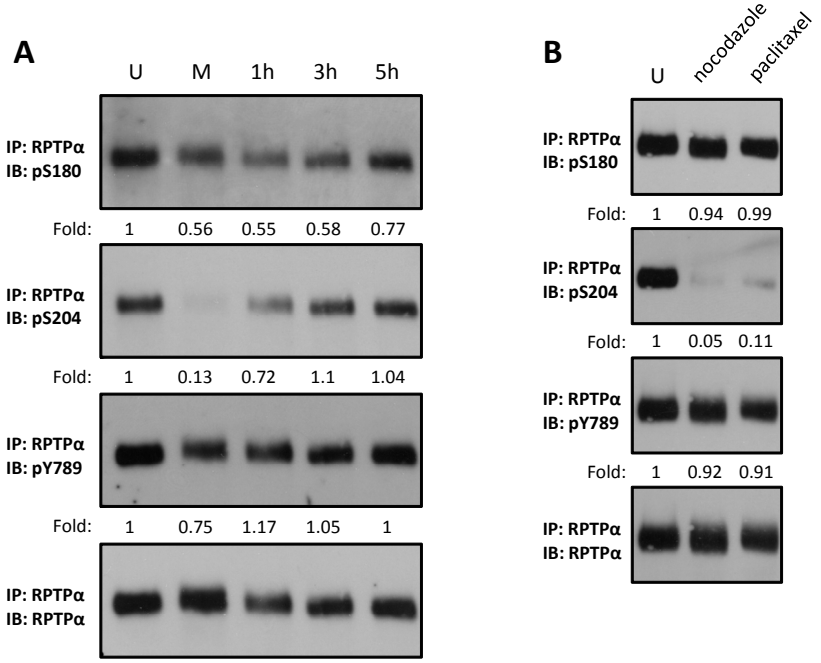
To investigate whether dephosphorylation of RPTPα pSer204 is a mitotic effect and not a nocodazole induced effect we used paclitaxel to arrest NIH3T3 cells in mitosis. After a 14h paclitaxel treatment the cells were harvested by mitotic shake-off, lysed and RPTPα was immunoprecipitated. The results were similar for the nocodazole and paclitaxel treated cells for all three RPTPα phosphorylation sites. Both types of mitotic arrest induced drastic dephosphorylation of RPTPα pSer204, but not pSer180 or pTyr789 (Fig. 2B).

Mitotic Src activation

To investigate Src tyrosine-phosphorylation in the cell cycle, the same lysates were analyzed by immunoblotting using antibodies for the autophosphorylation site of Src, pTyr416 and for the dephosphorylated form of the C-terminal phosphorylation site, npTyr527. In mitotic cells the levels of dephosphorylated Src Tyr527 were highly increased and decreased within 1 h to the levels from the unsynchronized cells after release from mitotic arrest (Fig. 3A). Surprisingly, in the mitotic arrested cells the levels of Src pTyr416 were reduced as well and they increased slowly (1 - 3h) after release from the mitotic block to the level in unsynchronized cells (Fig. 3A).

We observed that in the mitotic arrested cells Src pTyr527 was dephosphorylated, the first step in activation of Src. Phosphorylation of Src Tyr416 which is necessary for full activation was reduced in mitosis. To understand the cumulative effect of Tyr416 and Tyr527 dephosphorylation we assayed *in vitro* kinase

activity of Src from unsynchronized and mitotic arrested NIH3T3 cells using acid-denatured enolase as substrate (Fig. 3B). We found that immunoprecipitated Src from mitotic arrested cells was 2.3 times more active than Src from unsynchronized cells, which is consistent with previous results (Zheng and Shalloway 2001). Hence, the net result of pTyr527 and pTyr416 dephosphorylation in mitosis is a modest increase in Src kinase activity.



G1	66	2	25	45	45
S	20	2	12	12	14
G2/M	13	94	55	31	32

Cell cycle distribution (%)

Fig.2 Mitotic dephosphorylation of RPTPα. A, Endogenous RPTPα was immunoprecipitated from NIH3T3 lysates (0.5mg total protein) from unsynchronized (U), mitotic (M) and replated cells after mitotic arrest (1h, 3h, 5h). The immunoprecipitates were boiled in reducing sample buffer and the samples were run on a 7.5% SDS-polyacrylamide gel. The proteins were transferred to PVDF and the membranes were probed with anti-pS204 antibody and subsequently, after stripping, with anti-pS180, anti-pY789 and anti-RPTPα. The cell cycle distribution (%) of the NIH3T3 cells used to prepare each sample is shown in the table beneath. B, Unsynchronized NIH3T3 cells and cells obtained by mitotic shake-off after nocodazole and paclitaxel treatment were lysed and endogenous RPTPα was immunoprecipitated from approximately 1mg total protein/sample. The samples were processed as described in A. The blots were quantified and the phosphorylation levels were normalized for the total amount of RPTPα (bottom panel) and expressed as the ratio to the phosphorylation levels in the unsynchronized cells. The values are presented under each lane. Western blot quantification was performed using Quantity One software (BioRad). All the experiments were repeated at least three times with similar results and representative experiments are presented in this figure.

RPTPα and Src phosphorylation in HeLa cells

To see if RPTPα pSer204 mitotic dephosphorylation is a general mechanism, we investigated the phosphorylation state of human RPTPα in HeLa cells. Unsynchronized and nocodazole arrested cells were lysed and half of the lysates was used for immunoprecipitation of RPTPα, while the other half was used for immunoprecipitation of Src. Immunoblotting with the phospho-specific antibodies indicated that phosphorylation of RPTPα Ser180 and Tyr789 was not significantly affected, whereas Ser204 was completely dephosphorylated (Fig. 4A). In case of Src both pTyr416 and pTyr527 levels were decreased in the mitotic arrested HeLa cells (Fig. 4B). These results demonstrate that mitotic dephosphorylation of RPTPα pSer204 and concomitant dephosphorylation of Src pTyr416 and pTyr527 was similar in mouse NIH3T3 cells and human HeLa cells.

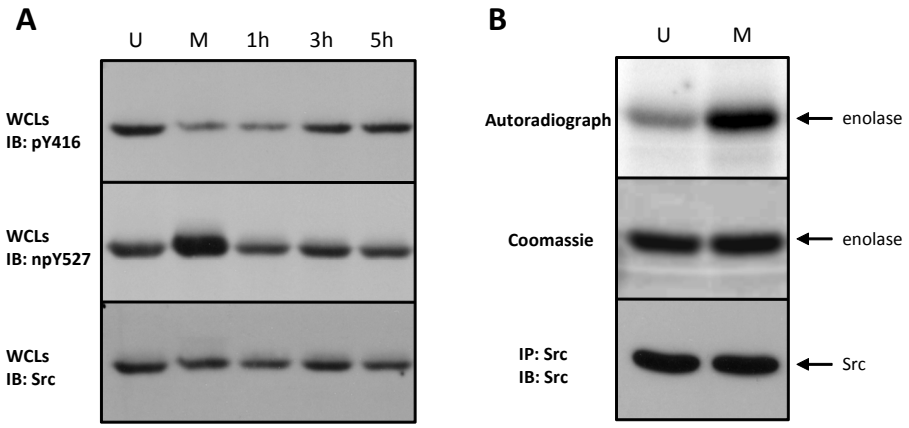


Fig.3 Mitotic phosphorylation and activation of Src. A, A fraction of the lysates used for RPTPα immunoprecipitation (Fig. 2A) was boiled in SDS sample buffer and the samples were run on a 7.5% SDS-polyacrylamide gel. The proteins were transferred to PVDF and the membranes were probed with anti-pY416 antibody and subsequently, after stripping, with anti-npY527 and anti-Src. B, Endogenous Src was immunoprecipitated with cross-linked antibodies to Protein A beads from unsynchronized and mitotic NIH3T3 cells. Half of the immunoprecipitate was subjected to an *in vitro* kinase assay, using enolase as substrate. The other half was used for immunoblotting with anti-Src antibody followed by ECL (bottom panel). The amount of incorporated phosphate was visualized by autoradiography (top panel). The positions of enolase and Src are indicated by arrows.

PP2A dephosphorylates RPTPα in vitro and in vivo

RPTPα pSer204 was almost completely dephosphorylated in mitotic arrested NIH3T3 cells (Fig. 2A, B) and HeLa cells (Fig. 4A), whereas pSer180 levels were much less affected under these conditions. We set out to identify the phosphatase(s) responsible for mitotic dephosphorylation of pSer204. The main

serine phosphatases that are activated in mitosis are PP1, PP2A and Cdc25 (Trinkle-Mulcahy and Lamond 2006). *In vitro* phosphatase assays were performed to assess which of these phosphatases dephosphorylated RPTPα pSer204. RPTPα was immunoprecipitated from NIH3T3 cell lysates and the immunoprecipitates were incubated with fresh NIH3T3 lysates, resulting in dephosphorylation of pSer204, compared to control RPTPα that was not incubated with lysate (Fig. 5A). Na_3VO_4 (V) did not affect RPTPα serine dephosphorylation, whereas NaF blocked pSer204 dephosphorylation almost completely (Fig. 5A). These results indicate that Cdc25, a cysteine based dual specificity phosphatase that is sensitive to Na_3VO_4, was not responsible for RPTPα dephosphorylation. However, the pSer204 phosphatase(s) was/were NaF-sensitive.

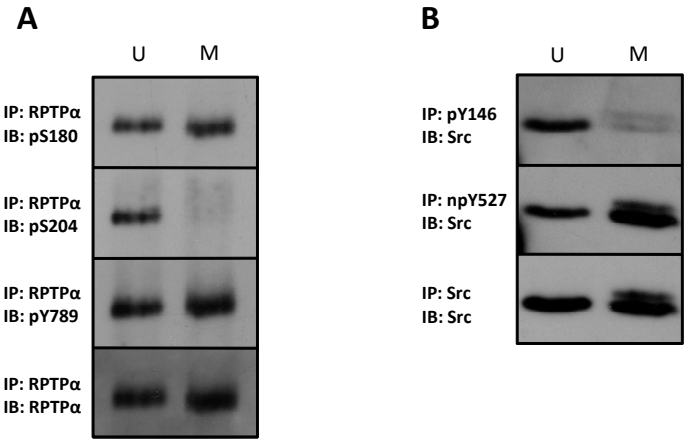


Fig.4 RPTPα and Src phosphorylation in HeLa cells. Unsynchronized (U) and mitotic (M) HeLa cell lysates (1mg total protein) were split in half. One half was used to immunoprecipitate endogenous RPTPα (A). The samples and the western blots were prepared as described in Fig. 2A. B, The second half was used for Src immunoprecipitation. Cross-linked Src antibodies were used for this purpose. The immunoprecipitates were fractionated by 7.5% SDS-PAGE and after transfer the membranes were probed with anti-pY416 antibody and subsequently, after stripping, with anti-npY527 and anti-Src. The experiment was performed at least three times with similar results and a representative experiment is shown here.

To test whether the pSer204 phosphatase activity was regulated during the cell cycle, RPTPα immunoprecipitates were incubated with lysates of unsynchronized or nocodazole arrested NIH3T3 cells. Both lysates readily dephosphorylated RPTPα pSer204 (Fig. 5B). Addition of a cocktail of PP1/PP2A inhibitors (okadaic acid, calyculin A and tautomycin) inhibited dephosphorylation of pSer204 (Fig. 5B). It appeared that the inhibitor cocktail was less efficient in inhibiting the phosphatases in the mitotic lysates, suggesting that the phosphatase activity was higher in lysates

from mitotic cells than from unsynchronized cells.

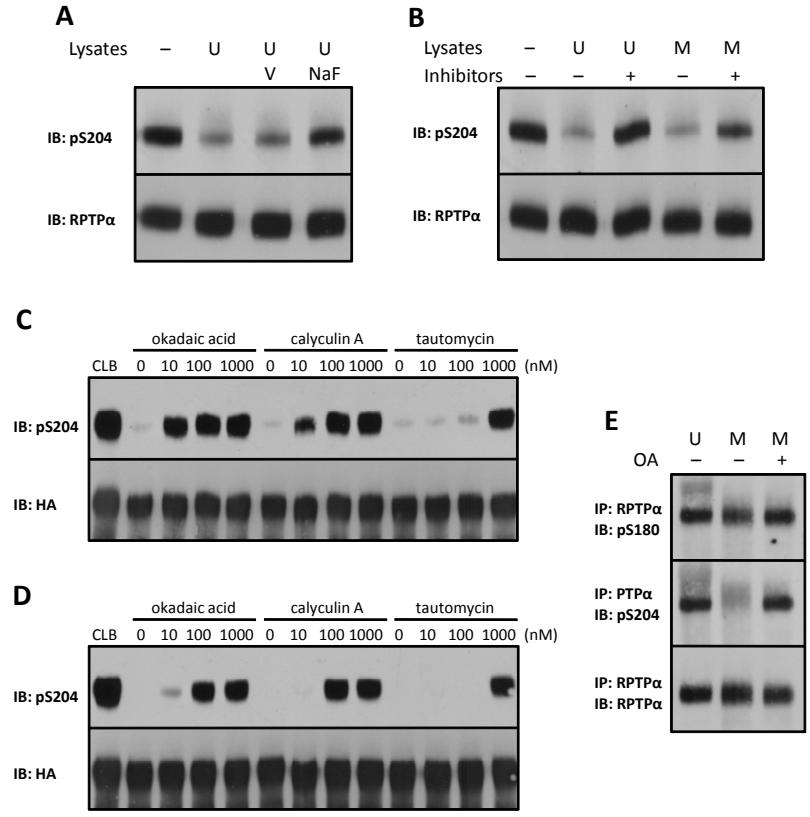


Fig.5 PP2A dephosphorylates RPTPα *in vitro* and *in vivo*. Endogenous RPTPα was immunoprecipitated from unsynchronized NIH3T3 cells (A and B). The immunoprecipitates were incubated with fresh lysates of unsynchronized (U) or mitotic (M) NIH3T3 cells in the presence of orthovanadate (V), sodium fluoride (NaF) or a cocktail of PP1/PP2A inhibitors containing okadaic acid, calyculin A and tautomycin (100 nM each). After the reactions were terminated, the proteins were separated by 7.5% SDS-PAGE and transferred to PVDF membranes. The blots were probed with anti-pS204 antibody and subsequently, after stripping, with anti-RPTPα antibody. HA-RPTPα overexpressed in COS1 cells was immunoprecipitated and incubated with unsynchronized (C) or mitotic (D) NIH3T3 lysates in the presence of increasing amounts of the PP1/PP2A inhibitors as indicated. The lysates were removed and the samples were processed as in A. Finally, the blots were probed with anti-pS204 antibody and subsequently, after stripping, with anti-HA tag antibody. E, NIH3T3 cells arrested with nocodazole were treated with 100 nM okadaic acid (OA). RPTPα was immunoprecipitated, blotted and the blots were probed with pS180, pS204 and RPTPα antibodies. These experiments have been done at least three times and representative blots are shown.

To investigate which of the two serine phosphatases, PP1 or PP2A, was responsible for RPTPα dephosphorylation, *in vitro* phosphatase assays were done in the presence of increasing concentrations of okadaic acid, calyculin A or tautomycin. Okadaic acid, an inhibitor with higher specificity for PP2A than for PP1

(IC_{50} PP2A = 0.1 nM, IC_{50} PP1 = 10-15 nM), blocked dephosphorylation of RPTPα already at 10 nM. Tautomycin, an inhibitor with higher efficiency for PP1 (IC_{50} PP2A = 10 nM, IC_{50} PP1 = 1 nM), blocked dephosphorylation only at 1000 nM. Calyculin A (IC_{50} PP2A = 0.5-1 nM, IC_{50} PP1 = 2 nM) had an intermediate effect (Fig. 5C). Based on these results, we conclude that PP2A is the main RPTPα pSer204 phosphatase in NIH3T3 cells. Similar results were obtained with lysates from nocodazole arrested cells (Fig. 5D), in that okadaic acid and calyculin A were the most potent inhibitors of serine phosphatase activity. Higher concentrations of inhibitors were required to fully block phosphatase activity in the lysates of mitotic arrested cells, compared to unsynchronized cells, suggesting that total pSer204 phosphatase activity was elevated in mitotic cells.

To confirm that PP2A is the phosphatase responsible for the mitotic dephosphorylation of pSer204 *in vitro*, mitotic arrested NIH3T3 cells were treated with okadaic acid for 14h. Endogenous RPTPα was immunoprecipitated and pSer180 and pSer204 levels were probed. RPTPα Ser204 phosphorylation was greatly reduced in mitotic cells, compared to unsynchronized cells and okadaic acid completely reversed this effect. pSer204 levels in okadaic acid-treated mitotic cells were as high as in the unsynchronized cells (Fig. 5E). Interestingly, pSer180 levels were not affected following the okadaic acid treatment. These results indicate that pSer180 and pSer204 are differentially dephosphorylated and that PP2A is the phosphatase responsible for pSer204 dephosphorylation in mitosis.

RPTPα catalytic activity is not significantly influenced by serine phosphorylation

We have shown that RPTPα Ser204 is completely dephosphorylated in mitotic cells (Fig. 2) concomitant with Src activation (Fig. 3). Hence, dephosphorylation of RPTPα pSer204 might activate intrinsic RPTPα catalytic activity. To investigate this, we assayed phosphatase activity of RPTPα serine mutants *in vitro*. HA-tagged wild-type RPTPα, RPTPα serine mutants and C433S mutant were expressed in COS1 cells, immunoprecipitated and PTP activity was determined using phosphorylated MBP as a substrate. Mutation of Ser204 did not activate PTP activity, nor did mutation of Ser180, whereas mutation of the catalytic Cys433 rendered RPTPα inactive (Fig. 6A). Expression of RPTPα and mutants was monitored by immunoblotting (Fig. 6B). The PTP activity of RPTPα from mitotic cells was not significantly enhanced compared to RPTPα activity from unsynchronized cells in *in vitro* phosphatase assays (Fig. 6C). We also tested the ability of RPTPα Ser mutants to dephosphorylate Src *in vitro*. RPTPα

and mutants dephosphorylated Src pTyr527 to similar extents and had modest activity towards Src pTyr416 (Fig. 6D). As expected, only the catalytically inactive RPTPα C433S did not dephosphorylate Src pTyr527. These results demonstrate that dephosphorylation of pSer204 in mitosis did not enhance intrinsic catalytic activity of RPTPα and therefore cannot explain enhanced Src dephosphorylation.

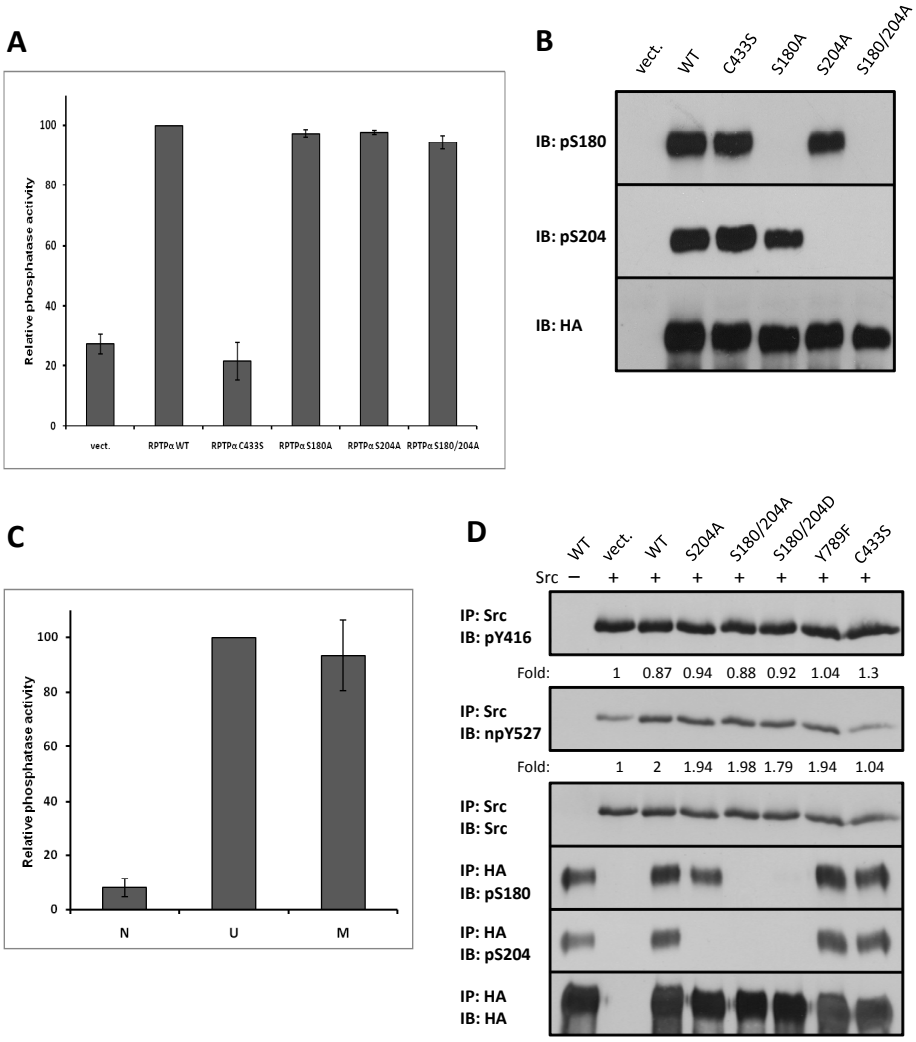


Fig.6 RPTPα catalytic activity is not significantly influenced by serine phosphorylation. A, WT HA-RPTPα, HA-RPTPα C433S and serine mutants of HA-RPTPα (S180A, S204A and S180A/S204A) were immunoprecipitated from transfected COS1 cells and their ability to release [^{32}P]-phosphate from phosphorylated MBP was detected. The combined results from three independent experiments are depicted here. B, For one of the experiments, the serine phosphorylation levels for each overexpressed protein are shown. C, Endogenous RPTPα was immunoprecipitated from lysates of unsynchronized (U) and mitotic (M) NIH3T3 cells (1mg total protein). For the negative control (N) the anti-RPTPα antibody was not added during immunoprecipitation. The ability to dephosphorylate MBP

is depicted in the graph. Each bar represents the average of three independent experiments ± S.D. The phosphatase activity of WT HA-RPTPα (A) and endogenous RPTPα from unsynchronized cells (C) was set to 100% and the other data was calculated relative to these values. D, HA-tagged RPTPα WT and the mutants indicated were immunoprecipiated from transfected COS1 cells and incubated with Src immunoprecipitated from transfected SYF cells. After incubation the samples were boiled in SDS sample buffer and resolved by 7.5% SDS-PAGE. The blots were probed for Src phosphorylation as well as for the amounts of HA-RPTPα present in each reaction. Ser phosphorylation levels of RPTPα are also shown. Src phosphorylation levels were quantified and normalized for the total amount of Src and expressed as the ratio to the phosphorylation levels of Src incubated with immunoprecipitates from empty vector transfected cells. The values are presented under each corresponding sample.

Phosphorylation of RPTPα Ser204 prohibits Src binding

Binding of Src to RPTPα is an important determinant in Src activation. To examine the mechanism underlying Src activation by RPTPα in mitosis, we investigated co-immunoprecipitation of endogenous Src with RPTPα in unsynchronized and mitotic NIH3T3 cells. Clearly, binding of Src to RPTPα was induced in mitotic cells (Fig. 7A), concomitant with Ser204 dephosphorylation. PSer180 and pTyr789 levels were determined in the same samples and were similar in unsynchronized and mitotic cells as observed before (Fig. 2A, 7A).

Previously Zheng et al. (2000) suggested that RPTPα-mediated Src activation requires phosphorylation of RPTPα Tyr789 and displacement of the adaptor protein GRB2 that is bound to pTyr789. We found that GRB2 binding to RPTPα was only modestly reduced in mitosis (23% reduction compared to unsynchronized cells) which cannot account for enhanced Src binding and activation (Fig. 7A). Interestingly, mutation of Ser180, Ser204 or both did not affect GRB2 binding in pull-down assays (data not shown), indicating that serine phosphorylation of RPTPα does not affect GRB2 binding. Several reports showed that RPTPα can activate Src in the absence of the Tyr789 phosphorylation site (Lammers et al. 2000; Yang et al. 2002; Chen et al. 2006). To determine definitively whether Src can bind to RPTPα in the absence of pTyr789 we immunoprecipitated Src from SYF cells co-transfected with Src and RPTPα WT or Y789F mutant. We found that mutant RPTPα-Y798F co-immunoprecipitated with Src, albeit to a lesser extent than wild type RPTPα (Fig. 7B), demonstrating that RPTPα pTyr789 is not strictly required for Src binding to RPTPα.

Next, we investigated whether Ser204 phosphorylation has a decisive role in the Src-RPTPα interaction. Okadaic acid treatment completely recovered the phosphorylation of RPTPα Ser204 in the mitotic arrested cells and concomitant with Ser204 re-phosphorylation, Src binding to mitotic RPTPα was lost completely (Fig. 7C), indicating that Ser204 phosphorylation prohibits Src binding. Okadaic

acid treatment did not significantly affect RPTPα Ser204 or Ser180 phosphorylation in unsynchronized NIH3T3 cells and Src binding to RPTPα was not detected in unsynchronized cells under these circumstances (Fig. 7C).

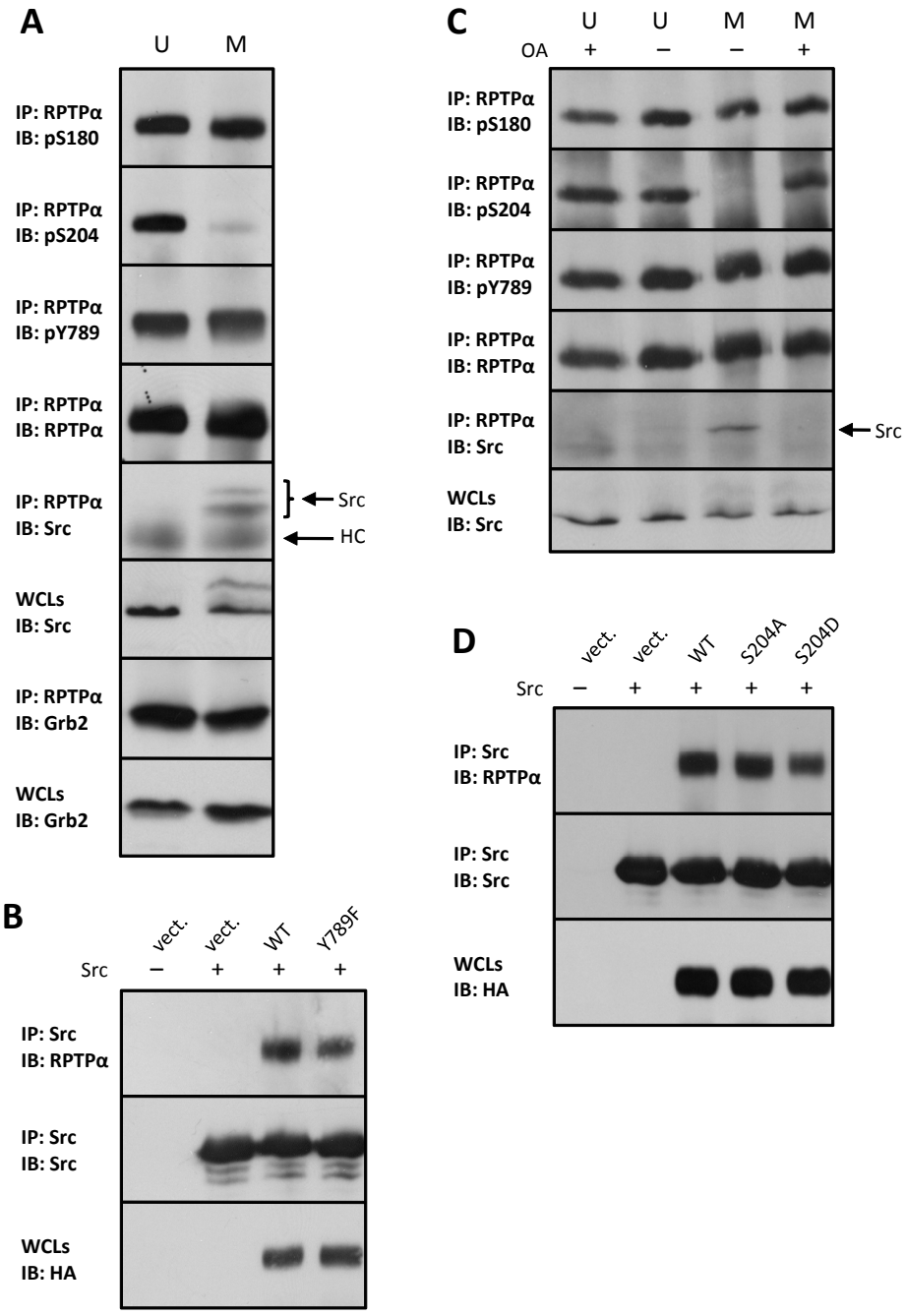


Fig.7 Src binding to RPTPα is induced in mitosis by RPTPα pSer204 dephosporyation, independently of GRB2. A, Unsynchronized (U) and mitotic (M) cells were lysed (3 mg total protein) and endogenous RPTPα was immunoprecipitated. The immunoprecipitates were fractionated on 12.5% SDS-polyacrylamide gel and tested for co-immunoprecipitated Src and GRB2. Phosphorylation of immunoprecipitated RPTPα was also probed. Src and the heavy chain (HC) of the antibody are indicated by arrows. The input levels of Src and GRB2 in the lysates (3% of the total lysate run on the same gel) are shown. B, Src and RPTPα WT, Y789F mutant or empty vector were co-transfected into SYF cells. The cells were lysed and Src was immunoprecipitated with cross-linked anti-Src antibodies. The samples were fractionated on a 7.5% SDS-polyacrylamide gel, transferred to PVDF membranes and immunoblotted with anti-RPTPα serum (top panel) and anti-Src MAb 327 (middle panel). Whole cell lysates were monitored for HA-RPTPα expression (bottom panel). C, Unsynchronized (U) and mitotic (M) NIH3T3 cells were treated with 100nM okadaic acid (OA) or left untreated. The samples were processed as in A. To reduce the signal from the antibody heavy chain, the blot showing the amount of co-immunoprecipitated Src was probed with HRP-coupled Protein A, which was less sensitive and hence did not allow detection of the slower migrating Src band. D, SYF cells were co-transfected with Src and HA-RPTPα WT, mutants (S204A and S204D) or empty vector (vect.). Co-immunoprecipitation of (mutant) RPTPα with Src was detected as described in B. The experiments were repeated at least three times with similar results and representative experiments are shown here.

We further investigated the effect of Ser204 phosphorylation on RPTPα binding to Src. SYF cells were cotransfected with Src and RPTPα or Ser mutants, the cells were lysed and Src was immunoprecipitated. Subsequently, the immunoprecipitates were probed for co-immunoprecipitated RPTPα. RPTPα-S204A bound to Src to a similar extent as WT RPTPα (Fig. 7D). Given our hypothesis that phosphorylation of Ser204 prohibits Src binding, we expected to find enhanced binding of RPTPα-S204A to Src, compared to WT RPTPα. Under these overexpression conditions RPTPα-S204A binding to Src apparently was maximal. Binding of RPTPα-S204D with a phosphomimicking mutation replacing Ser204 was reduced 30-50% (Fig. 7D). Binding of Src to RPTPα-S204D was not completely abolished. However, substitution of Ser by an Asp residue mimics pSer to some extent, not completely. The reduction in binding of RPTPα-S204D to Src is consistent with our conclusion that phosphorylation of Ser204 inhibits RPTPα binding to Src.

Based on our results, we conclude that RPTPα Ser204 phosphorylation is reduced in mitosis, allowing Src to bind to mitotic RPTPα, resulting in dephosphorylation of Src pTyr527 and pTyr416.

Discussion

In this study, we explored how endogenous RPTPα in NIH3T3 and HeLa cells is regulated by serine phosphorylation in mitosis and how these mitotic changes reflect the ability of RPTPα to activate Src. We developed phospho-specific antibodies directed at the known serine phosphorylation sites of RPTPα (Ser180 and

Ser204) (Tracy et al. 1995). In contrast to the model of Zheng et al. in which serine hyperphosphorylation drives RPTPα activation (Zheng and Shalloway 2001; Zheng et al. 2002), we demonstrate that Ser204 was almost completely dephosphorylated in mitosis. We established that pSer204 phosphatase activity is enhanced in mitotic NIH3T3 cells and using inhibitors, we identified PP2A as the pSer204 phosphatase *in vitro* and *in vivo* (Fig. 5). It appeared that PP2A was already active in unsynchronized cells and the moderate increase in PP2A activity in mitosis perhaps cannot account for the complete dephosphorylation of pSer204. We believe that dephosphorylation of pSer204 in mitosis is the net result of the combined effects of activation of PP2A and inactivation of the responsible kinase. Several kinases may phosphorylate Ser204, among which PKA, because (i) the flanking sequences of Ser204 form a consensus PKA phosphorylation site (Neuberger et al. 2007), (ii) PKA phosphorylates Ser204 *in vitro* (data not shown) (iii) pharmacological PKA inhibitors reduced Ser204 kinase activity in NIH3T3 cell lysates (data not shown) and (iv) cyclic AMP (cAMP) levels and hence PKA kinase activity are decreased in mitosis (Abell and Monahan 1973; Grieco et al. 1996). Additional experiments are required to definitively establish the identity of the Ser204 kinase.

We and others have shown previously that serine phosphorylation may regulate RPTPα activity directly. PKC-mediated enhanced serine phosphorylation of RPTPα leads to activation of RPTPα and dephosphorylation of RPTPα *in vitro* reduces its activity to pre-stimulation levels (den Hertog et al. 1995). Bacterially expressed RPTPα fusion protein phosphorylated *in vitro* using CaMKIIα enhanced RPTPα catalytic activity, which appeared to be mediated mostly by Ser180 (Bodrikov et al. 2008). Here, we demonstrate that mutation of Ser180 and Ser204 did not significantly affect intrinsic catalytic activity of RPTPα when MBP or Src were used as substrates. Moreover, the activity of endogenous nonphosphoSer204 RPTPα from mitotic NIH3T3 cells was not significantly different from the activity of RPTPα from unsynchronized cells (Fig. 6). These results indicate that mitotic activation of Src is unlikely caused by changes in intrinsic catalytic activity of RPTPα.

When looking at endogenous Src phosphorylation in mitotic cells we noticed that not only Tyr527 was dephosphorylated but also Tyr416. Previous studies have shown that overexpressed RPTPα dephosphorylates both tyrosine residues (Zheng et al. 1992; den Hertog et al. 1993; Harder et al. 1998). RPTPα has similar activity towards phosphorylated peptides coding for these two Src

tyrosine phosphorylation sites (Ng et al. 1997). Dephosphorylation of both pTyr416 and pTyr527 led to a modest net increase in Src activity (~2.3 fold) in mitosis (Fig. 3B), which is consistent with previously reported mitotic Src activation (Zheng and Shalloway 2001). Moreover, RPTPα overexpression results in modest activation of Src as well (Zheng et al. 1992; den Hertog et al. 1993; Zheng et al. 2000). Mutation of Tyr527 in Src results in full activation of Src (~20-fold higher activity than wild type Src) (Hunter 1987), concomitant with autophosphorylation of Src on Tyr416. Apparently, dephosphorylation of both pTyr527 and pTyr416 prevents inadvertent overactivation of Src.

Here, we demonstrate for the first time using endogenously expressed proteins that Src binding to RPTPα is induced in mitosis. Previously, detection of Src binding to RPTPα relied on overexpression of Src and RPTPα (Zheng and Shalloway 2001). Binding of Src to RPTPα was suggested to be mediated by binding of the Src SH2 domain to pTyr789 in RPTPα. The Src SH2 domain has suboptimal affinity for phosphorylated Tyr789 (the affinity for a peptide encoding the C-terminus of RPTPα, AFSDpYANFK, being more than 10 times weaker than the affinity for the optimal peptide sequence pYEEI of Src SH2) (Songyang and Cantley 1995; Sonnenburg et al. 2003). Phosphorylation of RPTPα Tyr789 forms a consensus binding site for GRB2 and it is hard to imagine that Src and GRB2 compete for binding to this site. Nevertheless, the FAK/p130Cas/Src complex dissociates in mitosis following serine phosphorylation of FAK (Yamakita et al. 1999), increasing the amount of mitotic Src that can bind to RPTPα. GRB2 binding is only marginally reduced in mitotic NIH3T3 cells (Fig. 7A). Tyr789 is essential for GRB2 binding, but appears to be dispensable for Src binding (Fig. 7B), consistent with several studies showing that RPTPα lacking Tyr789 is still able to dephosphorylate and activate Src (Lammers et al. 2000; Yang et al. 2002; Chen et al. 2006; Kapp et al. 2007). Therefore, it is unlikely that the underlying mechanism for mitotic activation of Src involves competition for binding of Src and GRB2 to RPTPα pTyr789. Our data suggest that Src binding is mediated by (a) different region(s) of RPTPα and that phosphorylation of Ser204 prohibits binding. Close to Ser204 in RPTPα is a putative SH3 binding site RKYPPLP (residues 207-213). RPTPα is constitutively phosphorylated on Ser204 and this could impair access of SH3 domains to this region. We observed enhanced binding of RPTPα to Src in mitosis and mitotic dephosphorylation of Ser204 may open up the SH3 binding site, thus triggering binding to Src. Mutation of Ser204 to Ala, prohibiting

phosphorylation of this site, did not affect GRB2 binding (data not shown), nor Src binding (Fig. 7D). Src binding to the RPTPα Ser204 phosphomimicking mutant (S204D) was reduced in co-immunoprecipitation assays (Fig. 7D), corroborating the hypothesis that phosphorylation of Ser204 prohibits Src binding.

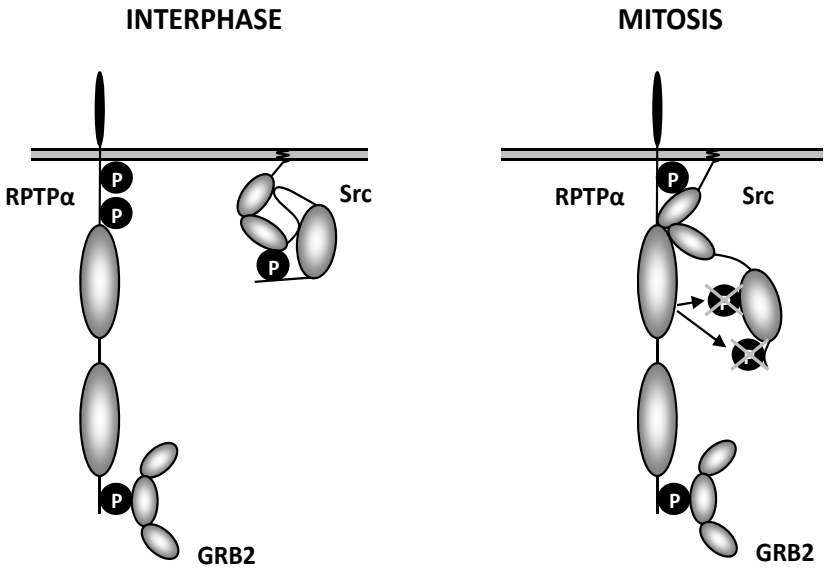


Fig.8 RPTPα-mediated activation of Src in mitosis. RPTPα is phosphorylated constitutively on Ser180, Ser204 and Tyr789. At interphase, phosphorylation of Ser204 prohibits binding of Src and as a result, Src is not activated. In mitosis, pSer204 is dephosphorylated due to activation of PP2A. NonphosphoSer204 binds Src through yet to be identified regions in Src and RPTPα, while remaining phosphorylated on Tyr789 and bound to the adaptor protein GRB2. Src binding to RPTPα in mitosis leads to dephosphorylation of pTyr527 and pTyr416 in Src, resulting in modest activation of Src kinase activity. Following release from mitosis, Ser204 is rapidly phosphorylated and Src phosphorylation reverts.

RPTPα Ser204 phosphorylation site is positioned in the proximity of the wedge structure, an element shown to play an important role in RPTPα dimerization (Bilwes et al. 1996). Phosphorylation or dephosphorylation of Ser 204 might influence the architecture of the juxtamembrane domain. Therefore, we also considered that RPTPα dimerization might be affected by pSer204 dephosphorylation, resulting in enhanced RPTPα activity and subsequent Src dephosphorylation. However, we did not observe any differences between RPTPα and serine mutants in co-immunoprecipitation and accessibility assays (van der Wijk et al. 2003) to assess differences in dimerization or quaternary structure of RPTPα (data not shown). However, Ser204 dephosphorylation might have subtle effects on dimerization (e.g.

on stability of the dimers), which might not be detected in the standard dimerization assays.

We cannot exclude the possibility that serine/threonine phosphorylation of Src has a role in mitotic activation of Src as well. Src is phosphorylated on Thr34, Thr46 and Ser72 in mitosis by Cdc2, which leads to an increase in exposure of pTyr527 (reviewed in Roskoski 2005). Serine/threonine phosphorylation of mitotic Src causes reduced electrophoretic mobility. There is no obvious difference in co-immunoprecipitation of the slower migrating forms of Src with RPTPα (Fig. 7A), making it unlikely that serine/threonine phosphorylation of Src has a decisive role in the Src-RPTPα interaction.

Our data led us to propose a model for mitotic activation of Src (Fig. 8). In interphase, Ser180, Ser204 and Tyr789 are phosphorylated. In mitosis, PP2A is activated, resulting in pSer204 dephosphorylation while phosphorylation of Ser180 and Tyr789 is hardly affected. This results in Src binding to RPTPα without significant effects on GRB2 binding. Subsequently, both pTyr416 and pTyr527 in Src are dephosphorylated, resulting in modest activation of Src. After release from mitosis, Ser204 is phosphorylated again, leading to release of Src and subsequent phosphorylation of Tyr527. This model is different from the model proposed by Zheng and Shalloway (Zheng and Shalloway 2001; Zheng et al. 2002) at several points: Zheng and Shalloway suggested that serine phosphorylation was enhanced in mitosis. In contrast, we demonstrate that pSer204 is dephosphorylated in mitosis. They proposed as underlying mechanism an increase in intrinsic RPTPα catalytic activity. We found no evidence for activation of RPTPα catalytic activity in mitosis and the catalytic activity of mutant RPTPα that cannot be phosphorylated was not significantly different from wild type RPTPα. Finally, we did not find evidence for a displacement mechanism in which GRB2, bound to pTyr789, is displaced by Src. Yet, we demonstrate that binding of Src to RPTPα was induced in mitosis, which we believe mediates the increase in Src activity. The discrepancy between our data and the results of Zheng and collaborators (Zheng and Shalloway 2001; Zheng et al. 2002) regarding the mitotic RPTPα phosphorylation may be caused by the different setup of the experiments. We followed the phosphorylation of endogenous RPTPα while Zheng et al. used RPTPα overexpressing NIH3T3 cells for most of their experiments. RPTPα overexpression in NIH3T3 cells reportedly results in metabolic changes (Lammers et al. 2000) which may affect cell behavior. Importantly, the molecular

ratios between RPTPα, Src and the other proteins involved in this mechanism are crucial and overexpression of one of these factors will severely impact on the balance of these factors.

In conclusion, we propose a new model for mitotic activation of Src by RPTPα in mitosis. Dephosphorylation of RPTPα pSer204 leads to Src binding. Whether Src binds directly to RPTPα close to dephosphorylated Ser204 remains to be determined. Concomitant dephosphoryation of pTyr527 and pTyr416 results in modest activation of Src, prohibiting Src to display its oncogenic potential.

Materials and methods

Materials and antibodies

12CA5 Anti-HA-tag, 327 anti-Src and 5478AP anti-RPTPα antibodies were prepared as previously described (den Hertog et al. 1993; den Hertog et al. 1994). The anti-RPTPα 1951AP antibody was obtained following the same procedure as for 5478AP antibody. Briefly, rabbits were immunized with bacterially expressed cytoplasmic domain of RPTPα fused with GST. The polyclonal antiserum was first cleared of anti-GST antibodies using a GST affinity column, then the anti-RPTPα antibodies were purified using a second affinity column loaded with GST-PTPα protein. Anti-RPTPα-pY789 and anti-Src-npY527 were from Cell Signaling. Anti-Src-pY418 was from Biosource and anti-rabbit and anti-mouse secondary antibodies were from BD Biosciences. Polyethylenimine (PEI), nocodazole, paclitaxel and glutathione-Sepharose were from Sigma Life Science. Okadaic acid, calyculin A and tautomycin were from Calbiochem. Total protein concentration in the lysates was detected using a BCA kit (Sigma).

Phospho-specific antibodies

Anti-phospho-Ser180 and anti-phospho-Ser204 antibodies were raised against two synthetic peptides corresponding to the known sequences containing the serine phosphorylation sites in RPTPα. Extra N-terminal cysteines were added to the peptides to allow coupling of the peptides to the carrier (in this case keyhole limpet hemocyanin). Anti-phospho-Ser180 antibody was raised against CSN**pS**FRLSNG peptide and anti-phospho-Ser204 antibody against CSP**pS**TNRKYP. The production of the peptides, the immunization of the rabbits and the affinity purification of the antibodies was carried out by Eurogentec.

DNA constructs

The constructs used for the expression of HA-RPTPα WT (den Hertog et al. 1993), HA-RPTPα-Y789F, Src WT and Src-Y527F were previously described (den Hertog et al. 1994). HA-RPTPα-S180A, HA-RPTPα-S204A and HA-RPTPα-S180A/S204A were generated by PCR-mediated site-directed mutagenesis using HA-RPTPα WT as template and the following oligonucleotides: 5'-AGTCATTCCAACGCTTTCCGCCTGTCA-3' for S180A and 5'-GCCAGGTCCCCAGCCACCAACAGGAAG-3' for S204A. The constructs were verified by sequencing.

Cell culture, mitotic arrest and FACS analysis

For the experiments described in this study we used NIH3T3, HEK293, COS1 and SYF cells. HEK293 and COS1 cells were grown in DF medium (Dulbecco's modified Eagle's medium/Ham's F-12 medium) supplemented with 7.5% fetal calf serum (FCS). NIH3T3 cells were gown in DMEM medium supplemented with 7.5% newborn calf serum (NCS). SYF cells were grown in DMEM medium supplemented with 7.5% FCS. The mitotic arrest of NIH3T3 cells was achieved after nocodazole treatment. 70-80% confluent NIH3T3 cells were treated with 0.6μg/ml nocodazole and grown for another 12-14 hours. Finally the mitotic cells were harvested by rinsing the monolayer repeatedly with a stream of medium or by mitotic shake-off. The collected cells were centrifuged and washed twice with PBS solution. Part of the cells was replated and harvested at different time points. The remaining cells were either used for FACS analysis or lysed. The FACS analysis was performed on a Becton-Dickinson FACSCalibur. The cell cycle distribution was determined by quantifying the amount of propidium iodide (PI) incorporated in the nuclei. The cells were fixed in 70% ice-cold ethanol and kept at 4°C for at least 12 hours. After fixation PBS solution was added to the tubes and the cells were centrifuged. Afterwards the cells were washed once more with PBS solution and centrifuged. The supernatant was removed and the cells were resuspended in small volume of PBS solution containing 250μg/ml RNAse and 1μg/ml PI. The cells were incubated for 15 minutes at 37°C then kept at 4°C until the analysis was performed.

SYF cells were transfected with FuGene6 (Roche) according to the protocol provided by the manufacturer. HEK293 and COS1 cells were transiently transfected with empty vector or HA-RPTPα using polyethylenimine (PEI). After transfection the cells were grown for 16 hours in serum containing medium and then the medium was changed with serum free medium and the cells were grown for additional 24

hours. The cells were lysed for 20 minutes 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 β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin and 1 µg/ml aprotinin). The lysates were collected using a rubber policeman and centrifuged for 10 minutes at 13000 rpm. Samples from the lysates were collected and boiled after being mixed with equal volumes of 2X 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.

Immunoprecipitation and immunobloting

Nocodazole treated or untreated NIH3T3 cells were lysed for 20 minutes on ice in RIPA buffer containing 20 mM Tris-HCl 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, 5 mM NaF, 5mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin and 1 µg/ml aprotinin. For immunoprecipitation of RPTPα the lysates were first incubated with the anti-RPTPα antibody (5478AP) for 1 h at 4°C and then with Protein A Sepharose for 1 h. For Src immunoprecipitation the lysates were incubated for 1 h at 4°C with anti-Src monoclonal antibody 327 cross-linked to Protein A Sepharose. The immunoprecipitates were extensively washed with RIPA buffer. Each immunoprecipitate was divided into two equal fractions of which one was immunobloted using anti-Src antibody and the other was subjected to kinase assay. The fraction used for kinase assay was washed once in kinase buffer before the assay.

Kinase assays

Src kinase assays were performed in 40 µl kinase reaction buffer (50 mM HEPES pH 7.5 and 10 mM $MgCl_2$), containing 10 µCi [γ-^{32}P]ATP and 3.5 µg acid-denatured enolase. Reactions were incubated at 30°C for 30 min, stopped by the addition of 2X SDS sample buffer and resolved by 7.5% SDS-PAGE. Results were visualized by autoradiography. Similarly Src Y527F was used to phosphorylate MBP for use as substrate in RPTPα phosphatase assays.

Phosphatase assays

The phosphatase assays were performed following the instruction for the Protein Tyrosine Phosphatase Assay System from New England Biolabs. Endogenous RPTPα from NIH3T3 cells and overexpressed RPTPα from transfected COS1 cells

were immunoprecipitated as mentioned above. After washing, the beads were incubated with the lysates of unsynchronized or mitotic NIH3T3 cells in cell lysis buffer for 1 hour at 30°C. The beads were washed 4 times with HNTG buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol) and boiled in 2X SDS sample buffer.

The variation in activity of RPTPα and mutants was determined *in vitro* using phosphorylated MBP or Src as substrates. Overexpressed RPTPα was immunoprecipitated from transfected COS1 cells as previously described. The beads were washed 3 times with HNTG buffer and one time with phosphatase buffer (20 mM MES pH 6, 150 mM NaCl, 2.5 mM DTT, 1mM EDTA and 1mg/ml BSA). Half of the immunoprecipitated RPTPα was used for the phosphatase assay and the other half for monitoring the amount of RPTPα in the samples. The reactions were performed in a total volume of 40 µl containing immunoprecipitated RPTPα, phosphatase buffer and 3 µg phosphorylated MBP. The mixtures were incubated for 1 hour at 30°C and the reaction was stopped by adding 200 µl TCA 20% to each tube. The tubes were kept on ice for 5 minutes and then centrifuged for 10 minutes at 12000 g and 4°C. 200 µl from each tube was added to vials containing scintillation fluid and the samples were measured in a scintillation counter. The activity of endogenous RPTPα from unsynchronized and mitotic NIH3T3 cells was detected similarly. The only difference was that after immunoprecipitation of RPTPα the beads were washed 3 times with RIPA buffer prior to phosphatase buffer washing.

When Src was used as substrate RPTPα was immunoprecipitated from transfected COS1 cells as described above and the beads were incubated for 1h at 30°C with beads containing Src immunoprecipitated from transfected SYF cells. The reactions were conducted in a buffer containing 50 mM HEPES pH 7.4, 150 mM NaCl, 2 mM DTT and 1 mM EDTA. The phosphorylation levels of Src Tyr416 and Tyr527 were used as readout for RPTPα activity.

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Chapter 3

RPTPα wedge structure is implicated in Src regulation

Andrei M. Vacaru[1] and Jeroen den Hertog[1,2]

[1]Hubrecht Institute – KNAW and University Medical Center, Utrecht, the Netherlands
[2]Institute of Biology Leiden, Leiden University, the Netherlands

Abstract

Previously, we have shown that dephosphorylation of RPTPα pSer204 leads to increased Src binding in mitosis, indicating that the region surrounding Ser204 may play a role in this interaction. RPTPα presents interesting features close to Ser204 that may explain the implication of Ser204 phosphorylation in Src binding: a putative SH3 binding domain and the so-called wedge structure that is involved in dimerization-induced inactivation. P210/211L mutations in RPTPα destroy the putative SH3 binding site and at the same time affect the stability of the wedge. We discovered that Src binding to RPTPα-P210/211L was impaired and the ability of RPTPα-P210/211L to activate Src was reduced compared to wild type RPTPα. Mutant Src with inactive SH3 domains (Src-W118A and Src-P133L) presented higher affinity for RPTPα than WT Src indicating that Src SH3 domain did not mediate Src binding to RPTPα. These results suggest that destabilization of the wedge in RPTPα-P210/211L affected the RPTPα-Src interaction. RPTPα with a mutation in the wedge, D228R, showed increased binding to Src, confirming that the wedge has an important role in the interaction. Finally, we compared the ability of two constitutively dimeric mutants of RPTPα to bind Src and we found that the open, active RPTPα dimer bound better to Src than the closed, inactive RPTPα dimer. We conclude that the quaternary structure of RPTPα is important for Src binding and activation.

Introduction

Tyrosine phosphorylation is one of the most important signal transduction mechanisms in eukaryotic organisms. The combined action of protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs) keeps Tyr phosphorylation at functional levels. Often, the equilibrium of phosphorylation in cells is also maintained by direct interactions between kinases and phosphatases. A well known PTK – PTP interaction is the activation of Src by RPTPα.

Protein-tyrosine kinase Src, a protein encoded by the first discovered proto-oncogene, is ubiquitously expressed (Brown and Cooper 1996). Src consists of an SH4, SH3, SH2 and a catalytic domain. While the SH4 domain contains the signals for Src lipid modification, resulting in myristoylation of Gly at position 2, thus targeting Src to the cellular membranes, the SH3 and SH2 domains are involved in intra- and intermolecular interactions (Thomas and Brugge 1997). Under basal conditions Src adopts an inactive closed conformation, in which the SH2 domain binds the C-terminal phosphorylated Tyr (Tyr527 in chicken Src) and the SH3 domain attaches to the linker between the SH2 domain and the kinase domain (Xu et al. 1999). Several PTPs were shown to activate Src by dephosphorylating pTyr527 (reviewed in Roskoski 2005), among which the transmembrane PTP, RPTPα (Zheng et al. 1992; den Hertog et al. 1993). The mechanism underlying RPTPα-mediated activation of Src is not completely understood.

RPTPα contains a small highly glycosylated extracellular domain (Daum et al. 1994) and like most of the receptor protein-tyrosine phosphatases (RPTPs) has two phosphatase domains, with the membrane proximal domain being responsible for most of the catalytic activity (Lim et al. 1997; Wu et al. 1997). The mechanism proposed by Zheng et al. (Zheng et al. 2000) for RPTPα mediated Src activation suggests that pTyr789 of RPTPα is required to displace the Src SH2 domain from pTyr527, allowing RPTPα to dephosphorylate this site. In contrast to this model, several studies show that RPTPα that lacks the Tyr789 phosphorylation site dephosphorylates and activates Src (Lammers et al. 2000; Yang et al. 2002; Chen et al. 2006; Kapp et al. 2007). Additionally, some of the PTPs that activate Src do not contain a phosphorylated Tyr that can bind Src SH2 domain. In this category is RPTPλ which is more efficient in activating Src than RPTPα (Fang et al. 1994).

Src has the highest activity during mitosis (Chackalaparampil and Shalloway 1988; Shenoy et al. 1989; Bagrodia et al. 1991; Roche et al. 1995) and RPTPα was

found to be responsible for this effect (Zheng and Shalloway 2001; Zheng et al. 2002). Zheng and his collaborators proposed that in mitosis RPTPα is hyperphosphorylated on two Ser residues (Ser180 and Ser204) in the juxtamembrane region. This induces an increase in activity and detachment from GRB2, a scaffolding protein that occupies most of the Tyr phosphorylated RPTPα through its SH2 domain (den Hertog et al. 1994; den Hertog and Hunter 1996; Su et al. 1996). In these conditions Src binds free pTyr789 and is further dephosphorylated by activated RPTPα, hence resulting in activation of Src. We confirmed that Src is activated in mitosis. However, Tyr789 phosphorylation is not required for Src binding or Src activation and in contrast to the model described above, we demonstrate using phospho-specific antibodies RPTPα pSer204 was completely dephosphorylated in mitotic cells, which triggered Src binding and activation (Vacaru and den Hertog, submitted). This warranted further investigation of the mechanism underlying the RPTPα-Src interaction.

Here, we confirm that neither RPTPα pTyr789 nor the Src SH2 domain were required for the RPTPα-Src interaction. We report that a putative SH3 binding site in RPTPα and the Src SH3 domain are also not involved in the interaction. Instead, a helix-loop-helix wedge structure in RPTPα appears to be required for Src binding. Our results suggest that differences in the quaternary structure of RPTPα regulate RPTPα-mediated activation of Src.

Results

Src and RPTPα interact in the absence of pTyr789 or a functional SH2-domain

We investigated the involvement of RPTPα pTyr789 and the Src SH2 domain in Src binding to RPTPα. To this end, WT Src was cotransfected with WT RPTPα or RPTPα-Y789F mutant into SYF cells. Src was immunoprecipitated from the SYF lysates and the co-immunoprecipitated RPTPα was detected using anti-RPTPα serum. As seen before (Vacaru and den Hertog, submitted) Src binds to RPTPα in the absence of the Tyr789 phosphorylation site (Fig. 1A). Further, we tested if Src with a defective SH2 domain can still bind to RPTPα. We cotransfected Src-R175L, a mutant shown to be unable to interact with phosphotyrosine-containing substrates (Bibbins et al. 1993), with RPTPα and we found that in the absence of a functional SH2 domain Src is able to bind RPTPα (Fig. 1B). These results indicate that other elements than pTyr789 and SH2 domain are implicated in the RPTPα-Src interaction.

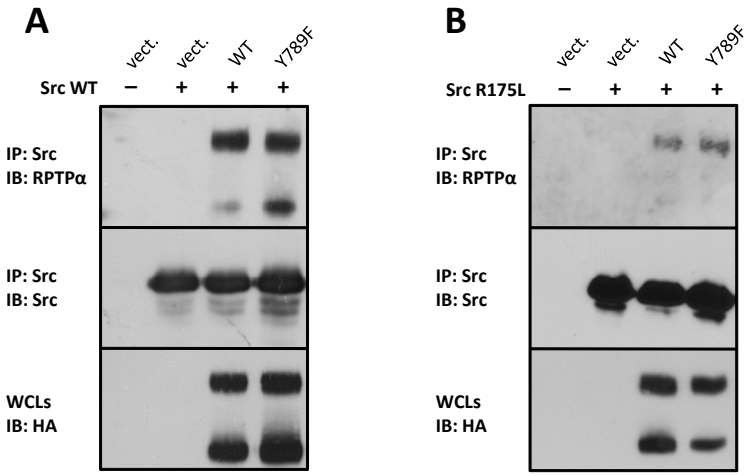

Fig.1 RPTPα interacts with Src in the absence of Tyr789 phosphorylation or SH2 domain activity. SYF cells were cotransfected with plasmids encoding for WT Src (A) or Src-R175L (B) and empty vector (vect.), HA-RPTPα WT or HA-RPTPα-Y789F. The cells were lysed and Src was immunoprecipitated with cross-linked monoclonal anti-Src antibodies. The samples were fractionated on a 7.5% SDS-polyacrylamide gel, transferred to PVDF membranes and immunoblotted with anti-RPTPα serum (top panels). The amounts of immunoprecipitated Src (middle panel) and HA-RPTPα in the lysates (bottom panel) are also shown.

RPTPα-P210/211L (LLLP) mediated Src activation is reduced

In mitosis, complete dephosphorylation of RPTPα pSer204 promotes Src binding and activation (Vacaru and den Hertog, submitted). In the proximity of RPTPα Ser204 there are two elements that could influence Src binding: a putative SH3 binding domain (PPLP) and the helix-loop-helix wedge structure that has a role in dimerization-induced inactivation of RPTPα. We proceeded to investigate the role of these two elements in the RPTPα-Src interaction using RPTPα containing P210/211L mutations that eliminate the putative SH3 binding domain and concomitantly destabilize the wedge structure (Jiang et al. 2000). Src was immunoprecipitated from SYF cells cotransfected with Src and RPTPα-P210/211L and the amount of RPTPα bound to Src was evaluated. We found that WT Src bound less RPTPα-P210/211L than WT RPTPα (Fig. 2A).

To assess the effect of the reduced Src binding to RPTPα-P210/211L on Src activation, we cotransfected the RPTPα expressing constructs with WT Src and after Src immunoprecipitation we performed an *in vitro* kinase assay using enolase as substrate. Wild type RPTPα induced a significant increase in Src activity and RPTPα-P210/211L only modestly affected Src activity (Fig. 2B). Quantification of three

independent experiments demonstrated that Src activation by RPTPα-P210/211L was significantly reduced (~1.5 fold) compared to activation of Src by WT RPTPα (~2.3 fold) (Fig. 2C). These results indicate that the integrity of the RPTPα region containing Pro210 and Pro211 is important for Src binding and activation.

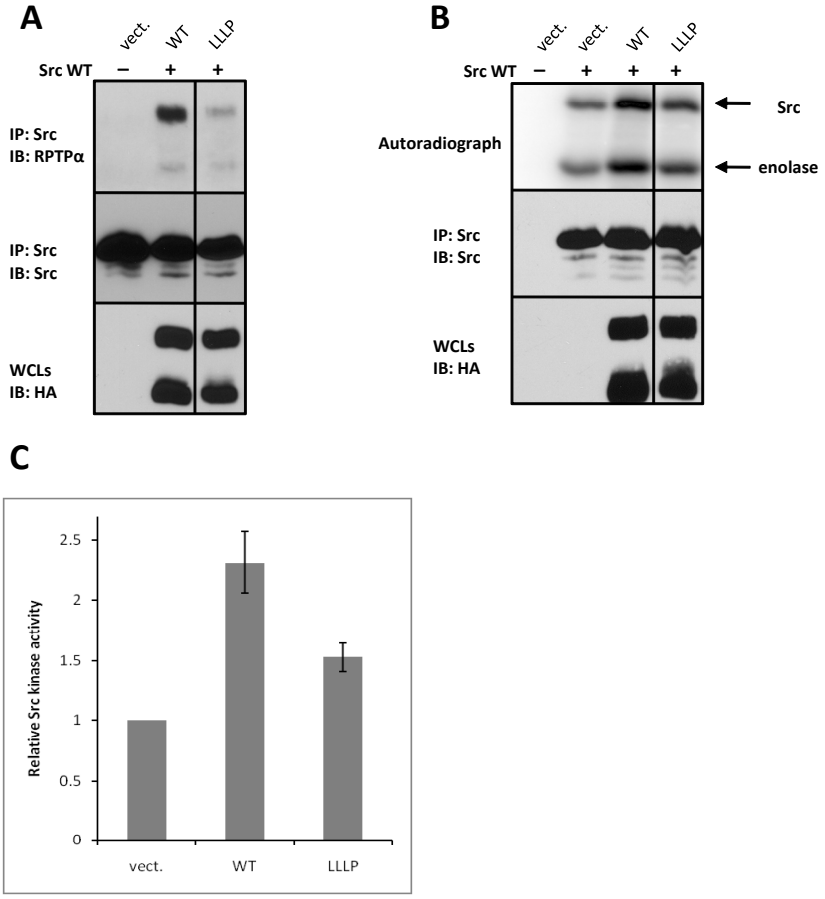

Fig.2 Decreased ability of RPTPα P210/211L (LLLP) to bind and activate Src. A, Anti-Src monoclonal cross-linked antibodies were used to immunoprecipitate Src from SYF cells cotransfected with WT Src constructs and empty vector, WT RPTPα or LLLP mutant. The samples were run on 7.5% SDS-polyacrylamide gels and after transfer, the PVDF membranes were probed for co-immunoprecipitated RPTPα using anti-RPTPα serum. The levels of immunoprecipitated Src and total HA-RPTPα in the lysates were also evaluated. Parts of the same blot from the same experiment were spliced together. The black line dividing lanes 2 and 3 was used to indicate this. B, Src was immunopecipitated from SYF cells contransfected with Src and RPTPα constructs (WT and LLLP). Half of the immunoprecipitated Src was subjected to an *in vitro* kinase assay, using enolase as 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 immunoprecipitated Src was fractionated by 7.5% SDS-PAGE, blotted, probed with anti-Src antibody and developed with ECL (middle panel). Total HA-RPTPα levels in the lysates were also monitored (bottom panel). C, Relative Src kinase activity. Each bar represents the average of three independent experiments ± SD, relative to Src immunoprecipitated from cells cotransfected with empty vector, which was set to one.

P210/211L mutations in RPTPα impair Src binding in a SH3-domain independent manner

The PPLP motif encompassing Pro210 and Pro211 may act as an SH3 binding site. To investigate if the Src SH3 domain is involved in Src interaction with RPTPα, we cotransfected RPTPα with a Src mutant with an inactive SH3 domain (W118A) (Erpel et al. 1995). In contrast to what we expected, Src-W118A had higher affinity for WT RPTPα than WT Src (Fig. 3A). Moreover, mutant Src-W118A still bound to RPTPα-P210/211L (Fig. 3A), indicating that the Src-RPTPα interaction was not mediated by a Src-SH3 - RPTPα-PPLP interaction. To confirm that the Src SH3 domain is not involved in RPTPα binding we used another Src mutant (P133L) that is reported to have an inactive SH3 domain (Erpel et al. 1995). Src-P133L, like Src-W118A mutant showed the same increased binding to RPTPα and still bound to RPTPα-P210/211L (Fig. 3B). These results demonstrate that the Src SH3 domain is not directly involved in the interaction with RPTPα and suggest that the effect of the P210/211L mutations on RPTPα wedge stability may affect the interaction with Src.

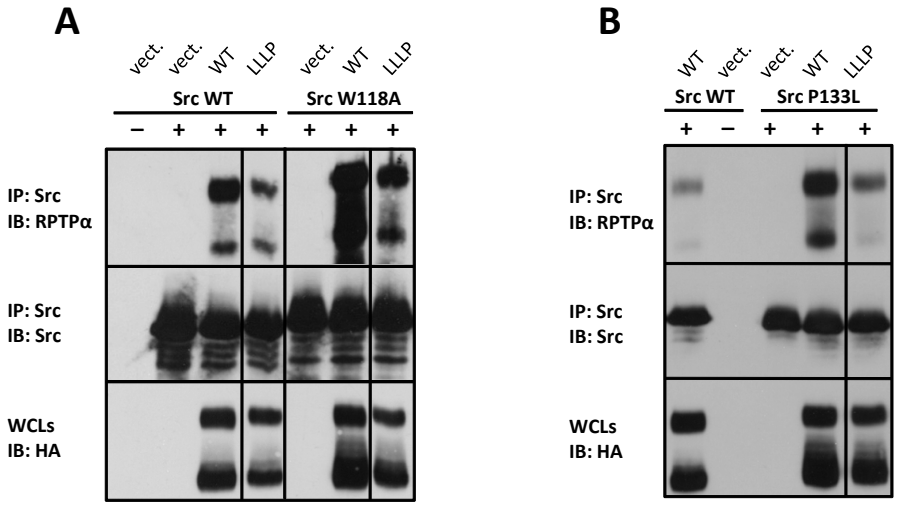

Fig.3 Src SH3 domain is not directly involved in Src binding to RPTPα. A, SYF cells cotransfected with Src (WT or W118A) and HA-RPTPα (WT or LLLP) constructs were lysed and Src was immunoprecipitated. The samples were resolved by 7.5% SDS-PAGE and transferred to PVDF membrane. Co-immunoprecipitated RPTPα was detected using anti-RPTPα serum. The levels of immunoprecipitated Src and total RPTPα in the lysates were also monitored. B, Cotransfected SYF cells with HA-RPTPα (WT, LLLP and LLLP/Y789F) and Src (WT or P133L) were processed as described in A. In both, A and B, parts of the same blots from the same experiments were spliced together this being indicated by vertical black lines.

RPTPα dimerization influences Src binding

To investigate whether the wedge of RPTPα has a role in Src binding we

used a mutant of RPTPα with a mutation at the tip of the wedge (D228R). A similar mutation in CD45 disrupts dimerization-induced inactivation of CD45 and has far-reaching consequences in knock-in mice in vivo (Majeti et al. 1998; Majeti et al. 2000). RPTPα-D228R was cotransfected with WT Src in COS1 cells and Src was immunoprecipitated. The amount of co-immunoprecipitated RPTPα-D228R was highly increased in comparison with WT RPTPα or RPTPα-P210/211L (Fig. 4A). As observed before, the wedge mutant RPTPα-P210/211L presented the same reduced binding to Src when compared to WT RPTPα.

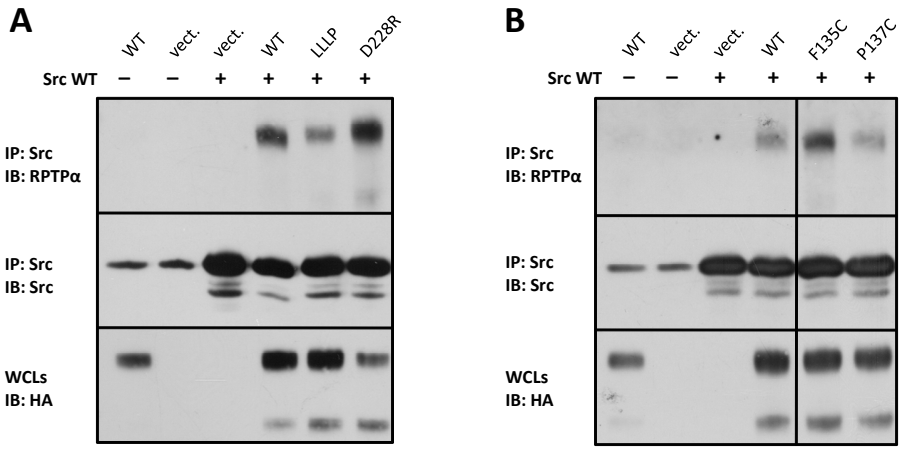

Fig.4 Conformation of RPTPα dimers affects Src binding. WT Src was cotransfected into COS1 with either empty vector (vect.), HA-RPTPα WT, HA-RPTPα-P210/211L and HA-RPTPα-D228R (A) or empty vector (vect.), HA-RPTPα WT, HA-RPTPα-F135C and HA-RPTPα-P137C (B). Src was immunoprecipitated from the cell lysates using cross-linked anti-Src antibodies. The samples were separated by 7.5% SDS-PAGE, transferred to PVDF membranes and probed with anti-RPTPα serum (top panels). Immunoprecipitated Src levels are shown in the middle panel and total HA-RPTPα amounts in the lysates are shown in the bottom panel. Parts of the same blot from the same experiment were spliced together. The black line dividing lanes 4 and 5 was used to indicate this.

It is well established that the wedge structure plays an important role in RPTPα dimerization (Bilwes et al. 1996; Jiang et al. 1999; Jiang et al. 2000). To test whether RPTPα dimerization influences the interaction with Src we used two RPTPα mutants that form constitutive dimers and have different catalytic activities: the RPTPα-F135C dimers are active whereas RPTPα-P137C dimers are inactive (Jiang et al. 1999). WT Src was cotransfected into COS1 cells with F135C or P137C RPTPα mutants and the affinity for each of these mutants was tested by co-immunoprecipitation assay. The mutant forming active dimers, RPTPα-F135C, had increased affinity for Src compared to WT RPTPα whereas RPTPα-P137C binding to Src was similar to WT

RPTPα (Fig. 4B). These results suggest that RPTPα dimerization affects Src binding and the conformation of the RPTPα dimers is important for this process.

Discussion

Mitotic dephosphorylation of RPTPα pSer204 leads to an increase in Src binding and to Src activation (Vacaru and den Hertog, submitted). In the proximity of RPTPα Ser204 there are two elements that could influence Src binding: (i) a putative SH3 binding domain and (ii) the so-called wedge structure. Using different Src and RPTPα mutants we investigated the role of these structural elements in the RPTPα-Src interaction. We reconfirmed that Src can bind to RPTPα in the absence of pTyr789. RPTPα-P210/211L mutant that lacks the putative SH3 binding site and the stability of the wedge, showed decreased binding to Src and did not activate Src effectively. Src SH3 domain mutants (W118A and P133L) exhibited increased binding to RPTPα compared to WT Src. These results indicate that the Src SH3 domain was not directly involved in the interaction with RPTPα and further suggested that the wedge of RPTPα plays an important role in Src binding and activation.

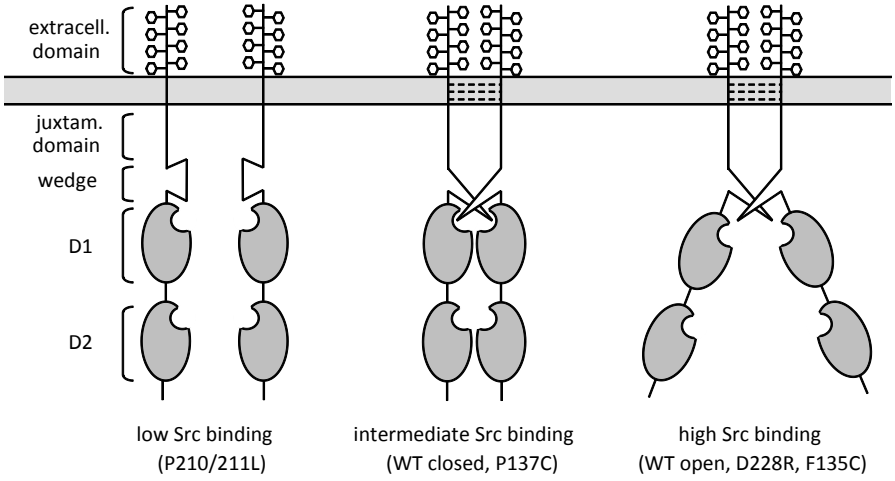

Fig.5 Influence of RPTPα dimer conformation on Src binding.

Mutation of the Src SH2 domain did not affect RPTPα binding, which corroborated earlier results that RPTPα Tyr789 is dispensable for Src binding (Fig. 1). These data contradict the model suggested by Zheng et al. (Zheng et al. 2000) who proposed a displacement mechanism in which RPTPα pTyr789 binds to the Src SH2 domain substituting pTyr527 which can then be dephosphorylated. Other

studies have also shown that mutant RPTPα-Y789F can activate Src to the same extent as WT RPTPα (Lammers et al. 2000; Yang et al. 2002; Chen et al. 2006; Kapp et al. 2007). Our results clearly demonstrate that the RPTPα-Src interaction is independent of pTyr789 and the Src SH2 domain.

We showed decreased affinity of Src for RPTPα P210/211L, a mutant that eradicates the consensus SH3 binding site (PPLP) and destabilizes the wedge structure (Fig. 2A). Introduction of SH3 domain inactivating mutations (W118A and P133L) did not lead to a reduction of Src affinity for RPTPα, on the contrary, they enhanced the interaction. This indicates that the Src SH3 domain is not involved in the interaction with RPTPα. Enhanced binding of the SH3 mutants is likely caused by opening up of the Src structure, exposing the RPTPα binding domains. Whereas all RPTPs contain the so-called helix-turn-helix "wedge" structure (Barr et al. 2009), only for a small number of them this motif was proven to be functional. Since RPTPα-D1 crystallized as a dimer in which the tip of the wedge from each monomer occluded the catalytic site of the other monomer (Bilwes et al. 1996), it was proposed that the wedge is responsible for the dimerization-induced inhibition of RPTPα. The importance of the wedge for RPTPα dimerization was demonstrated using P210/211L mutations which recovered the ability of RPTPα-P137C, a mutant that forms constitutively inactive dimers, to activate Src (Jiang et al. 1999). These indications bolster the idea that a destabilized wedge in mutant RPTPα-P210/211L is responsible for reduced Src binding. The role of the wedge in Src binding is confirmed by the increased binding of Src to mutant RPTPα-D228R (Fig. 4A). The importance of the wedge was corroborated by the use of the corresponding mutant of CD45, CD45-E613R. A CD45 chimeric molecule containing the extracellular domain of the epidermal growth factor receptor (EGFR) and the transmembrane and cytoplasmic domain of CD45 was functionally inactivated by EGF. However, EGF-induced inactivation was abolished in CD45-E613R (Majeti et al. 1998). Moreover, the CD45-E613R knock-in mouse phenotype was consistent with CD45 activation (Majeti et al. 2000). Altered binding of RPTPα wedge mutant to Src promotes the idea that RPTPα dimerization has a role in the interaction of RPTPα with Src. Distinct interactions of the two constitutively dimeric RPTPα mutants, F135C and P137C, with Src supports this hypothesis and further suggests that the conformation of the dimers is important for this interaction.

The model that is emerging from our results is that a functional wedge might

be required for proper binding to Src (Fig. 5). RPTPα-P210/211L was previously shown to be incapable of forming dimers (Jiang et al. 2000) and we show that these mutations impaired Src binding. RPTPα-D228R may not affect dimerization per se, but instead may adopt an active, open conformation, resembling the RPTPα-F135C conformation. Our results are consistent with a model in which dimeric RPTPα is required for Src activation. One RPTPα molecule in the dimer would bind Src and present it to the second RPTPα molecule for dephosphorylation and activation. Taken together, our data provide evidence that the wedge structure is involved in the RPTPα-Src interaction and they imply that RPTPα dimerization may have a role in Src activation as well.

Materials and methods

Materials and antibodies

Anti-HA-tag (12CA5), anti-Src (327) antibodies and anti-RPTPα serum (5478) were prepared as previously described (den Hertog et al. 1993; den Hertog et al. 1994). HRP-coupled anti-rabbit and anti-mouse secondary antibodies were from BD Biosciences. FuGene6 transfection reagent was from Roche.

Table1 *Primers used for Src mutagenesis*

Mutation	Primers	
W118A	For	5'- CAC GGA GGG AGA CGC GTG GCT GGC ACA C - 3'
	Rev	5'- GTG TGC CAG CCA CGC GTC TCC CTC CGT G - 3'
P133L	For	5'- CAG ACC GGT TAC ATC CTC AGC AAC TAT GTG GCG - 3'
	Rev	5'- CGC CAC ATA GTT GCT GAG GAT GTA ACC GGT CTG - 3'
R175L	For	5'- GAC CTT CCT CGT GCT GGA GAG TGA GAC C - 3'
	Rev	5'- GGT CTC ACT CTC CAG CAC GAG GAA GGT C - 3'

DNA constructs

The constructs used for the expression of HA-RPTPα WT (pGS-HA-RPTPα), HA-RPTPα mutants (P210/211L, C433S, C723S, F135C and P137C) and Src WT (pSLX-Src) were previously described (den Hertog et al. 1993; den Hertog et al. 1994; Jiang et al. 2000). Src mutant constructs were obtained by circular site-directed mutagenesis using pSLX-Src as template and the primers listed in Table 1. The construct expressing HA-RPTPα-Y798F was obtained by HindIII/BglII directional cloning into the pSG vector of the PCR product resulted from using pSG-HA-RPTPα WT as template and

the following primers: For 5'-TAC AGA TCT CCC GGG TCA CTT GAA GTT GGC AAA ATC TGA AAA GGC GTC-3' and Rev 5'-TAG AAG CTT CTC GAG CCC GGG ATG GAT TCC TGG TTC ATT CTT G-3'. HA-RPTPα-D228R mutant was obtained with the same method using pSG-HA-RPTPα WT as template and the primers: For 5'- CCG GAG AAT GGC TGA TCG CAA TAA GCT CTT CAG AG – 3' and Rev 5' – CTC TGA AGA GCT TAT TGC GAT CAG CCA TTC TCC GG – 3'.

Cell culture and transfection

SYF cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal calf serum (FCS). The cells were transfected with FuGene6 following the instructions of the manufacturer. After transfection the cells were grown for 16 hours in complete medium then the medium was replaced with serum free medium and the cells were grown for an additional 24 hours. COS1 cells were grown in DMEM/ Ham's F-12 supplemented with 7.5% FCS. The COS1 cells were transfected using polyethylenimine (PEI) and subsequently processed like the SYF cells.

Immunoprecipitation

SYF cells were lysed for 20 minutes 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 µg/ml leupeptin and 1 µg/ml aprotinin). The lysates were collected using a cell scraper and centrifuged at 13000 rpm and 4°C for 10 minutes. To test the protein expression, samples from the lysates were collected and boiled after being mixed with equal volumes of 2X 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. The remaining of the lysates was incubated for 1 h at 4°C with anti-Src monoclonal antibody 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% TritonX-100 and 10% glycerol), boiled for 5 min. in 2X SDS sample buffer and separated on a 7.5% SDS-polyacrylamide gel.

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 (50 mM HEPES pH 7.5, 10 mM $MgCl_2$, 5 mM NaF and 1 mM Na_3VO_4) and divided into two equal

fractions of which one was used for the kinase assay and the other was used to quantify the protein amounts. The assay was performed in 40 µl kinase reaction buffer containing 10 µCi [γ-^{32}P]ATP and 3.5 µg acid-denatured enolase. The reactions were incubated at 30°C for 30 min, stopped by the addition of 2X SDS sample buffer, boiled for 5 min. and resolved by 7.5% SDS-PAGE. Results were visualized by autoradiography and quantified using the basic mode of Quantity One software.

Acknowledgements

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Chapter 4

Catalytically active membrane-distal phosphatase domain of RPTPα is required for Src activation

Andrei M. Vacaru[1] and Jeroen den Hertog[1,2]

[1]Hubrecht Institute – KNAW and University Medical Center, Utrecht, the Netherlands
[2]Institute of Biology Leiden, Leiden University, the Netherlands

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Abstract

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 turned out not to be linked to the disease. Here, we report that 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 to 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, with an inactive D2. Again Src binding was reduced and Tyr527 phosphorylation enhanced. Our results suggest that a catalytically active D2 is required for RPTPα to bind and dephosphorylate its well characterized substrate, Src.

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 (Brady-Kalnay and Tonks 1995; Alonso et al. 2004; Tonks 2006). The ectodomains may play an important role in the regulation of the RPTPs following cell-cell, cell-matrix contacts or upon interaction with specific extracellular ligands (den Hertog et al. 2008; Tabernero et al. 2008). Besides the extracellular domain the majority of the RPTPs present another interesting feature: tandem catalytic domains. RPTP-D1s and -D2s are highly conserved, in that the D2s contain a PTPase signature motif, like the D1s (Andersen et al. 2001). In addition, the three-dimensional structures are conserved between D1s and D2s (Nam et al. 1999; Sonnenburg et al. 2003; Barr et al. 2009). However, most RPTP-D2s have very low or no catalytic activity (Streuli et al. 1990; Wang and Pallen 1991; Gebbink et al. 1993). In case of LAR and RPTPα the absence of two residues in the D2 domain was shown to be responsible for decreased activity: the tyrosine from the pTyr recognition loop (also known as the KNRY motif) and the aspartic acid from the WPD loop. Upon substitution of these two residues, the catalytic activity of the D2 domains was greatly improved (Lim et al. 1997; Buist et al. 1999; Nam et al. 1999). RPTPε-D2, but not CD45-D2, regained catalytic activity upon changing the two amino acids mentioned above (Lim et al. 1999).

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* (Streuli et al. 1990; Wang and Pallen 1991). In addition, the D2 domains were shown to be involved in inter- and intra-molecular interactions as well as in homodimerization and oligomerization of RPTPs (Felberg and Johnson 1998; Wallace et al. 1998; Blanchetot and den Hertog 2000; Hayami-Noumi et al. 2000; Blanchetot et al. 2002; Toledano-Katchalski et al. 2003; Barr et al. 2009). Based on the crystal structure of LAR and CD45, another role of the D2 domain was proposed: stabilization of the D1 domain (Nam et al. 1999; Nam et al. 2005). 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 (Debant et al. 1996), Abl kinase and Enabled (Wills et al. 1999), β-Catenin (Kypta et al. 1996) and Liprin-α (Serra-Pages

et al. 1995). Another study indicated that an acidic region from CD45-D2 is required for regulation of TCR-mediated calcium signaling pathways (Wang et al. 2000). The involvement of D2 domains in substrate recognition was observed for CD45-D2 which seems to mediate the interaction with Lck (Felberg et al. 2004).

The D2 domain of RPTPα is the only known membrane-distal domain with considerable catalytic activity (Wang and Pallen 1991; Lim et al. 1997; Wu et al. 1997). It was shown that its activity is comparable or even higher than the activity towards pNPP of dual specificity phosphatases like cdc25, VH1 and YPTP1 (Lim et al. 1997). One of the main roles that surfaced so far for RPTPα-D2 is that of redox sensor. This function is dependent on its catalytic cysteine (Cys723). Following H_2O_2 induced oxidation this cysteine mediates the stabilization of RPTPα dimers followed by complete inactivation of the enzyme and rotational coupling of the extracellular domain (Blanchetot et al. 2002; van der Wijk et al. 2003). RPTPα-D2 is essential for RPTPα homodimerization in the absence of oxidizing reagents (Jiang et al. 2000) and has a role in pervanadate induced tyrosine phosphorylation of RPTPα (Buist et al. 2000) showing that D2 is involved in protein-protein interactions. Besides the interaction with other phosphatase domains, RPTPα-D2 binds to calmodulin, leading to inactivation of D2 (Liang et al. 2000).

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. Src Tyr527 dephosphorylation and activation was also reduced in response to these mutations, compared to wild type RPTPα. A third mutant, RPTPα-R729K, with impaired catalytic activity in D2 confirmed that a catalytically active D2 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 Noonan syndrome patients contain dominant activating mutations in *PTPN11* (Tartaglia et al. 2001). Other genes that are associated with Noonan syndrome encode factors in the Ras-MAPK pathway: SOS1, KRAS, BRAF and RAF1 (Bentires-Alj et al. 2006; Razzaque et al. 2007; Tartaglia et al. 2007; Sarkozy et al. 2009). To assess whether RPTPα is involved in Noonan syndrome as well, 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 turned out 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 been identified in other Noonan patients as well and we therefore concluded that Noonan syndrome in this patient was most likely caused by the missense mutation in *SOS1*, not by a mutation in *PTPRA*. Nevertheless, biochemically RPTPα-R554H behaved differently than wild type RPTPα.

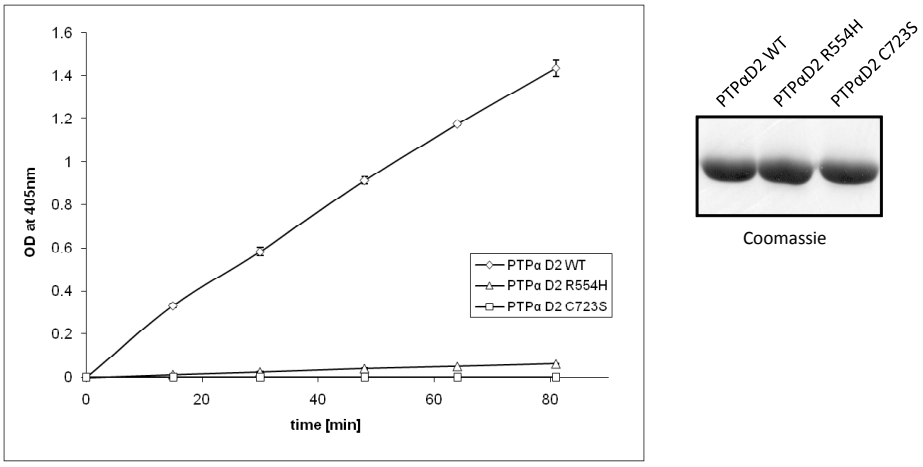

Fig.1 R554H mutation abolished RPTPα-D2 catalytic activity. The catalytic activity of GST fusion proteins containing the D2 domain of RPTPα WT, R554H or C723S was tested *in vitro* using pNPP as substrate. The error bars represent ± SD of three independent phosphatase activity determinations. Equal amounts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

R554H mutation abolished catalytic activity of RPTPα-D2

RPTPα Arg554 is an absolutely conserved residue in the pTyr recognition loop (the KNRY motif). This Arg residue is important for electrostatic attraction of ligands and is a putative substrate binding site (Andersen et al. 2001). We tested whether the R554H mutation had an influence on the catalytic activity of RPTPα-D2 in vitro using GST-fusion proteins and pNPP as a substrate. The phosphatase activity of D2-R554H was compared to WT-D2 and to 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 to 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.

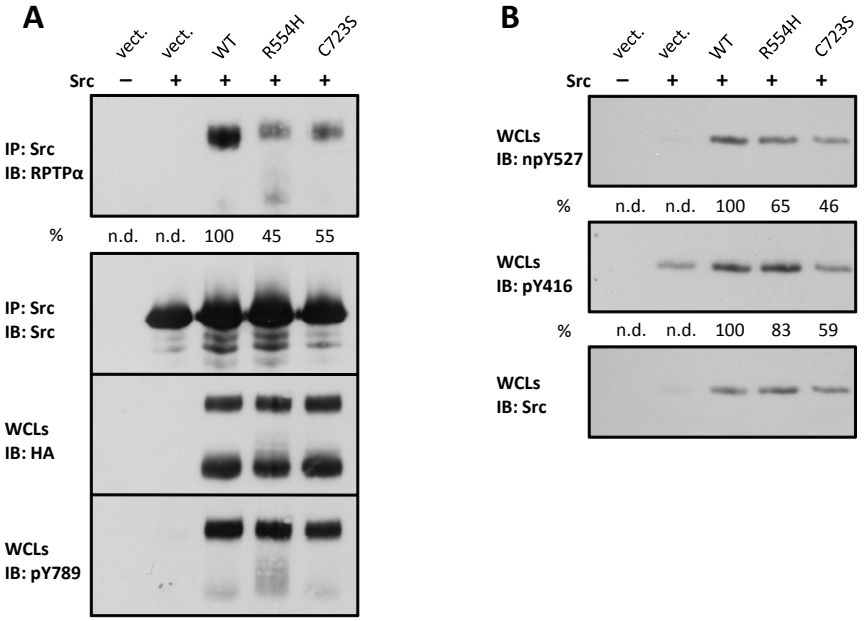

Fig.2 Src association with RPTPα-R554H and RPTPα-C723S mutants is reduced. A, SYF cells were cotransfected withSrc and HA-RPTPα WT, R554H or C723S, lysed and Src was immunoprecipitated with cross-linked monoclonal anti-Src antibodies. The samples were fractionated on a 7.5% SDS-polyacrylamide gel, transferred to PVDF membranes and immunoblotted with anti-RPTPα serum and anti-Src MAb 327. Whole cell lysates were probed with anti-HA MAb 12CA5 to monitor HA-RPTPα expression and 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 and the membranes were probed with anti-npY527 antibody and subsequently, after stripping, with anti-pY416 and anti-Src 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.

Catalytic activity of 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 (Felberg and Johnson 1998; Wallace et al. 1998; Blanchetot and den Hertog 2000; Blanchetot et al. 2002; Felberg et al. 2004). We investigated if the introduction of inactivating mutations in RPTPα-D2 (R554H and C723S) affected the interaction with the well established substrate of RPTPα, Src. For this purpose SYF cells that lack endogenous Src, Fyn and Yes, were transiently cotransfected with constructs encoding Src and HA-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 (~50%) reduced when compared to WT RPTPα (Fig. 2A). The interaction of Src with RPTPα-C723S mutant was similarly decreased. The interaction between RPTPα and Src may be mediated by pTyr789. Therefore, we tested whether phosphorylation of Tyr789 was affected in R554H and C723S mutants and we did not observe significant differences in pTyr789 levels. These results indicate a role for RPTPα-D2 in the interaction with Src independent of phosphorylation of Tyr789.

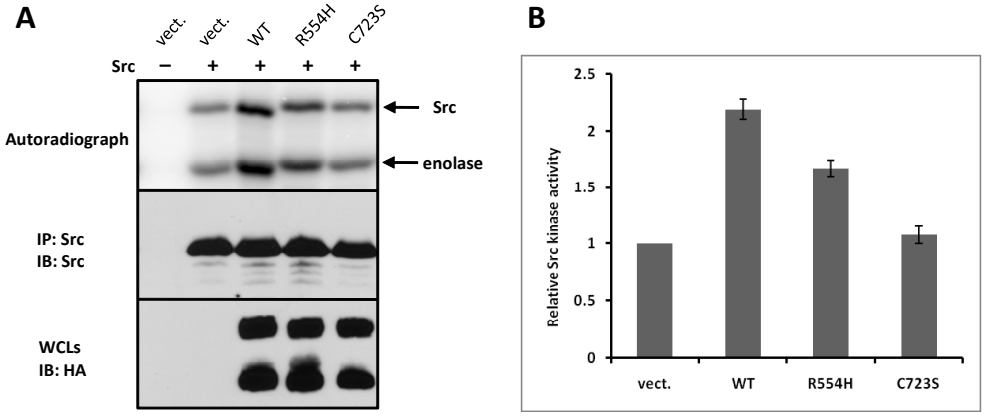

Fig.3 Reduced activation of Src by RPTPα-R554H and RPTPα-C723S. A, Src was immunoprecipitated from SYF cells contransfected with Src and RPTPα constructs. Half of the immunoprecipitates was subjected to an *in vitro* kinase assay, using enolase as substrate and the amount of incorporated phosphate was visualized by autoradiography (top panel). The positions of enolase and Src are indicated. The other half of the immunoprecipitate was fractionated by 7.5% SDS-PAGE, blotted, probed with anti-Src antibody and developed with ECL (middle panel). Part of the lysates was resolved on 7.5% SDS-polyacrylamide gel, transferred to PVDF 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 empty vector, which was set to one.

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 and autophosphorylation of Tyr416 was enhanced. 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 ~65% of pTyr527 dephosphorylation in wild type RPTPα cotransfected cells. Cotransfection of RPTPα-C723S with Src led to a more significant decrease in Src pTyr527 (~46% of wild type RPTPα). Src Tyr416 autophosphorylation was less prominent in R554H and C723S transfected cells, 83% and 59% of wild type cotransfected cells, respectively (Fig. 2B). These results suggest that RPTPα-mediated Src dephosphorylation was impaired in RPTPα mutants with inactive D2 domains.

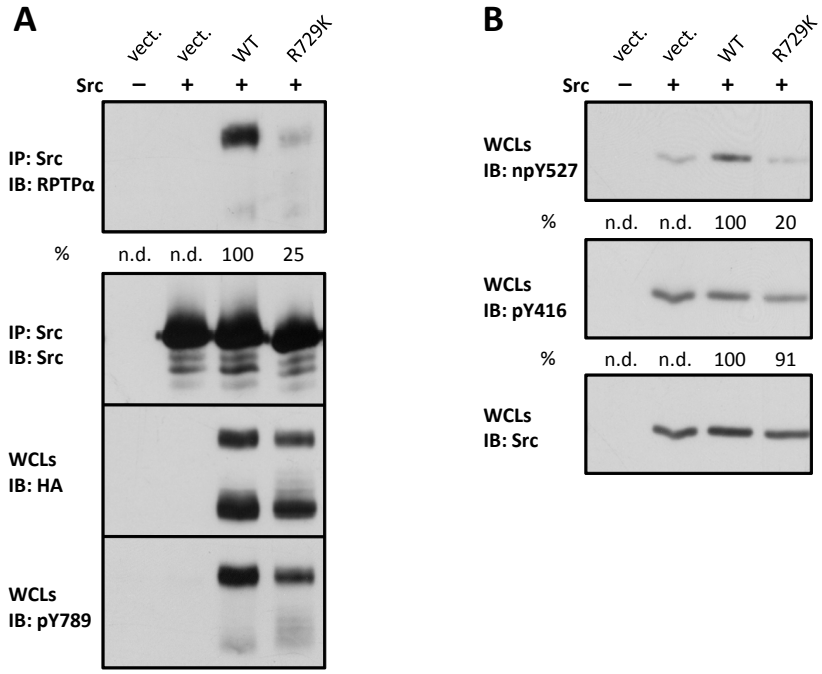

Fig.4 Impaired Src binding to the inert RPTPα-R729K mutant. A, SYF cells cotransfected with Src and empty vector, HA-RPTPα WT or HA-RPTPα-R729K were lysed and Src was immunoprecipitated. The samples obtained were run on 7.5% SDS-polyacrylamide gels, transferred to PVDF membranes and probed for co-immunoprecipitated 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.

To establish the ability of RPTPα-D2 mutants to activate Src, we tested *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 to the activity of Src cotransfected with WT RPTPα. RPTPα-C723S hardly activated Src under these circumstances (Fig. 3A). Three independent experiments 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 RPTPα-D2 catalytic activity plays an important role in Src activation.

To confirm that the RPTPα-D2 domain catalytic activity is required for Src binding and activation we used an RPTPα-D2 mutant with an Arg to Lys mutation in the PTPase signature motif in D2, RPTPα-R729K. This Arg residue has an essential role in catalysis in PTPs and mutation of this residue in PTPs results in inactivation of catalytic activity, but does not result in substrate trapping mutants. Likewise, mutant RPTPα-D2-R729K has no D2 catalytic activity (Buist et al. 2000). Src was coexpressed in SYF cells with RPTPα-R729K and Src was immunoprecipitated. RPTPα-R729K mutant showed ~75% reduction in Src binding compared to RPTPα WT (Fig. 4A). In the presence of RPTPα-R729K, Src pTyr527 dephosphorylation was strongly decreased 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 *de novo* Noonan syndrome patient 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 mutation does not have a dominant effect over the wild type RPTPα allele. It is noteworthy that heterozygous RPTPα knock-out mice are indistinguishable from wild type siblings (Ponniah et al. 1999; Petrone et al. 2003), corroborating the

conclusion that a single RPTPα allele suffices for mammalian life.

Mutant RPTPα-D2-R554H was almost completely inactive, compared to 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 (Buist et al. 1999). The conserved arginine residue was proposed to be involved in the electrostatic attraction of the substrate (Andersen et al. 2001) and mutation of the corresponding arginine (Arg45) to Ala in PTP1B led to very low catalytic activity most probably due to structural perturbation of the catalytic site (Guo et al. 2002). As seen from the crystal structure of RPTPα-D2 (Sonnenburg et al. 2003), 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 since the D2 domain only marginally contributes to the overall catalytic activity of RPTPα (Wang and Pallen 1991; Lim et al. 1997; Wu et al. 1997).

Src binding to three different RPTPα-D2 inactive mutants was impaired. Moreover, the ability of these mutants to activate Src was reduced when compared to WT RPTPα. According to the current model, Src binds to phosphorylated Tyr789 of RPTPα via its SH2 domain (Zheng et al. 2000). We did not observe significant changes in Tyr789 phosphorylation of the D2-inactive mutants compared to WT RPTPα (Fig. 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). Taken together, phosphorylation of Tyr789 is not the only determinant in Src binding. Instead, other features of RPTPα-D2 may mediate Src binding.

Ever since their discovery, it has been speculated that RPTP-D2s may have a role in substrate binding and substrate presentation to the catalytically active D1 (Hunter 1998). 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 (Tsujikawa et al. 2001). 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 (Felberg et al. 2004). CD45-D2 is also critical for substrate recruitment of TCR-zeta *in vivo*, since

replacement of the membrane-distal domain of CD45 with LAR D2 domain abolishes binding of TCR-zeta (Kashio et al. 1998).

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. We cannot exclude the possibility that the mutations disrupt the structure of RPTPα-D2 and hence result in the inability to target Src.

Materials and methods

Materials and antibodies

Anti-HA-tag (12CA5), anti-Src (327) and anti-RPTPα (5478AP) antibodies were prepared as previously described (den Hertog et al. 1993; den Hertog et al. 1994). Anti-Src-npY527 was from Cell Signaling and anti-Src-pY418 was from Biosource. HRP-coupled anti-rabbit and anti-mouse secondary antibodies were from BD Biosciences. Glutathione-Sepharose was from Sigma Life Science and FuGene6 transfection reagent was from Roche.

DNA constructs

The constructs used for the expression of HA-RPTPα WT (den Hertog et al. 1993), Src WT (den Hertog et al. 1994) and HA-RPTPα-R729K (Buist et al. 2000) were previously described. HA-RPTPα-R554H was obtained by site-directed mutagenesis using HA-RPTPα WT as 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 membrane distal catalytic domain (D2) fusion proteins were obtained by directional cloning of PCR fragments digested with NcoI and HindIII into the pGEX-KG vector digested with the same restrictions 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% FCS. The cells were transfected with FuGene6 according to the protocol provided by the

manufacturer. After transfection the cells were grown for 16 hours in complete medium then the medium was replaced with serum free medium and the cells were grown for an additional 24 hours.

Recombinant proteins

The constructs encoding GST-fusion proteins were transformed into BL21 bacteria. Expression of fusion proteins was induced with 0.1 mM IPTG for 5 h at 25°C. The bacteria were harvested by centrifugation, resuspended in TBS solution containing 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 mg/ml lysozyme and incubated 10 minutes at room temperature. The suspension was sonicated on ice, supplemented with 1% TritonX-100, kept 10 minutes 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 minutes at 4°C. The beads were washed three times with ice cold TBS. 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 TBS containing 10% glycerol.

Immunoprecipitation and immunobloting

SYF cells were lysed for 20 minutes 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 µg/ml leupeptin and 1 µg/ml aprotinin). The lysates were collected using a cell scraper and centrifuged for 10 minutes at 13000 rpm. Samples from the lysates were collected and boiled after being mixed with equal volumes of 2X 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 anti-Src monoclonal antibody 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% TritonX-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 divided into two equal fractions of which one was immunobloted using anti-Src antibody and the other was subjected to kinase assay. The reactions were performed in 40 µl

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 [γ-^{32}P]ATP and 3.5 µg acid-denatured enolase. Reactions were incubated at 30°C for 30 min, stopped by the addition of 2X SDS sample buffer, boiled for 5 min. and resolved by 7.5% SDS-PAGE. Results were visualized by autoradiography.

Phosphatase assay

The activity of RPTPα-D2 domain was investigated *in vitro* using GST-fusion proteins and pNPP as 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 DTT and 10 mM pNPP. The reaction was initiated by the addition of fusion protein and incubated at 30°C for the times indicated. 1 ml of 1 N NaOH was added to quench the reaction and the formation of p-nitrophenol was detected with a spectrophotometer at OD 405 nm.

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Chapter 5

Approaches to discover new RPTPα substrates

Andrei M. Vacaru[1], Simone Lemeer[1,2], Albert Heck[2] and Jeroen den Hertog[1,3]

[1]Hubrecht Institute – KNAW and University Medical Center, Utrecht, the Netherlands
[2]Department of Biomolecular Mass Spectrometry, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, the Netherlands
[3]Institute of Biology Leiden, Leiden University, the Netherlands

Abstract

RPTPα is a receptor protein-tyrosine phosphatase with a small highly glycosylated extracellular domain. It contains two cytoplasmic catalytic domains, D1 and D2, of which D1 is ~1000-fold more active than D2. Previously described substrates for RPTPα include the related protein-tyrosine kinases Src and Fyn as well as p130cas and the potassium channel Kv1.2.

The identification of substrates of protein-tyrosine phosphatases is probably the most important step in understanding the physiological role of these enzymes. The aim of this study was to discover new RPTPα substrates. We started by looking at the changes that occur in *in vivo* tyrosine phosphorylation levels in the absence of RPTPα. Using anti-phosphotyrosine antibodies we compared the phosphotyrosine-containing protein profiles of wild type and RPTPα knock-out mouse embryo fibroblasts. Next, we used a substrate-trapping mutant to pull-down putative substrates from pervanadate treated mouse embryo fibroblasts. Finally, using quantitative mass spectrometry, we assessed tyrosine phosphorylation of proteins from wild type and RPTPα knock-out mouse embryo fibroblasts in response to PDGF treatment. Using these approaches we identified new candidate RPTPα substrates that remain to be validated. Our results confirm that RPTPα has other substrates than the ones discovered to date.

Introduction

Tyrosine phosphorylation is a vital signaling mechanism for all eukaryotic cells. In eukaryotes the balance of tyrosine phosphorylation is kept by the antagonistic activity of the protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs). The PTPs catalyze the removal of the phosphate from the phosphotyrosyl residues (Denu and Dixon 1998). The classical PTPs are divided into cytoplasmic and receptor protein-tyrosine phosphatases (RPTPs) based on their subcellular localization (Tonks 2006). Identifying the substrates of these enzymes is one of the most important steps in understanding their function.

We are trying to get more insight into the role of RPTPs using RPTPα as a prototypical model. RPTPα has a small, highly glycosylated ectodomain (Daum et al. 1994) and like most of the RPTPs, RPTPα contains two phosphatase domains, with the membrane proximal domain being responsible for most of the catalytic activity (Lim et al. 1997; Wu et al. 1997). RPTPα function is mainly linked to its ability to activate Src and Fyn kinases by dephosphorylating their C-terminal inhibitory phosphotyrosine (Zheng et al. 1992; den Hertog et al. 1993; Bhandari et al. 1998; Ponniah et al. 1999; Su et al. 1999). Evidence that RPTPα dephosphorylates other substrates is scarce and only two other substrates were identified to date: the potassium channel Kv1.2 (Tsai et al. 1999) and p130Cas (Buist et al. 2000).

It is known that the *in vitro* specificity of the PTPs is poor but they show excellent specificity *in vivo* (Tonks and Neel 2001). This specificity is achieved through different controlling mechanisms at different levels. Presence of functional domains, subcellular localization and posttranslational modifications are only few of the ways that confer the PTPs their substrate specificity (den Hertog et al. 2008). An exhaustive search of the human genome revealed there are 107 genes encoding members of the four PTP families. From these only 81 are catalytically active and can dephosphorylate phosphotyrosines (Alonso et al. 2004). This number is close to the total number of active PTKs, 85 (Manning et al. 2002) indicating that the PTPs and the PTKs numerically have similar specificities for their substrates. In a recent study, Rikova et al. using a phosphoproteomics approach identified 4551 tyrosine phosphorylation sites on more than 2700 proteins from non-small cell lung cancer cell lines and tumors (Rikova et al. 2007). A rough estimation shows that each PTP may be responsible for the dephosphorylation of more than 50 unique phosphotyrosines, much more than the number of substrates discovered for any

known PTP.

Using three different approaches we tried to identify new RPTPα substrates. Each method provided new RPTPα candidate substrates. Our preliminary results suggest that RPTPα is able to dephosphorylate other proteins than the substrates discovered so far.

Results and discussion

Study of the phosphorylation profile of RPTPα -/- and +/+ MEFs using specific anti-phospho-tyrosine antibodies

Recently, we and other research groups developed RPTPα knock-out mice (Ponniah et al. 1999; Su et al. 1999; Bodrikov et al. 2005). PTP knock-out mice are a very powerful tool for the discovery of new substrates. Next step in harvesting the advantage conferred by these genetically modified animals was the isolation of RPTPα knock-out (-/-) mouse embryo fibroblasts (MEFs). We isolated and immortalized -/- and WT (+/+) MEFs by retroviral insertion of *TBX2* (Jacobs et al. 2000) and we used these cell lines to try and identify new substrates. RPTPα -/- and +/+ cells were lysed and tyrosine phosphorylated proteins were immunoprecipitated using anti-pTyr antibodies. The samples were processed and after separation by SDS-PAGE and transfer, the membranes were probed with the same anti-pTyr antibodies used for immunoprecipitation (Fig. 1A). Tyrosine-phosphorylated proteins were not very abundant in the whole cell lysates of unstimulated RPTPα -/- and +/+ MEFs. However, a protein with an apparent molecular weight of approximately 190,000 (called p190) was detected in the RPTPα -/- cell lysates and apparently the same protein was observed in the anti-pTyr immunoprecipitates from RPTPα -/- MEFs. The protein concentration in the lysates was normalized as seen on the Coomassie stained membranes, therefore the differences in phosphorylation cannot be caused by unequal loading or differences in protein concentration of the lysates. The same result was obtained for E6 and E9 cells, a different set of RPTPα -/- and +/+ MEFs generated in Jan Sap's group (Su et al. 1999) (Fig. 1B). We cannot exclude the possibility that p190 protein expression is upregulated in the knock-out cells giving the impression of increased phosphorylation.

Mass spectrometry was used to identify p190. The anti-pTyr IP was scaled up and after SDS-PAGE and Coomassie staining, the area encompassing p190 was excised from the gel. After in-gel digestion, the samples were analyzed by mass

spectrometry. The protein that was identified with this approach was MYH9 (non-muscle myosin heavy chain 9), a very abundant protein in MEF cells. The anti-pTyr immunoprecipitates were probed with anti-MYH9 antibodies but no MYH9 was not detected (Fig 1C). Moreover, the molecular weight of MYH9 is bigger than that of p190 and further tests indicated that MYH9 is the 200,000 protein that co-precipitates in the anti-pTyr immunoprecipitates.

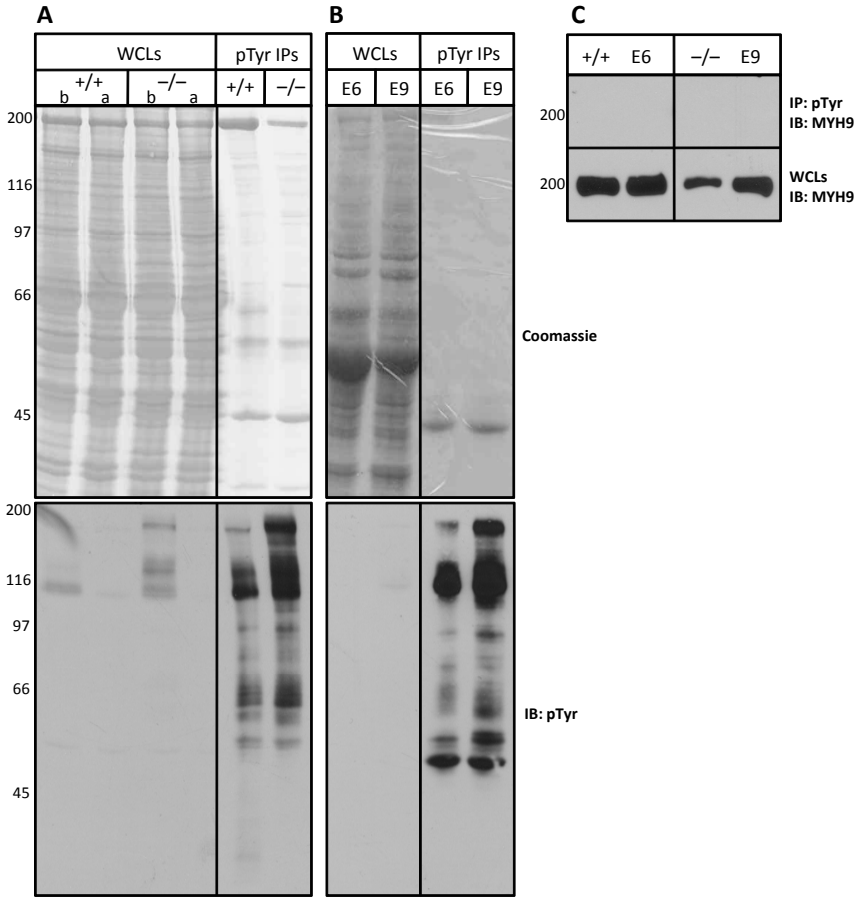

Fig.1 Tyrosine phosphorylation profiles of wild type and RPTPα -/- MEFs. A, WT and RPTPα -/- MEFs were lysed. The lysates were first incubated with anti-pTyr antibodies and then with Protein A Sepharose. Subsequently the samples were separated on 7.5% SDS-polyacrylamide gels and transferred to PVDF membranes. The Tyr phosphorylation levels were probed with the anti-pTyr antibody PY20. Part of the lysates was resolved by 7.5% SDS-PAGE to test the pTyr levels in the lysates. For the untreated cells samples from the lysates before (b) and after (a) immunoprecpitation were used to test the efficiency of the immunoprecipitation. The Coomassie stained membranes are shown in the upper panels. B, The phosphorylation profile of another pair of WT (E6) and RPTPα -/- (E9) MEFs was tested as described for A. C, Anti-pTyr immunoprecipitates from WT and RPTPα -/- MEFs lysates were fractionated on 7.5% SDS-polyacrylamide gels and transferred to PVDF membranes. The immunoprecipitates were probed for MYH9 (upper panel). The amount of MYH9 in the lysates was monitored (lower panel). The numbers on the left side of the panels represent the protein markers in kDa.

Pull-down of candidate substrates using substrate trapping mutants

The use of substrate trapping mutants is a widespread method used for identifying new PTP ligands. The vast majority of PTP substrates was discovered or confirmed using substrate trapping mutants (Blanchetot et al. 2005). We used for our experiment RPTPα-C433S mutant which was employed with success to identify p130Cas as RPTPα substrate (Buist et al. 2000). Pervanadate treatment of RPTPα -/- and +/+ MEFs was used to enrich for protein tyrosine phosphorylation. It was suggested that the membrane distal catalytic domain could work as a substrate trapping mutant by itself. To assess for the effect of D2 domain on binding Tyr phosphorylated proteins we also used RPTPα lacking D2 domain. The GST-fusion proteins used for this substrate trapping experiment are depicted in Fig. 2.

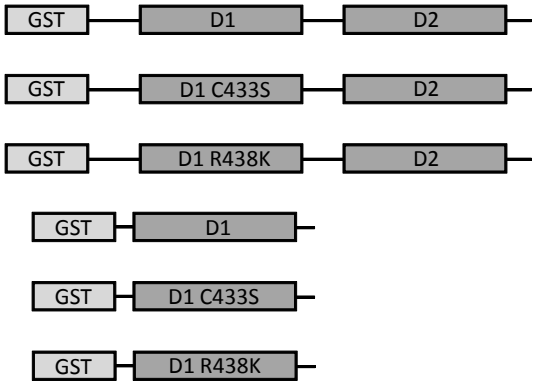

Fig.2 Schematic representation of the GST-fusion proteins used for the substrate trapping experiment. GST-fusion proteins containing the full length cytoplasmic domain or the D1 domain of RPTPalpha were used for the substrate trapping assay. bPTPalpha-C433S is a D1 catalytic inactive and substrate-trapping mutant while bPTPalpha-R438K is a mutant lacking both the D1 catalytic activity and the substrate trapping ability.

RPTPα -/- and +/+ MEF lysates were incubated with GST-fusion proteins immobilized on beads. The samples were resolved by 10% SDS-PAGE. After transfer, the membranes were probed with anti-pTyr antibody PY20 for detection of putative RPTPα substrates. We did not detect any Tyr phosphorylated proteins in the pull-down from untreated RPTPα -/- and +/+ cells (data not shown). Pervanadate treatment resulted in a similar increase in Tyr phosphorylation and Tyr phosphorylated proteins from both +/+ and -/- MEF lysates bound to the RPTPα-C433S substrate trapping mutant (Fig. 3A and B). These proteins did not bind to WT RPTPα, to the catalytic dead RPTPα-R438K mutant or to GST alone. Only minor differences in Tyr phosphorylated protein binding were detected when RPTPα D1 domain or the entire cytoplasmic domain of RPTPα were used. When comparing the pull-down from +/+ and -/- cells we observed that a higher number of Tyr phosphorylated proteins from the -/- MEFs bound to the substrate trapping mutant. This may be the

result of increased kinase activity in the -/- MEF cells.

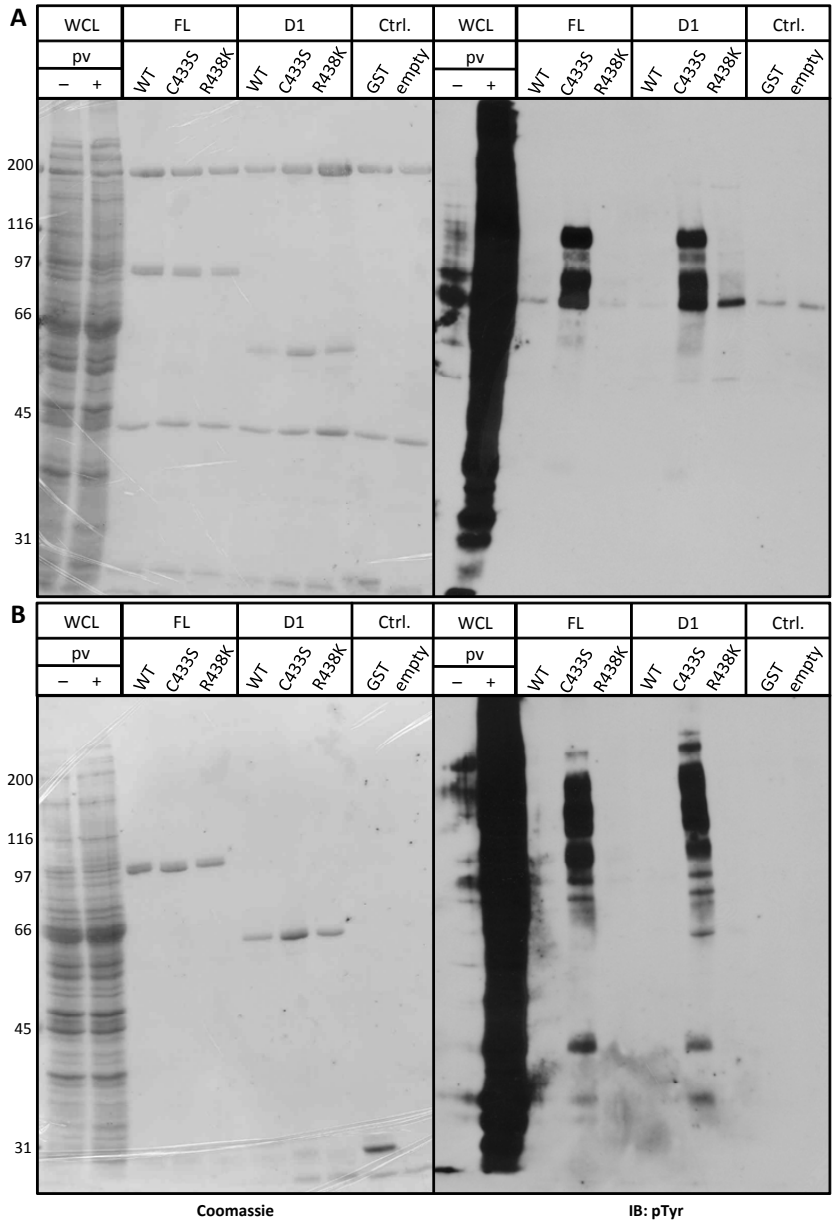

Fig.3 RPTPalpha substrate candidates pull-down from pervanadate treated MEFs. GST-fusion proteins representing the full length cytoplasmic domain of RPTPalpha (FL) and RPTPalpha D1 (D1) bound to glutathione-agarose beads were incubated with lysates of pervanadate (pv) treated wild type (A) or RPTPalpha -/- (B) MEFs. The samples were fractionated by 10% SDS-PAGE, transferred to PVDF membranes and probed for pTyr containing proteins. GST alone and empty beads were used as controls. Coomassie staining of the membranes is shown to indicate the amounts of fusion proteins used in each reaction. The protein markers in kDa are indicated.

Here we show that a number of Tyr phosphorylated proteins bound to RPTPα C433S substrate trapping mutant but not to WT RPTPα nor to RPTPα R438K mutant. These proteins may be bona-fide RPTPα substrates. Scale up of these experiments combined with protein mass spectrometry could lead to the identification of new RPTPα substrates.

Comparison of the RPTPα -/- and +/+ MEFs Tyr phosphorylation profiles using quantitative mass spectrometry

In a recent study a quantitative proteomics method based on stable isotope labeling by amino acids in cell culture (SILAC) was used to identify new PTP1B substrates (Mertins et al. 2008). Using a similar quantitative proteomics method based this time on stable isotope dimethyl labeling we tried to identify new RPTPα substrates. Dimethyl labeling was chosen because it is a fast and inexpensive labeling method compared to SILAC or isobaric tagging for relative and absolute quantification (iTRAQ) (Boersema et al. 2009). In this experiment we used RPTPα reconstitution in RPTPα -/- MEF cells next to RPTPα -/- and +/+ MEF cells that were used before. The level of RPTPα in the reexpressor cells was 2-3 fold lower than endogenous RPTPα in wild type MEF cells.

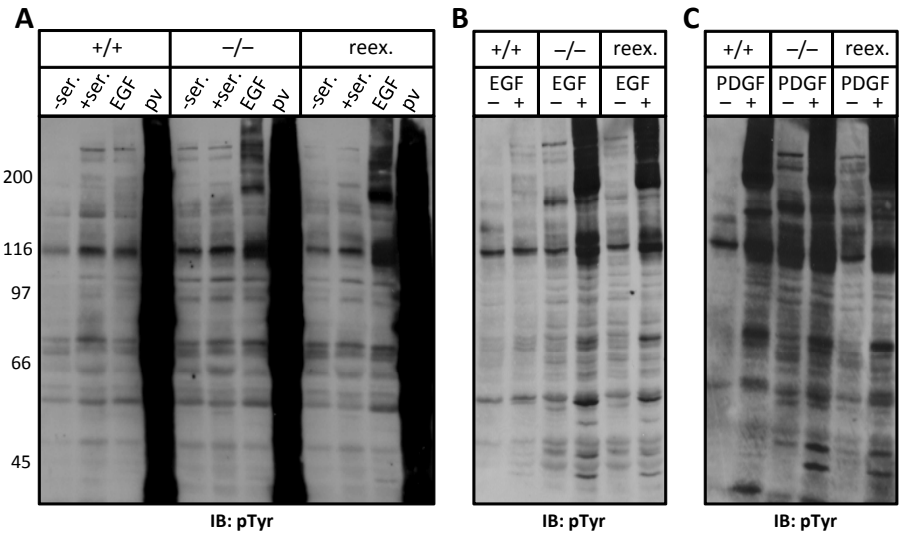

Fig.4 Methods to stimulate Tyr phosphorylation in MEFs. RPTPalpha+/+, -/- and RPTPalpha reexpressor MEFs were starved and then stimulated with serum, EGF, pervanadate or left untreated (A), stimulated with EGF(B) or stimulated with (PDGF).Then, the cells were lysed and samples from the lysates were run on 7.5% SDS-polyacrylamide gels. After transfer to PVDF membranes the samples were probed for pTyr.

As shown above, Tyr phosphorylated proteins were not detected under control conditions. To increase the amount of Tyr phosphorylation we decided to stimulate the cells. Several ways of cell stimulation were tested. First, we followed the Tyr phosphorylation response to serum, EGF and pervanadate (Fig. 4A). Serum stimulation did not have a significant effect on the total amount of Tyr phosphorylation, whereas pervanadate treatment, as previously shown resulted in extreme Tyr phosphorylation that made the comparison of proteins derived from different cell lines impossible. EGF treatment had an intermediate effect but only on the -/- MEFs and on the RPTPα reexpressors. For unknown reasons RPTPα +/+ cells did not respond to EGF stimulation making it impossible to use EGF for this experiment (Fig. 4B). Further, we tested the response of the cells to PDGF. All cell types showed increased Tyr phosphorylation following PDGF stimulation. Based on these results, we decided to use PDGF stimulation to increase the amount of Tyr phosphorylation in the cells.

After stimulation the cells were lysed and the proteins were digested with trypsin. Subsequently, the peptides were labeled and the Tyr phosphorylated peptides were immunoprecipitated using specific anti-pTyr antibodies. The enriched pTyr containing peptides were analyzed by LC-MS/MS and the data were processed as described in “Materials and methods”.

In total we identified 137 Tyr phosphorylated proteins (Table 1). Sixteen of these showed a significant increase in Tyr phosphorylation (≥ 2 fold) in RPTPα -/- cells compared to RPTPα +/+ cells. Unfortunately, Src, Fyn and p130Cas were not among the identified candidate substrates and we were not able to quantify the phosphorylation of these known RPTPα substrates. However, Yes auto-phosphorylation was 2.7-fold higher in wild type cells than in RPTPα -/- cells. Auto-phosphorylation of this site takes place after dephosphorylation of the C-terminal inhibitory Tyr (Tyr527 in chicken Src) and RPTPα is known to dephosphorylate this site in several Src family kinases. This increase in Yes phosphorylation indicates indirect activation of Yes, which may result from RPTPα-mediated dephosphorylation of the inhibitory C-terminal phosphorylation site.

It is noteworthy that MYH9 was also identified here. Surprisingly MYH9 tyrosine phosphorylation is enhanced in RPTPα +/+ cells compared to RPTPα -/- cells (Table 1). We did not detect MYH9 phosphorylation directly in MEF cells (Fig. 1C). However, in the initial experiments we repeatedly observed that MYH9 was

more abundant in RPTPα +/+ cells than in RPTPα -/- cells. These differences in MYH9 expression could account for the observed increase in Tyr-phosphorylation in RPTPα +/+ MEFs.

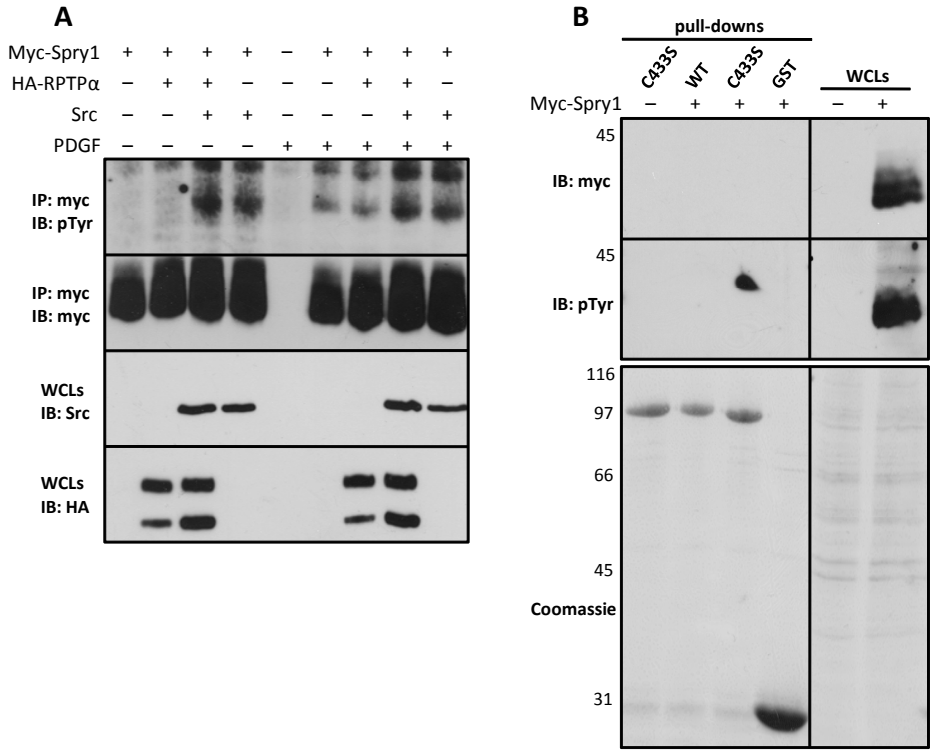

Fig.5 RPTPalpha does not influence Spry1 Tyr phosphorylation. A, SYF cells were cotransfected with constructs encoding for myc-Spry1, HA-RPTPalpha and Src and were treated with PDGF or left untreated. The cells were lysed and Spry1 was immunoprecipitated using anti-myc antibodies. The samples were fractionated by 10% SDS-PAGE, transferred to PVDF membranes and the immunoprecipitates were probed for pTyr. The amounts of Src and HA-RPTPalpha in the lysates were monitored (lower panels). B, GST-fusion bPTPalpha-C433S coupled to glutathione-agarose beads was used to pull-down myc-Spry1 from lysates of SYF cells cotransfected with myc-Spry1 and Src. The samples were separated on 10% SDS-polyacrylamide gels, transferred to PVDF membranes and probed for myc-Spry1. Subsequently the membranes were stripped and reprobed for pTyr containing proteins. Coomassie staining of the membrane is shown to indicate the amounts of fusion proteins used in each reaction. The amounts of myc-Spry1 and Tyr phosphorylated myc-Spry1 were monitored (right panels). The protein markers in kDa are indicated.

Sprouty1 (Spry1) showed the highest Tyr phosphorylation ratio in the RPTPα -/- MEFs compared to WT MEFs. We tested if RPTPα could dephosphorylate Spry1 directly by co-transfection experiments, but we did not find an effect of RPTPα co-transfection on Spry1 tyrosine phosphorylation (Fig. 5A). PDGF treatment induced Spry1 tyrosine phosphorylation under these conditions, but RPTPα did not reduce

Spry1 phosphorylation. Likewise, Src co-transfection enhanced Spry1 tyrosine phosphorylation, but again RPTPα did not reverse this effect (Fig. 5A). Moreover, we were not able to detect Spry1 binding to the RPTPα-C433S substrate trapping mutant (Fig. 5B). The differences in Tyr phosphorylation ratios between RPTPα -/- and +/+ MEFs may have other explanations than the direct catalytic activity of RPTPα. Apparently, RPTPα indirectly affects Spry1 tyrosine phosphorylation. Nevertheless, Table 1 contains many candidate substrates of RPTPα and it will be interesting to validate these.

The p190 protein remains an interesting candidate substrate of RPTPα. Further mass spectrometric determinations may reveal the identity of this elusive protein. The use of substrate trapping mutants does not seem to be very efficient for detecting new substrates from unstimulated cells. Nevertheless, this method could be a very useful tool to confirm candidates identified using other methods or to identify new candidates from cells with stimulated Tyr phosphorylation. Quantitative mass spectrometry was the most efficient method for identifying new candidate substrates of RPTPα. However, the most probable candidate, Spry1, does not seem to be an RPTPα substrate. These results warrant careful validation of the candidate substrates. Taken together, these results indicate that RPTPα has other substrates than the ones identified so far.

Table 1 *Tyrosine phosphorylation ratios of proteins from +/+, -/- and RPTPα reexpressor cells*

IPI	Protein description	Ratio +/+ / -/-	Ratio reex. / -/-
IPI00135614	Spry1 (Protein sprouty homolog 1)	0.077	0.207
IPI00754489	Papss2 (Bifunctional 3'-phosphoadenosine 5'-phosphosulfate synthetase 2)	0.097	1.282
IPI00117172	Mapk12 (Mitogen-activated protein kinase 12)	0.113	2.282
IPI00117039	Abl2 (Protein-tyrosine kinase Abl2)	0.114	1.631
IPI00136618	Tollip (Toll interacting protein)	0.138	1.696
IPI00124182	Epha3 (Isoform of Ephrin type A receptor 3 precursor)	0.172	0.714
IPI00124240	Cdk2 (Isoform beta of Cell division protein kinase 2)	0.198	0.464
IPI00626176	Myh10 (Nonmuscle myosin heavy chain B homolog)	0.405	0.628
IPI00266006	Cblb (Isoform 2 of E3 ubiquitin-protein ligase CBL-B)	0.423	0.882
IPI00761717	Mpzl1 (Isoform 1 of Myelin protein zero-like protein 1 precursor)	0.423	0.700
IPI00124453	Crim1 (Cysteine-rich motor neuron 1 protein precursor)	0.433	0.601
IPI00121827	Pdgfrb (PDGF protein-tyrosine kinase receptor beta)	0.442	0.826
IPI00129220	Epha2 (Ephrin type A receptor 2 precursor)	0.445	0.837

IPI	Protein description	Ratio +/+ / -/-	Ratio reex. / -/-
IPI00126248	Acly (ATP citrate lyase)	0.522	1.781
IPI00331568	Hgs (HGF regulated tyrosine kinase substrate)	0.523	1.654
IPI00124709	Rps27l (40S ribosomal protein S27-like protein)	0.549	1.084
IPI00126861	Tgm2 (Protein-glutamine gamma-glutamyltransferase 2)	0.556	2.951
IPI00323683	Efnb2 (Ephrin B2)	0.574	1.926
IPI00109650	Cdk3 (Isoform 1 of Cell division protein kinase 3)	0.588	0.589
IPI00169899	Afap1l2 (Isoform 1 of Actin filament-associated protein 1-like 2)	0.594	0.209
IPI00117869	Dyrk1c (YRK fragment)	0.678	1.235
IPI00122131	Rin1 (Ras and Rab interactor 1)	0.691	3.371
IPI00133158	Hipk1 (Isoform 3 of Homeodomain-interacting protein kinase 1)	0.696	1.350
IPI00125319	Gsk3b (Glycogen synthase kinase 3 beta)	0.706	1.126
IPI00126449	Mapk7 (Mitogen-activated protein kinase 7)	0.709	1.473
IPI00127976	Grb10 (Isoform 3 of Growth factor receptor-bound protein 10)	0.715	1.975
IPI00319076	Hipk3 (Homeodomain-interacting protein kinase 3)	0.733	0.906
IPI00622322	Mapk11 (Mitogen-activated protein kinase 11)	0.781	1.271
IPI00130754	Efnb1 (Ephrin B1 precursor)	0.786	0.372
IPI00117829	Cav1 (Isoform alpha of Caveolin-1)	0.790	0.907
IPI00480321	Grlf1 (Glucocorticoid receptor DNA-binding factor 1)	0.792	1.251
IPI00114855	Ophn1 (Oligophrenin 1)	0.794	2.279
IPI00130442	Ephb3 (Ephrin type-B receptor 3 precursor)	0.806	0.501
IPI00314240	Hmga1 (Isoform of High mobility group protein HMG-I/HMG-Y)	0.820	N.D.
IPI00116381	Prpf4b (PRP4 pre-mRNA processing factor 4 homolog beta)	0.891	1.247
IPI00230277	Mapk3 (Mitogen-activated protein kinase 3)	0.902	1.460
IPI00135971	Tjp1 (Tight junction protein ZO-1)	0.903	1.041
IPI00468418	Stam2 (Isoform 1 of Signal transducing adapter molecule 2)	0.911	1.718
IPI00187275	Cdv3 (Isoform 2 of protein CDV3)	0.982	2.543
IPI00123410	Usp24 (Ubiquitin carboxyl-terminal hydrolase)	1.021	1.558
IPI00119663	Mapk1 (Mitogen-activated protein kinase 1)	1.031	1.225
IPI00125298	Shc1 (Isoform p66 of SHC-transforming protein 1)	1.063	1.502
IPI00416163	Ephb4 (Ephrin receptor B4 isoform B)	1.137	0.781
IPI00116554	Ptpn11 (Isoform 1 of Protein-tyrosine phosphatase non-receptor type 11)	1.147	1.665
IPI00112346	Mapk14 (Isoform 1 of Mitogen-activated protein kinase 14)	1.153	1.209
IPI00336844	Epn2 (Isoform 1 of Epsin-2)	1.164	3.213
IPI00110850	Actb (Actin, cytoplasmic 1)	1.171	1.095
IPI00229315	Trafd1 (Isoform 1 of TRAF-type zinc finger domain-containing protein 1)	1.213	1.011

IPI	Protein description	Ratio +/+ / -/-	Ratio reex. / -/-
IPI00127755	Nedd9 (Enhancer of filamentation 1)	1.225	0.638
IPI00330958	Hnrpd (Isoform 1 of Heterogeneous nuclear ribonucleoprotein D0)	1.229	N.D.
IPI00330333	Ankrd13a (Ankyrin repeat domain-containing protein 13A)	1.229	2.128
IPI00123967	Map3k7 (Mitogen-activated protein kinase kinase kinase 7, isoform CRA_B)	1.261	0.724
IPI00462072	Eno1 (Alpha enolase)	1.315	2.002
IPI00406794	Gab1 (GRB2-associated binding protein 1)	1.333	1.413
IPI00776145	Myo6 (Myosin 6)	1.338	2.175
IPI00319994	Ldha (L-lactate dehydrogenase A chain)	1.363	1.749
IPI00377738	Tnk2 (Tyrosine kinase non-receptor 2)	1.372	1.324
IPI00322839	Rbck1 (RanBP-type and C3HC4-type zinc finger containing 1)	1.381	1.723
IPI00307837	Eef1a1 (Elongation factor 1-alpha 1)	1.391	1.969
IPI00405227	Vcl (Vinculin)	1.412	1.231
IPI00113348	Gm944 (Isoform 1 of protein FAM59A)	1.459	2.447
IPI00309224	Pik3ca (Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha isoform)	1.488	2.956
IPI00409405	EG620772 (similar to Cofilin-1 isoform 1)	1.509	1.679
IPI00468996	Wasl (Neural Wiskott-Aldrich syndrome protein)	1.620	2.029
IPI00222827	Itsn2 (Isoform 2 of Intersectin-2)	1.637	1.739
IPI00622390	Eps8 (Epidermal growth factor receptor pathway substrate 8)	1.641	2.930
IPI00113563	Ptk2 (Isoform 1 of Focal adhesion kinase 1)	1.690	1.534
IPI00136572	Zc3hav1 (Zinc finger CCCH type, antiviral 1)	1.749	2.799
IPI00224626	Sept7 (Cell division cycle 10 homolog)	1.752	2.197
IPI00117159	Pik3r2 (Phosphatidylinositol 3-kinase regulatory subunit beta)	1.784	2.043
IPI00403823	Ctnnd1 (Catenin, delta 1 isoform 1)	1.798	1.259
IPI00154012	Usp15 (Isoform 1 of Ubiquitin carboxyl-terminal hydrolase 15)	1.855	1.428
IPI00229647	Tln2 (Talin 2)	1.903	3.491
IPI00126124	St5 (Isoform 1 of Suppression of tumorigenicity 5)	1.949	1.183
IPI00227299	Vim (Vimentin)	1.951	2.414
IPI00319973	Pgrmc1 (Membrane-associated progesterone receptor component 1)	1.951	N.D.
IPI00266188	Cfl2 (Cofilin 2)	1.986	1.850
IPI00122143	Jak2 (Janus kinase 2)	2.012	0.987
IPI00113783	Sh2b3 (SH2B adapter protein 3)	2.013	1.671
IPI00229786	Ttc23 (Isoform 1 of Tetratricopeptide repeat protein 23)	2.057	3.257
IPI00114982	Stat5b (Signal transducer and activator of transcription 5B)	2.065	1.182
IPI00127364	Epn3 (Epsin 3)	2.098	2.612
IPI00415684	Pdlim5 (Isoform 2 of PDZ and LIM domain protein 5)	2.133	1.939

IPI	Protein description	Ratio +/+ / -/-	Ratio reex. / -/-
IPI00464280	Epn2 (Epsin 2)	2.185	2.984
IPI00125534	Dok1 (Docking protein 1)	2.230	2.023
IPI00121514	Stip1 (Stress-induced-phosphoprotein 1)	2.243	1.964
IPI00135514	Dapp1 (Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide)	2.266	4.042
IPI00113099	Ccdc50 (Isoform 1 of Coiled-coil domain-containing protein 50)	2.275	1.175
IPI00123181	Myh9 (Myosin 9)	2.313	0.994
IPI00109334	Fert2 (Isoform 1 of Proto-oncogene protein-tyrosine kinase FER)	2.383	2.420
IPI00467841	Calm1 (Calmodulin 1)	2.447	1.935
IPI00117689	Ptrf (Polymerase I and transcript release factor)	2.449	2.255
IPI00136350	Dlg3 (Disks large homolog 3)	2.563	1.419
IPI00377592	Sec31a (SEC31-like 1)	2.671	2.690
IPI00229509	Plec1 (Isoform PLEC-1I of Plectin 1)	2.680	2.835
IPI00109672	Yes1 (Proto-oncogene protein-tyrosine kinase Yes)	2.752	1.153
IPI00136917	C230081A13Rik (Protein-tyrosine kinase-protein kinase SgK269)	2.809	1.183
IPI00330773	Phldb2 (Isoform 1 of Pleckstrin homology-like domain family B member 2)	2.825	4.580
IPI00121089	Plcg1 (1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-1)	2.845	2.320
IPI00139383	Tbc1d10a (Novel protein, ortholog of human EBP50-PDZ interactor EPI64)	2.874	3.005
IPI00227814	Stat3 (Isoform Stat3B of Signal transducer and activator of transcription 3)	2.963	1.545
IPI00128703	Pxn (Isoform alpha of Paxillin)	3.122	1.112
IPI00622946	Palld (Isoform 4 of Palladin)	3.139	2.052
IPI00118205	Fhl2 (Four and a half LIM domains protein 2)	3.202	1.770
IPI00322033	Tom1l2 (Isoform 1 of TOM1-like protein 2)	3.241	3.199
IPI00109932	Ddx6 (Probable ATP-dependent RNA helicase DDX6)	3.310	2.476
IPI00123376	Snrpc (U1 small nuclear ribonucleoprotein C)	3.367	2.868
IPI00378438	Tns1 (Tensin 1)	3.396	1.544
IPI00848816	LOC100048522 similar to Cofilin 1	3.403	2.436
IPI00753792	Stat3 (Isoform Del-701 of Signal transducer and activator of transcription 3)	3.505	1.805
IPI00229848	Plcg2 (Phospholipase C gamma 2)	3.579	3.725
IPI00116649	Cnn2 (Calponin-2)	3.720	3.924
IPI00227152	Tdrd3 (Tudor domain-containing protein 3)	3.780	2.419
IPI00125855	Prkcd (Isoform 1 of Protein kinase C delta type)	3.815	0.984
IPI00312188	Stambpl1 (Isoform 1 of AMSH-like protease)	4.048	6.622
IPI00849830	Shb (Src homology 2 domain-containing transforming protein B)	4.136	0.640
IPI00130621	Rasa1 (RAS p21 protein activator 1)	4.152	2.348

IPI	Protein description	Ratio +/+ / -/-	Ratio reex. / -/-
IPI00453999	Nck1 (Non-catalytic region of tyrosine kinase adaptor protein 1)	4.202	3.403
IPI00762785	Tenc1 (Isoform 1 of Tensin-like C1 domain-containing phosphatase)	4.371	2.822
IPI00626106	Tagln2 (Transgelin 2)	4.398	4.613
IPI00221494	Lpp (Isoform 1 of Lipoma-preferred partner homolog)	4.417	1.584
IPI00664670	Flnc (Isoform 1 of Filamin-C)	4.539	2.433
IPI00663627	Flnb (Filamin-B)	5.005	2.111
IPI00226515	Tagln (Transgelin)	5.123	1.656
IPI00420480	Gak (Isoform 1 of Cyclin G-associated kinase)	5.160	2.423
IPI00112648	Csk (Protein-tyrosine kinase CSK)	5.296	1.959
IPI00309481	Epb4.1l2 (Band 4.1-like protein 2)	5.514	2.597
IPI00137932	Syk (Protein-tyrosine kinase SYK)	5.781	2.024
IPI00465786	Tln1 (Talin 1)	5.829	2.358
IPI00553777	Hnrnpa1 (Heterogeneous nuclear ribonucleoprotein)	5.880	2.859
IPI00230395	Anxa1 (Annexin A1)	5.952	2.476
IPI00553798	Ahnak (AHNAK nucleoprotein isoform 1)	6.130	2.065
IPI00380418	Mtmr10 (Myotubularin related protein 10)	6.256	4.297
IPI00331334	Bag3 (BAG family molecular chaperone regulator 3)	6.979	2.573
IPI00109335	Stx4a (Syntaxin 4)	9.148	2.128
IPI00353237	Tns3 (Isoform 1 of Tensin 3)	11.170	2.680
IPI00122450	Cald1 (Caldesmon 1)	11.730	4.406

Materials and methods

Plasmids

The pGEX expression vectors for the GST-fusion intracellular domain of RPTPα and D1 domain wild type or having the C433S mutation were described before (den Hertog et al. 1994). The constructs encoding for RPTPα R438K GST-fusion proteins (full length and D1) were obtained by cloning the StuI-ClaI fragment containing the mutation from pSG-HA-RPTPα R438K (Buist et al. 2000) into the StuI-ClaI digested pGEX constructs mentioned above.

Recombinant proteins

The constructs encoding GST-fusion proteins were transformed into BL21 bacteria. Expression of fusion proteins was induced with 0.1 mM IPTG for 5 h at 25°C. The bacteria were harvested by centrifugation, resuspended in Tris-buffered saline (TBS) solution containing 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 mg/ml lysozyme

and incubated 10 minutes at room temperature. The suspension was sonicated on ice, supplemented with 1% TritonX-100, kept 10 minutes 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 minutes at 4°C. The beads were washed three times with ice cold TBS. 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 TBS containing 10% glycerol.

GST pull-down

The GST pull-down protocol was previously described (Blanchetot et al. 2005). Briefly, RPTPα -/- and +/+ MEFs were lysed on ice for 30 min. in lysis buffer A (20 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 5 mM iodoacetic acid, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1μM PMSF). 10mM DTT was added to the lysates and incubated for 15 min. at 4°C to inactivate any unreacted iodoacetic acid. The samples were centrifuged for 15 min. at 13000 rpm and 4°C. Subsequently, the supernatants were incubated with the corresponding GST-fusion proteins conjugated to Glutathione-Agarose beads overnight at 4°C. The beads were washed 4 times with RIPA buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM Na2HPO4, 5 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 10% glycerol, 1 μg/ml leupeptin and 1 μg/ml aprotinin), then resuspended in 2X SDS sample buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 2% β-mercaptoethanol and 0.04% bromophenol blue), boiled for 5 min and resolved by 10% SDS-PAGE.

Immunoprecipitation

Nearly confluent RPTPα -/-, RPTPα reexpressors and WT MEFs were lysed for 20 minutes 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, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 μg/ml aprotinin). The lysates were collected using a cell scraper and centrifuged for 10 minutes at 13000 rpm and 4°C. Samples from the lysates were collected and boiled after being mixed with equal volumes of 2X SDS sample buffer and resolved on 7.5% SDS-PAGE gels. To the remaining lysates PY20 anti-pTyr antibody (BD transduction Laboratories) was added to a final concentration of 1 μg/ml and the samples were incubated for 1 h at 4°C on a rotary shaker. Subsequently, Protein A Sepharose beads were added and the samples were incubated for an additional 1 h at 4°C. The immunoprecipitates were washed 4 times with HNTG buffer (20 mM

HEPES pH 7.4, 150 mM NaCl, 0.1% TritonX-100 and 10% glycerol) and boiled in 2X SDS sample buffer.

Cell culture, stimulation and protein digest preparation

RPTPα -/-, RPTPα reexpressors and WT MEFs were grown to 90% confluence in Dulbecco's modified Eagle's medium (DMEM) containing 7.5% fetal bovine serum (Invitrogen) and 0.05 mg/ml penicillin/streptomycin (Invitrogen). Cells were placed in serum-free medium 16 h before PDGF stimulation. Cells were stimulated with 50 ng/ml PDGF for 5 min or left untreated then washed with cold phosphate buffered saline (PBS). Before labeling and immunoprecipitation, cells were lysed in 8 M Urea/ 50 mM ammoniumbicarbonate, 5 mM sodium phosphate, 1 mM potassium fluoride, 1 mM sodium orthovanadate and EDTA-free protease inhibitor cocktail (Sigma). Samples were reduced with DTT at a final concentration of 10 mM at 56°C, subsequently samples were alkylated with iodoacetamide at a final concentration of 55 mM at RT. The samples were diluted to 2 M Urea/50 mM ammoniumbicarbonate and trypsin (1:100) was added. Digestion was performed overnight at 37°C.

Stable isotope labeling by amination of tryptic peptides

Tryptic peptides were desalted, dried *in-vacuo* and re-suspended in 100 µl of triethylammonium bicarbonate (100 mM). Subsequently, formaldehyde-H_2 (573 µmol) was added, vortexed for 2 minutes followed by the addition of freshly prepared sodium cyanoborohydride (278 µmol). The resultant mixture was vortexed for 60 min. at RT. Ammonia was added to consume the excess formaldehyde. Finally 100% formic acid was added to acidify the solution. For intermediate labels, formaldehyde-D2 (573 µmol) was used. For the heavy labeling, ^{13}C-D_2-formaldehyde (573 µmol) and freshly prepared cyanoborodeuteride (278 µmol) was used (Boersema et al. 2008; Boersema et al. 2009). The light, intermediate and heavy dimethyl labeled samples were mixed in 1:1:1 ratio based on total peptide amount, determined by running an aliquot of the labeled samples on a regular LC-MS run and comparing overall peptide signal intensities.

Peptide immunoprecipitation

Labeled peptides were mixed, desalted, dried down and re-dissolved in immunoprecipitation (IP) buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 1% NOG and 1x Complete mini cocktail of protease inhibitors (Roche Diagnostics). Prior to immunoprecipitation, pY99 agarose beads (Santa Cruz) were washed in IP buffer.

The labeled peptide mixture was added to the pY99 agarose beads and incubation was performed overnight at 4°C. Beads were washed 3 times with IP buffer and 2 times with water. Peptides were eluted by adding 0.15% TFA for 20 min at RT. Eluted peptides were desalted and concentrated on STAGE-tips as described previously (Boersema et al. 2009).

On-line nanoflow liquid chromatography

Nanoflow LC-MS/MS was performed by coupling an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) to a LTQ-Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) as described previously (Raijmakers et al. 2008). Dried fractions were reconstituted in 10 µl 0.1 M acetic acid and delivered to a trap column Aqua^{tm} C18, 5 µm, (Phenomenex, Torrance, CA, USA); 20 mm × 100 µm ID, packed in-house) at 5 µl/min in 100% solvent A (0.1 M acetic acid in water). Subsequently, peptides were transferred to an analytical column (ReproSil-Pur C18-AQ, 3 µm, Dr. Maisch GmbH, Ammerbuch, Germany; 40 cm × 50 µm ID, packed in-house) at ~100 nL/min in a 2 or 3 hour gradient from 0 to 40% solvent B (0.1 M acetic acid in 8/2 (v/v) acetonitrile/water). The eluent was sprayed via distal coated emitter tips (New Objective), butt-connected to the analytical column. The mass spectrometer was operated in data dependent mode, automatically switching between MS and MS/MS. Full scan MS spectra (from *m/z* 300-1500) were acquired in the Orbitrap with a resolution of 60,000 at *m/z* 400 after accumulation to target value of 500,000. The three most intense ions at a threshold above 5000 were selected for collision-induced fragmentation in the linear ion trap at a normalized collision energy of 35% after accumulation to a target value of 10,000.

Data analysis

All MS2 spectra were converted to single DTA files using Bioworks 3.3. Runs were searched using an in-house licensed MASCOT search engine (Mascot (version 2.1.0) software platform (Matrix Science, London, UK), http://www.matrixscience.com/) against the Mouse IPI database version 3.36 (51326 sequences) with carbamidomethyl cysteine as a fixed modification. Light, intermediate and heavy dimethylation of peptide N-termini and lysine residues, oxidized methionine and phosphorylation of tyrosine, serine and threonine were set as variable modifications. Trypsin was specified as the proteolytic enzyme and up to two missed cleavages were allowed. The mass tolerance of the precursor ion was set to 5 ppm and for fragment ions 0.6 Da. Individual MS/MS spectra from phosphopeptides were

accepted for a Mascot score ≥ 20. All identified phosphopeptides that were found to be differentially phosphorylated were manually validated.

Quantification

Quantification was performed using an in-house dimethyl-adapted version of MSQuant (http://msquant.sourceforge.net/), as described previously (Boersema et al. 2008). Briefly, peptide ratios were obtained by calculating the extracted ion chromatograms (XIC) of the "light", "intermediate" and "heavy" forms of the peptide using the monoisotopic peaks only. The total XIC for each of the peptide forms was obtained by summing the XIC in consecutive MS cycles for the duration of their respective LC-MS peaks in the total ion chromatogram using FT-MS scans. This total XIC was then used to compute the peptide ratio. Heavy and light labeled peptides were found to largely co-elute. Quantified proteins were normalized against the Log2 of the median of all peptides quantified. StatQuant, an in-house developed program (van Breukelen et al. 2009), was used for normalization, outlier detection and determination of standard deviation. Ratios of phosphotyrosine levels were normalized to the ratios of (non-specifically binding) non-phosphorylated peptides. Ratios derived from different charge states of the peptide and/or missed cleavages with the same phosphorylation sites were log averaged

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Chapter 6

Discussion

It is well established that tyrosine phosphorylation is one of the most important mechanisms in signal transduction. Early estimations suggested that Tyr phosphorylation is about 3000 times less abundant than Ser and Thr phosphorylation (Hunter and Sefton 1980). Recently approximately 2% of all phosphorylation events were attributed to Tyr phosphorylation, whereas Thr and Ser were credited with 12 and 84 percent respectively (Olsen et al. 2006). In a study dedicated to Tyr phosphorylation in non-small cell lung cancer cell lines and tumors, 4551 unique phosphorylation events were identified on more than 2700 proteins (Rikova et al. 2007), indicating that the previous estimations were on the low side with regard to Tyr phosphorylation. These numbers suggest that at least 10% of all proteins encoded by the human genome are Tyr phosphorylated. In human cells, all these Tyr phosphorylation events are regulated by approximately 90 protein tyrosine kinases (PTKs)(Manning et al. 2002) and as many protein tyrosine phosphatases (PTPs) (Alonso et al. 2004). The high abundance of pTyr in cells might change our view on the mechanisms required to control tyrosine phosphorylation. On the one side it seems that the PTKs and the PTPs are less specific in vivo than it was previously believed with each of these proteins being responsible for the phosphorylation or dephosphorylation of tens of targets. On the other side it is very well known that Tyr phosphorylation is a tightly controlled process and small changes could lead to disease, suggesting that more regulatory mechanisms work together to maintain the pTyr balance.

We studied tyrosine phosphorylation from the phosphatases point of view using the prototypical receptor protein-tyrosine phosphatase alpha (RPTPα). RPTPα investigation was done in tandem with its best characterized substrate, the non-receptor PTK Src. RPTPα activates Src by dephosphorylating pTyr527. In non-stimulated cells Src has a closed-inactive conformation with the SH2 domain bound to pTyr527 and the SH3 domain bound to the SH2-kinase domain linker. Following pTyr527 dephosphorylation Src adopts an open-active conformation. Open Src autophosphorylates itself on Tyr416 and becomes fully activated. A displacement mechanism was proposed for RPTPα-mediated Src activation in which Src SH2 domain replaces GRB2 adaptor protein from RPTPα pTyr789, this being followed by dephosphorylation of Src pTyr527 (Zheng et al. 2000). It was proposed that mitotic activation of Src was mediated by hyperphosphorylation of RPTPα on two serines (Ser180 and Ser204) in the juxtamembrane region. This event was proposed to lead

to loss of GRB2 and increased catalytic activity of RPTPα. Subsequently, Src binds free pTyr789 and is dephosphorylated by activated RPTPα, resulting in increased Src activity (Zheng and Shalloway 2001).

In Chapter2 we investigated the role of serine phosphorylation in RPTPα regulation. We developed phospho-specific antibodies that recognize RPTPα pSer180 and pSer204 and using these antibodies we followed the variation of endogenous RPTPα Ser phosphorylation in the cell cycle. We found that phosphorylation of Ser204 was almost completely abolished in mitosis whereas Ser180 phosphorylation was less affected. The same result was obtained for mouse RPTPα in NIH3T3 cells and human RPTPα in HeLa cells. Mitotic dephosphorylation of RPTPα pSer204 was concomitant with dephosphorylation of the Src inhibitory pTyr527 and Src activation and surprisingly with dephosphorylation of Src pTyr416 as well. The net result of these Src dephosphorylation events was a modest increase in Src activity. Our findings contradict the current model from Shalloway and co-workers (see above). We further discovered that mitotic PP2A activation is responsible for dephosphorylation of RPTPα pSer204. Moreover, mitotic RPTPα-Src interaction is increased and *in vivo* inhibition of PP2A impairs dephosphorylation of RPTPα pSer204 and concomitantly the interaction with Src. These results clearly show that dephosphorylation of RPTPα pSer204 leads to increased Src binding and activation. But how can this dephosphorylation event have such a big influence on RPTPα-Src interaction? Unfortunately the crystal structure of RPTPα-D1 does not reveal how Ser204 phosphorylation might affect the local conformation because the crystallized protein does not contain Ser204. An answer might come from a very recent study showing that Tyr phosphorylation in the C-terminal region of RPTPρ leads to increased dimerization and inactivation probably due to the effect of this modification on the flexibility of the wedge (Lim et al. 2009). RPTPα Ser204 is located in the vicinity of the wedge and similarly to the mechanism proposed for RPTPρ, phosphorylation of Ser204 might lead to a rigidization of the wedge and stabilization of the dimers. Another interesting aspect is that close to Ser204 there is a putative SH3 binding site and Ser204 phosphorylation might influence this site, hence regulating the RPTPα-Src interaction.

In Chapter3 we investigated the environment of Ser204 in RPTPα and found that the Src SH3 domain is not directly involved in the interaction with RPTPα, thus excluding a role for the N-terminal RPTPα putative SH3 binding site (PPLP-motif)

close to Ser204 in Src binding and activation. However, the wedge structure seems to be important for RPTPα-mediated Src activation. The mutation of two tandem prolines at the N-terminal end of the wedge (P210/211L), a modification that was shown to destabilize the wedge and abolish dimerization of RPTPα (Jiang et al. 2000), led to decrease in binding of RPTPα to Src. A mutation at the tip of the wedge (D228R) presented increased binding to Src. The corresponding mutation in CD45 (E613R) activated the EGF-induced inactive dimers of CD45 chimeric molecules containing the extracellular domain of the epidermal growth factor receptor (EGFR) and the transmembrane and cytoplasmic domain of CD45 (Majeti et al. 1998). This activation of CD45 was suggested to be the result of the loss of the interactions between the amino acids from the wedge and those within the catalytic site. Moreover, RPTPα-F135C, a mutant demonstrated to form stable active dimers (Jiang et al. 1999), also shows increased binding to Src confirming that the conformation of the RPTPα dimers is important for Src binding as well. This increase in Src binding was not observed for an RPTPα mutant (P137C) that forms constitutively inactive dimers suggesting that specific conformation of the dimers is required for efficient Src binding. Combined, the evidence from Chapter2 and Chapter3 suggest that RPTPα Ser204 phosphorylation might influence the conformation of the wedge, affecting the quaternary structure of RPTPα and altering the interaction with Src.

Whereas the Src SH3 domain does not seem to be required for direct binding to RPTPα we observed that Src containing SH3 domain inactivating mutations interacts more strongly with RPTPα (Chapter3). Thus, cellular processes that induce loss of binding between SH3 domain and the SH2-kinase domain linker may eventually lead to increased RPTPα-Src interaction.

So far there is no evidence for disease related RPTPα mutations. In Chapter4 we analyzed the role of a spontaneous mutation in RPTPα (R554H) on RPTPα function. Even though the genetic history of the patient with the R554H mutation revealed that the mutation was not directly associated with the disease, the study of this mutation led us to an interesting discovery: a catalytically active membrane-distal PTP domain (D2) in RPTPα is required for Src activation. We do not know if RPTPα-D2 dephosphorylates Src. Whereas the activity of D2 was shown to be only ~10 times lower than the activity of RPTPα membrane-proximal domain (D1) when pNPP was used as a substrate, the activity of RPTPα-D2 is at least three orders of magnitude lower that the activity of RPTPα-D1 towards tyrosine phosphorylated

peptides (Wu et al. 1997). Since the activity of RPTPα-D2 is so low towards pTyr-containing peptides, it is unlikely that D2 has catalytic function towards Src. Instead RPTPα-D2 may function as a pseudo-phosphatase with a role in Src binding.

Recently Barr and colleagues (Barr et al. 2009) proposed a new model for RPTPgamma dimerization. They showed that RPTPgamma dimers adopted a head-to-toe conformation and the dimers had reduced catalytic activity. Studies from our lab have shown that RPTPα-D1 can interact with RPTPα-D2 and this interaction is dependent on the presence of the wedge (Blanchetot and den Hertog 2000). Moreover, the crystal structure of RPTPα-D2 reveals that the D1-D2 linker is disordered, indicating a high flexibility of this region (Sonnenburg et al. 2003). Therefore, an interaction between the RPTPα-D1 and -D2 may occur in full lengths proteins. We cannot exclude the possibility of a head-to-toe, wedge RPTPα-D2 conformation in RPTPα dimers. This type of interaction would occlude the catalytic site of RPTPα-D2 which might impair binding to Src.

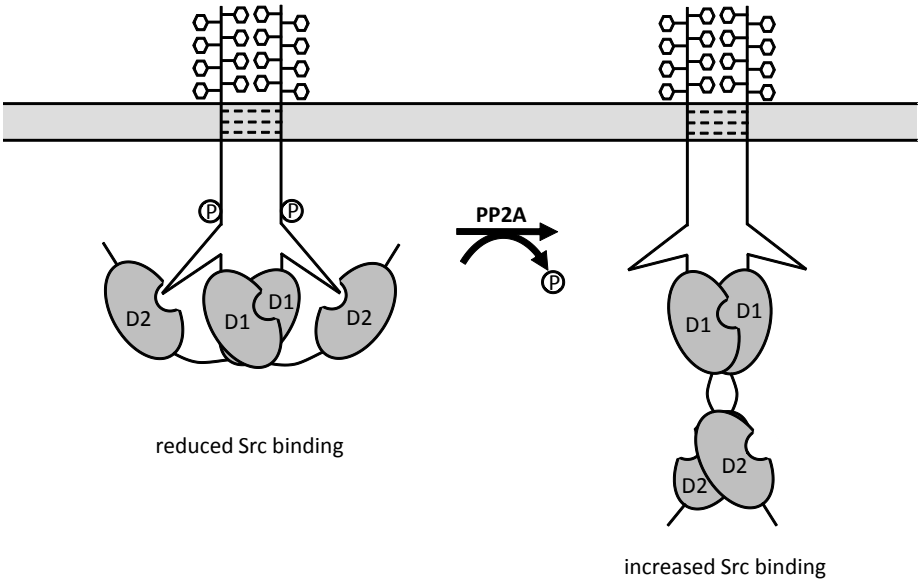

Fig.1 Relation between RPTPα dimer conformation and Src binding. See text for details.

A potential model emerging from the results in Chapters 2, 3 and 4 is depicted in Fig. 1. In this model Ser204 phosphorylation helps the wedge to adopt the proper conformation to be able to occupy RPTPα-D2 catalytic site of a neighboring molecule. In this situation the affinity for Src is reduced. Dephosphorylation of pSer204 by PP2A would lead to changes in the local conformation. Subsequently, the wedge

releases RPTPα-D2 which allows Src to bind. In this situation RPTPα dimers will be able to adopt the conformation required for Src activation.

In these studies we investigated the role of RPTPα in Src activation and the mechanism governing RPTPα-Src interaction. We discovered that Ser204 phosphorylation is essential for mitotic RPTPα-mediated Src activation. We found that RPTPα wedge probably through its influence on RPTPα dimer conformation is important for RPTPα-Src interaction. Finally, we discovered that a catalytic active RPTPα-D2 might be required for Src binding and activation. We conclude that RPTPα-mediated activation of Src is a complex mechanism that leads to a tight regulation of Src activity.

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Summary

One of the main signal transduction mechanisms in all eukaryotic organisms is tyrosine phosphorylation. The cellular levels of tyrosine phosphorylation are tightly controlled by the activity of two classes of enzymes with opposing activities: the protein-tyrosine kinases (PTKs) and the protein-tyrosine phosphatases (PTPs). Malfunction of these enzymes results in changes of the delicate tyrosine phosphorylation balance and leads to disease. We are interested in elucidating RPTP regulation and we use RPTPα as a prototypical RPTP. The best characterized substrate of RPTPα is Src and dephosphorylation of Src leads to Src activation.

We studied mitotic RPTPα phosphorylation using phospho-specific antibodies. We discovered that RPTPα phospho-Ser204 was almost completely dephosphorylated in mitotic NIH3T3 and HeLa cells, whereas phosphorylation of Ser180 was only slightly affected. Concomitantly, Src pTyr527 and pTyr416 were dephosphorylated, resulting in modest Src activation. Using inhibitors, we found that dephosphorylation of RPTPα phospho-Ser204 in mitosis is likely mediated by PP2A. Interestingly, binding of endogenous Src to RPTPα was induced in mitosis. Based on these results, we proposed a new model for mitotic activation of Src in which RPTPα pSer204 dephosphorylation facilitates Src binding, leading to RPTPα-mediated dephosphorylation of Src pTyr527 and pTyr416 and hence activating Src in a tightly controlled manner.

Next, we investigated how dephosphorylation of RPTPα phospho-Ser204 could lead to increased Src binding. In the vicinity of Ser204, RPTPα has two domains that might have a role: a putative SH3 binding site and a helix-turn-helix wedge motif. P210/211L mutations in RPTPα abolish the putative PXXP SH3 binding site and concomitantly affect the stability of the wedge. We discovered that Src binding to RPTPα-P210/211L is impaired and RPTPα-P210/211L mutant has decreased ability to activate Src. Mutations of the Src-SH3 domain (W118A or P133L) increased binding to RPTPα indicating that the Src-SH3 domain is not required for binding. Further, different RPTPα mutations affecting the wedge structure suggested that the wedge is required for the interaction with Src.

In a screen for disease-related RPTPα mutations we found a mutation in RPTPα consisting of an Arg to His substitution in the phospho-tyrosine recognition loop of the membrane-distal catalytic domain (D2) that turned out not to be associated with the disease. RPTPα-R554H mutation depletes the catalytic activity

of RPTPα-D2 without significantly affecting the total activity of RPTPα. In addition, R554H mutation impaired Src binding to RPTPα. RPTPα lacking the catalytic cysteine (Cys723) from the D2 domain had also diminished ability to bind Src. Decreased Src binding to R554H and C723S RPTPα mutants resulted in low Src activation. These results suggested that a catalytically active membrane-distal domain is required for a fully functional RPTPα that binds and activates Src.

Finally, we used three different approaches to identify new RPTPα substrates: (1) study of the tyrosine phosphorylation profiles of wild type and RPTPα knock-out mouse embryo fibroblasts, (2) substrate trapping experiments and (3) quantitative mass spectrometry. We discovered a number of interesting candidate substrates that remain to be confirmed.

The results described in this thesis led us to propose a new model for activation of Src by RPTPα.

Samenvatting

Wij zijn geïnteresseerd in het ontrafelen van receptor eiwit tyrosine fosfatase (RPTP) regulatie en gebruiken RPTPα als een prototypisch RPTP. Het best gekarakteriseerde substraat van RPTPα is Src en defosforylatie van Src leidt tot Src activering.

Wij hebben mitotische RPTPα fosforylering bestudeerd gebruikmakend van verschillende fosfo-specifieke antilichamen. We ontdekten dat RPTPα fosfo-Ser204 bijna geheel gedefosforyleerd is in mitotische NIH3T3 en HeLa cellen. Gelijktijdig waren Src pTyr527 en pTyr416 gedefosforyleerd, resulterend in een lichte Src activatie. We vonden dat defosforylatie van RPTPα fosfo-Ser204 tijdens mitose waarschijnlijk beïnvloed wordt door PP2A. Binding van endogeen Src aan RPTPα wordt geïnduceerd tijdens mitose. Gebaseerd op deze resultaten poneren wij een nieuw model voor mitotische activatie van Src waar RPTPα pSer204 defosforylatie Src binding vergemakkelijkt wat tot Src activatie leidt.

Vervolgens hebben we onderzocht hoe defosforylering van RPTPα fosfo-Ser204 zou kunnen leiden tot verhoogde Src binding. In de omgeving van Ser204 heeft RPTPα twee domeinen die hierbij mogelijk een rol kunnen hebben: een vermeende SH3 bindings-site en een helix-turn-helix wedge motief. Gebruik makend van verschillende Src en RPTPα mutanten vonden we dat RPTPα interactie met Src beïnvloed wordt door de wedge conformatie.

Voortvloeiend uit de studie van een spontane mutatie in RPTPα ontdekten we dat een katalytisch actief membraan-distaal domein onontbeerlijk is voor het volledig functioneren van RPTPα welke Src bindt en activeert.

Tenslotte hebben we verschillende methoden gebruikt om nieuwe RPTPα substraten te identificeren, en vonden een aantal interessante kandidaat substraten die nog bevestigd moeten worden.

Op basis van de resultaten in dit proefschrift stellen wij om een nieuw model voor de activatie van Src door RPTPα.

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Andrei

Curriculum Vitae

Andrei Mircea Vacaru was born on 11th of February 1979 in Bucharest, Romania. He finished his pre-university education in 1997 at “Eugen Lovinescu” theoretical high school from Bucharest, focusing on Biology and Chemistry. He continued his education at Faculty of Industrial Chemistry from University “Politehnica” of Bucharest where, in 2003 he obtained his Biochemical Engineer diploma under the supervision of Dr. Mariana Ferdes and Prof. dr. Ovidiu Muntean. After graduation he was employed as research assistant in the group of Prof. dr. Stefan Szedlacsek at the Institute of Biochemistry of Romanian Academy. In February 2005 he started his PhD studies at Hubrecht Institute under the supervision of Prof. dr. Jeroen den Hertog, research described in this book. After obtaining his PhD he will start as post-doc in Prof. dr. Margaret Baron’s group at Mount Sinai School of Medicine.

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