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Receptor protein-tyrosine phosphatase signalling in development

JEROEN DEN HERTOG*, CHRISTOPHE BLANCHETOT, ARJAN BUIST, JOHN OVERVOORDE, ASTRID VAN DER SAR and LEON G.J. TERTOOLEN

Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Utrecht, The Netherlands

ABSTRACT Receptor Protein-Tyrosine Phosphatases (RPTPs) belong to the superfamily of protein-tyrosine phosphatases and have the intrinsic ability to transduce signals across the cell membrane. We are beginning to understand the role of RPTPs in development of invertebrates, due to elegant genetic studies. In contrast, relatively little is known about the role of RPTPs in vertebrate development. Signalling by RPTPs has predominantly been studied in mammalian cell systems, which has led to important insights into potential ligands, into regulation of RPTP activity and into potential RPTP substrates. Here, we will introduce the RPTPs, and discuss the function of the LAR-subfamily of RPTPs. In addition, we focus on the function and signalling of the haematopoietic RPTP, CD45. Finally, we will discuss the structure and function of RPTPalpha, the RPTP that is the subject of our studies.

KEY WORDS: *receptor protein-tyrosine phosphatase, phosphotyrosine, signalling, development*

Introduction

Protein phosphorylation on tyrosine residues is one of the main eukaryotic cell signalling mechanisms, regulating cell proliferation, differentiation, migration and transformation. Cellular protein phosphotyrosine (P.Tyr) levels are regulated by the antagonistic activities of the protein-tyrosine kinases (PTKs) and protein-tyrosine phosphatases (PTPs) (Hunter, 1995). Over the past two decades much emphasis has been placed on elucidation of the biological function of PTKs. This has led to good insights into the function of PTKs in development and into the role of PTKs in cell signalling, as well as into the working mechanism of PTKs (van der Geer *et al.*, 1994). Although our insights into PTP-mediated signalling are not as good as into PTK-mediated signalling, it has been established definitively that several PTPs play crucial roles during development (reviewed by Van Vactor, 1998).

The Protein-Tyrosine Phosphatase superfamily

Following the purification of the first PTP, PTP1B (Tonks *et al.*, 1988b), and the identification of the first transmembrane PTP, CD45 (Tonks *et al.*, 1988a), at least 75 distinct PTPs have been cloned on the basis of sequence homology in the catalytic domain (reviewed by Neel and Tonks, 1997; Van Vactor *et al.*, 1998). PTPs have been identified in many different species, ranging from bacteria to human. Random sequencing projects suggest that the human genome encodes approximately 500 PTPs. All PTPs contain a homologous catalytic PTP domain of approximately 240 residues. The sequence identity between any two PTP domains is 35% or higher, and the PTP domain contains the "PTP-fingerprint"–sequence that defines PTPs, [I/V]HCXAGXXR[S/T]G, with the absolutely conserved catalytic site cysteine (Fischer *et al.*, 1991). The catalytic site cysteine is required for catalysis, since a cysteinyl-phosphate intermediate is formed during catalysis (Guan and Dixon, 1991; Pot and Dixon, 1992).

The PTP superfamily is structurally distinct from the Ser/Thr phosphatase family (Denu *et al.*, 1996), and consists of "classical" PTPs, dual specificity phosphatases (DSPs) and low molecular

Abbreviations used in this paper: P.Tyr, phosphotyrosine; PTK, Protein-Tyrosine Kinase; PTP, protein-tyrosine phosphatase; DSP, dual specificity phosphatase; LMW PTP, low molecular weight PTP; RPTP, Receptor PTP; FNIII, fibronectin type III; IG, immunoglobulin; MAM, meprin, A5-like, Mu; CA-like, carbonic anhydrase-like; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; SNb, segmental nerve b; ISN, intersegmental nerve; LIP-1, LAR-interacting protein-1; TCR, T cell receptor; BCR, B cell receptor; m1 mAChR, m1 muscarinic acetylcholine receptor; PKC, protein kinase C; ITAM, Immunoreceptor Tyrosine-based Activation Motif; SH2, Src homolgy 2.

***Address for reprints:** Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands. FAX: +31 30 251 6464. e-mail: hertog@niob.knaw.nl

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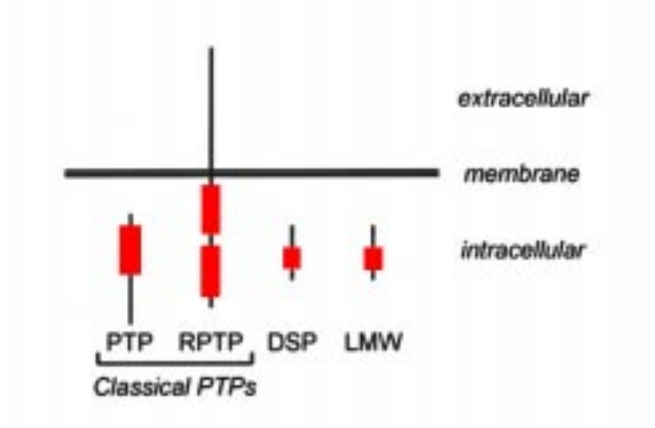

Fig. 1. The PTP superfamily. *Schematic representation of the PTP superfamily, including the "classical" PTPs, with cytoplasmic and transmembrane, "Receptor" PTPs (RPTP). The dual specificity phosphatases (DSPs) and low molecular weight PTPs (LMW) are cytoplasmically localized. A small part of the catalytic phosphatase domains from the classical PTPs, including the fingerprint sequence with the catalytic site cysteine, is conserved in the DSPs and LMW PTPs (red boxes).*

weight PTPs (LMW PTPs) (Fig. 1). The "classical" PTPs can be divided into two large groups, based on their overall structure, the cytoplasmic PTPs and the transmembrane PTPs, which are tentatively called Receptor PTPs (RPTPs). The DSPs and LMW PTPs are divergent from the classical PTPs in that the DSPs and LMW PTPs contain the PTP-fingerprint sequence with the catalytic site cysteine, but lack most of the other sequences that are conserved in the classical PTP domain (Fauman and Saper, 1996). Very little is known about the role of LMW PTPs in cell signalling or development. The DSPs dephosphorylate P.Tyr, P.Ser and P.Thr, and contain very important family members, including the MAPK phosphatase, MKP-1 (Sun *et al.*, 1993), the cell cycle phosphatases, CDC25A, -B and -C (reviewed by Millar and Russell, 1992), and the recently identified tumour suppressor gene, P-TEN (Li and Sun, 1997; Li *et al.*, 1997; Steck *et al.*, 1997). Recent evidence suggests that PTEN not only dephosphorylates proteins, but also a lipid, phosphatidylinositol 3,4,5-trisphosphate (Maehama and Dixon, 1998). In fact, lipid phosphatase activity is actually responsible for the tumour suppressing activity of P-TEN (Myers *et al.*, 1998). In this review, we will focus on classical PTPs, in particular RPTPs.

Receptor Protein-Tyrosine Phosphatases

The RPTPs have been categorized based on their overall structure, and at least six different types of RPTPs have been defined (Fig. 2). Especially the extracellular domains are diverse, ranging from very short (e.g. RPTPepsilon, 27 residues), to very extensive (e.g. LAR). Many different protein modules have been identified in the extracellular domains of RPTPs, including those reminiscent of cell adhesion molecules (fibronectin type III [FNIII]-like and immunoglobulin [Ig]-like domains [Streuli *et al.*, 1989]). In addition, the MAM (Meprin, A5-like, Mu) domain was identified in the ectodomain of RPTPs (Beckmann and Bork, 1993), as well as a domain with high homology to carbonic anhydrases (Krueger and Saito, 1992) (Fig. 2). The ectodomains of some RPTPs are cleaved off by specific proteases and remain bound extracellularly to the cytoplasmic region of these PTPs (e.g. LAR [Streuli *et al.*, 1992]). In addition, these ectodomains may be shed, and thus may have a function by themselves, acting as ligands, independently of the catalytic activity of the RPTP. The most appealing function of the ectodomains is that they may bind ligands that somehow induce an alteration of the intracellular PTP activity. It is noteworthy that ligand-induced dimerisation of chimeric epidermal growth factor receptor (EGFR)-CD45 results in functional inactivation of the RPTP CD45 (Desai *et al.*, 1993), suggesting that RPTPs may actually function as *bona fide* receptors, transducing extracellular signals intracellularly.

The extracellular domain of RPTPs

The extracellular domains of RPTPs are diverse and may bind to different classes of ligands. For instance, the cell adhesion molecule CD22 is a lectin that interacts with CD45 through N-linked alpha-2,6-sialylated oligosaccharides (Stamenkovic *et al.*, 1991). However, this interaction may be rather non-specific and whether the CD45-CD22 interaction is physiologically relevant remains to be determined. The first *bona fide* ligand that was identified for a RPTP was the ectodomain of RPTPmu that interacts with itself in a homophilic manner when expressed at high levels on apposing cells (Brady-Kalnay *et al.*, 1993; Gebbink *et al.*, 1993). Similarly, RPTPkappa interacted in a homophilic manner (Sap *et al.*, 1994). Detailed analysis of the interaction indicated that the MAM domain and the Ig-like domain of RPTPmu are involved in the interaction (Brady-Kalnay and Tonks, 1994; Zondag *et al.*, 1995). Despite the fact that RPTPmu and RPTPkappa are highly homologous, their extracellular domains did not interact with each other, suggesting that the interaction is highly specific (Zondag *et al.*, 1995). The extracellular domain of RPTPzeta/beta, and in particular the carbonic anhydrase-like domain, is a ligand for the neuronal Contactin/Caspr and Contactin/NrCAM receptor complexes (Peles *et al.*, 1995, 1997; Sakurai *et al.*, 1997). Both membrane-bound and soluble RPTPzeta/beta ectodomains bind and induce neurite outgrowth of tectal neurons, suggesting that PTP activity is not required for this effect. However, binding of the RPTPzeta/beta ectodomain to Contactin/protein complexes may induce bidirectional signalling, resulting in neurite outgrowth of the Contactin-expressing cell and a PTP-dependent, as yet to be identified, effect in the RPTPzeta/beta expressing cell. Recently, the Laminin/Nidogen complex was identified as a ligand for a specific isoform of the RPTP LAR (O'Grady *et al.*, 1998). Whether LAR activity is regulated by binding to this complex remains to be determined, but ligation of antibody to the extracellular domain induced internalisation of LAR, suggesting that the net LAR PTP activity at the cell membrane may be reduced by ligand-binding. In contrast, homophilic interactions of RPTPmu at cell-cell contact sites leads to accumulation of RPTPmu at these intercellular contact regions, which led to the hypothesis that RPTPmu signals at sites of cell-cell contact (Gebbink *et al.*, 1995). Taken together, ligands have been identified for several RPTPs, and ligand binding may affect the amount of these RPTPs in (particular locations of) the cell, but whether these interactions directly affect intracellular PTP activity remains to be determined.

The intracellular domain of RPTPs

Most RPTPs contain two cytoplasmic PTP domains (Fig. 2). The membrane proximal PTP domain of the tandem domain-containing RPTPs, D1, contains the majority of the catalytic activity. More-

over, the catalytic activity of D1 is essential for the function of several RPTPs (den Hertog *et al.*, 1993; Desai *et al.*, 1994; Kokel *et al.*, 1998). The membrane-distal domain, D2, contains residual activity, or no activity at all (Wang and Pallen, 1991; Wu *et al.*, 1997). In fact, some RPTP-D2s lack the catalytic site Cys, e.g. RPTPgamma, and thus have no catalytic activity whatsoever (Krueger *et al.*, 1990). However, overall, the sequences of RPTP-D2s are highly conserved in evolution. In addition, the 3D structure of RPTPalpha-D2 is highly similar to the structure of RPTPalpha-D1 (J. Noel and A. Bilwes, personal communication), suggesting the RPTP-D2s have important functions. Mutation of only two residues in RPTPalpha-D2 converts this domain into an active PTP, with catalytic activity close to, or even exceeding, RPTPalpha-D1 (Lim *et al.*, 1998; Buist *et al.*, 1999), demonstrating how closely some RPTP-D2s resemble RPTP-D1s. It is unlikely that residual RPTP-D2 activity is physiologically relevant. However, RPTP-D2s may be involved in defining the substrate specificity of RPTP-D1s by binding to substrates that are phosphorylated on multiple residues, thereby presenting the P.Tyr-containing substrates to RPTP-D1s (Hunter, 1998). Alternatively, RPTP-D2s may negatively regulate RPTP-D1 activity. For instance, RPTPdelta-D2 binds to RPTPsigma-D1 and inhibits RPTPsigma-D1 activity, presumably by blocking the catalytic site of RPTPsigma-D1 (Wallace *et al.*, 1998). In contrast, CD45-D2 binds to CD45-D1 in an intramolecular fashion and CD45-D2 appears to activate CD45-D1 by disrupting CD45 D1-D1 homodimers (Felberg and Johnson, 1998). The role of RPTP-D2s in regulation of RPTP-D1s is complex, since in RPTPalpha, D2 has neither positive nor negative effects on D1 activity, in that the catalytic activity of RPTPalpha-D1 is indistinguishable from RPTPalpha-D1/D2, containing both catalytic domains (Wu *et al.*, 1997). In conclusion, RPTP-D1 catalytic activity is essential for RPTP function and RPTP-D2 may play a modulatory role by presenting substrates to RPTP-D1, or by regulation of RPTP-D1.

Many RPTPs have been identified with a single PTP domain (type III and type VI, Fig. 2). Obviously, these RPTPs do not require a second PTP domain for regulation. Interestingly, some of the single PTP-domain containing RPTPs, including IA-2, do not display detectable PTP activity (Lu *et al.*, 1994), suggesting that these RPTPs do not function by dephosphorylation of cellular substrates, but instead may function by binding to P.Tyr-containing proteins.

Regulation of RPTP activity

Little is known about regulation of RPTP activity. Many single-membrane-spanning receptors are regulated by dimerisation (Weiss and Schlessinger, 1998). For instance, receptor PTKs are activated by dimerisation. Conversely, the RPTPs may be negatively regulated by dimerisation. Ligand-induced dimerisation of chimeric EGFR/CD45 results in functional inactivation, suggesting that dimerisation inhibits CD45 PTP activity (Desai *et al.*, 1993). Moreover, RPTPalpha-D1 formed dimers in two independent crystal structures. The crystal structure of RPTPalpha-D1 dimers suggests that they are inactive, since an N-terminal helix-turn-helix wedge-like structure of one monomer inserts into the catalytic site of the other monomer and *vice versa*, thereby blocking both catalytic sites in the dimer (Bilwes *et al.*, 1996). The wedge sequences are conserved in RPTP-D1s, but not in RPTP-D2s or cytoplasmic PTPs, suggesting that dimerisation-induced inhibition

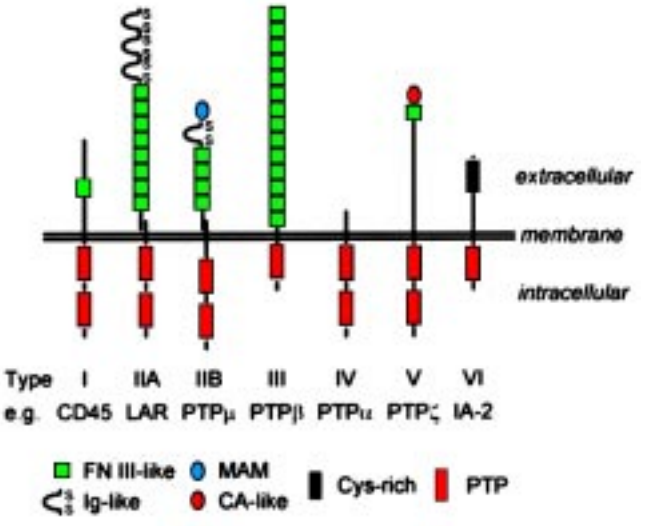

Fig. 2. Receptor Protein-Tyrosine Phosphatases. *Six distinct types of RPTPs have been defined, and a typical mammalian example of each type is depicted. The identity of the different domains is explained in the key at the bottom: FN III-like, fibronectin type III-like; Ig-like, immunoglobulin-like; MAM, Meprin-A5-Mu; CA-like, carbonic anhydrase-like; Cys-rich, cysteine-rich; PTP, protein-tyrosine phosphatase.*

is a general phenomenon of RPTPs. However, RPTPmu-D1 contains a wedge, but does not form dimers like RPTPalpha-D1 (Hoffmann *et al.*, 1997). It is noteworthy that RPTPmu contains an unusually long juxtamembrane region, suggesting that RPTPmu may be regulated differently. As described above, CD45-D1 homodimerises in solution (Felberg and Johnson, 1998), and it will be interesting to see whether the wedge blocks the catalytic sites in the CD45-D1 crystal structure as well. Mutation of a single residue in the wedge of the EGFR/CD45 chimaera abolishes ligand-induced inactivation, suggesting that the wedge plays a central role in regulation of RPTP activity (Majeti *et al.*, 1998).

RPTP substrates and associated proteins

In order to fully understand the function of RPTPs, it is crucial to know the identity of their substrates. Identification of specific RPTP substrates is extremely difficult and not many substrate/RPTP pairs are known. *In vitro* PTP assays, using phosphopeptides, indicate that there is substrate-specificity among the RPTPs. In addition, overexpression of RPTPs does not induce dephosphorylation of all P.Tyr-containing proteins, suggesting that RPTP-mediated dephosphorylation is specific. Paradoxically, RPTPs may enhance cellular P.Tyr levels, since several RPTPs dephosphorylate the inhibitory C-terminal P.Tyr residue in Src-family PTKs, thereby enhancing their PTK activity. Src and Src-family members are negatively regulated by Csk-mediated tyrosine phosphorylation of Tyr527 in its C-terminus (Nada *et al.*, 1991). The recently solved crystal structure of two Src-family members, Src and Hck, demonstrates that phosphorylation of Src Tyr527 induces an intramolecular “closed” conformation due to binding of the Src SH2 domain to this P.Tyr527 residue, which is further stabilised by the SH3 domain (Sicheri *et al.*, 1997; Xu *et al.*, 1997). Dephosphorylation of P.Tyr527 abolishes intramolecular SH2 domain binding, leading to an “open”, active conformation. CD45 dephosphorylates

and activates the Src-family members Lck and Fyn (Mustelin *et al.*, 1989; Ostergaard *et al.*, 1989; Shiroo *et al.*, 1992), while RPTPalpha dephosphorylates and activates Src and Fyn (Zheng *et al.*, 1992; den Hertog *et al.*, 1993; Bhandari *et al.*, 1998). It will be interesting to see whether different RPTPs dephosphorylate and activate distinct sub-sets of Src-family PTKs, thereby conferring specificity.

The specificity of RPTP-mediated dephosphorylation of substrates may be dictated by physical interactions between the substrate and the RPTP. For example, the cadherin-catenin complex binds to LAR-family PTPs, and beta-catenin is dephosphorylated *in vitro* by its associated PTP activity (Kypta *et al.*, 1996). Similarly, RPTPkappa binds to beta-catenin and plakoglobin (gamma-catenin) *in vivo* and *in vitro*, and recombinant RPTPkappa dephosphorylates beta-catenin (Fuchs *et al.*, 1996). The interaction between an RPTP and the cadherin-catenin complex was first described for RPTPmu (Brady-Kalnay *et al.*, 1995), but the interaction between RPTPmu and cadherins has been the subject of debate (Zondag *et al.*, 1996). However, Brady-Kalnay *et al.* (1998) recently provided definitive proof that RPTPmu indeed interacts with cadherins. Whether RPTPmu plays a role in tyrosine dephosphorylation of the cadherin-catenin complex remains to be determined.

Not all RPTPs have to bind stably to their substrates and the substrate-enzyme interactions may be highly transient and dynamic. A method that has been applied successfully to identify substrates relies on the fact that subtle mutations in the catalytic site of PTPs abolish PTP activity, and at the same time enhance binding to specific substrates. This "substrate-trapping" procedure has been used to identify substrates of several cytoplasmic PTPs (Garton *et al.*, 1996; Flint *et al.*, 1997), but not RPTPs. The combination of a substrate trapping procedure and analysis of the phosphorylation state of candidate substrates in PTP knock-out cell lines led to the identification of $p130^{cas}$ and its family members Hef1 and Sin as specific substrates of the cytoplasmic PTP-PEST (Côté *et al.*, 1998). Similar substrate trapping procedures may provide definitive proof as to the identity of specific, physiological substrates of RPTPs, which is crucial for elucidation of the function of RPTPs and will provide insight into the role of RPTPs in development *in vivo*.

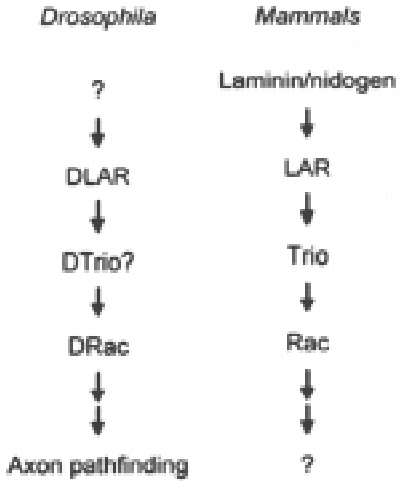

Fig. 3. Model for LAR signalling. *DLAR is involved in axon pathfinding and is genetically upstream of DRac. In mammals, the Laminin/Nidogen complex binds to the ectodomain of LAR, and may act as a ligand for LAR. The cytoplasmic domain of LAR binds to Trio, a multidomain protein that contains a guanine nucleotide exchange factor domain for Rac, suggesting that LAR may couple directly to Rac via Trio. Taken together, the genetic evidence in Drosophila and the biochemical evidence in mammals suggests a simple linear pathway, linking LAR to Rac. However, many question marks remain in this model, including the ones that have been indicated, and it will be a challenge to elucidate the LAR signalling pathway.*

The function of RPTPs *in vivo*

Since PTPs counteract PTK activity, it has been speculated for a long time that RPTPs play an equally important role as the RPTKs in development. Recent conclusive evidence strikingly confirms the importance of RPTPs in invertebrates (reviewed by Chien, 1996; Van Vactor, 1998; Van Vactor *et al.*, 1998; see below). In contrast, still relatively little is known about the role of RPTPs in vertebrates. The expression patterns of many RPTPs have been determined during vertebrate development. For instance, many RPTPs are highly expressed in the developing brain (reviewed by Stoker and Dutta, 1998), suggesting important functions for these RPTPs during brain development. However, gene targeting of several RPTPs disappointingly did not result in obvious developmental phenotypes and thus did not reveal the function of these RPTPs. For instance, RPTPalpha (J. Sap, personal communication), RPTPepsilon (A. Elson, personal communication), RPTPmu (M.F.B.G. Gebbink, personal communication) and RPTPkappa (Skarnes *et al.*, 1995) have been knocked out or targeted otherwise, which did not result in obvious developmental defects. Moreover, knock-out of several other RPTPs, including LAR, induced mild phenotypes at best (see below). Therefore, more rigorous approaches may be required to elucidate the function of RPTPs in vertebrates.

The function of the LAR-subfamily of RPTPs

The LAR-subfamily of RPTPs, i.e. the type IIA RPTPs (Fig. 2), is defined by multiple FNIII-like and Ig-like domains in the extracellular domain. The first transmembrane PTP that was reported in *C. elegans*, Clr-1, belongs to the LAR-subfamily of RPTPs. Clr-1 contains two immunoglobulin-like domains and one FNIII-like domain in its extracellular domain (Kokel *et al.*, 1998), and therefore it most closely resembles *Drosophila* DPTP69D (see below). Mutant embryos with mutations in *clr-1* express the severe *clear* phenotype that is characterised by extremely short, immobile and infertile animals. In addition, their pharynx and intestine appears to float within the pseudocoelom. Animals that are homozygous for the most severe alleles die during larval development, but most animals survive. *Clr-1* was identified in a screen for suppressors of the *egl-15* hypomorphic mutant phenotype. *Egl-15* encodes a *C. elegans* fibroblast growth factor receptor (FGFR) (DeVore *et al.*, 1995). *Clr-1* and *egl-15* mutually suppress each other, suggesting that this RPTP and this RPTK have opposing activities. Using null alleles, it was clear that the *clear* phenotype of *clr-1* mutants is dependent on integrity of *egl-15*, while the phenotype of *egl-15* mutants is not dependent on *clr-1*. Since catalytic activity of Clr-1 is required for its function, it is highly likely that Clr-1-mediated dephosphorylation acts as a negative regulator of egl-15 signalling (Kokel *et al.*, 1998).

One of the first indications that RPTPs play an important role in development came from the expression pattern of three RPTPs in *Drosophila*. DLAR, DPTP10D and DPTP99A are highly expressed in the growth cones of motor neuron axons, suggesting that these RPTPs are involved in axon guidance (Tian *et al.*, 1991; Yang *et al.*, 1991). Recently, genetic evidence confirmed the importance of RPTPs in axon guidance, since *Drosophila* mutants were identified with mutations in RPTPs that showed defects in axonal pathfinding (Desai *et al.*, 1996, 1997; Krueger *et al.*, 1996).

In DLAR mutant embryos, the segmental nerve b (SNb) and SNd axons bypass their normal muscle target region and continue to extend distally along the common pathway, following the intersegmental nerve (ISN) (Krueger *et al.*, 1996). DPTP69D and DPTP99A are also highly expressed on motor axons. DPTP69D mutant embryos show a complex axon pathfinding phenotype, but DPTP99A mutant embryos do not. In DPTP69D mutant embryos, the SNb axon stops growing before reaching its target muscle, or it follows incorrect pathways and bypasses its target (Desai *et al.*, 1996). Comparison of the phenotypes of mutant embryos lacking DLAR, DPTP69D, DPTP99A and combinations demonstrates that these RPTPs have partially redundant functions and that specific RPTPs are required for pathway decisions along the trajectories of the ISN and SNb motor nerves. DPTP99A is involved in axon pathfinding, even though DPTP99A mutant embryos show no phenotypes, since the penetrance and the severity of the SNb defects in mutant embryos that lack both DPTP69D and DPTP99A is enhanced compared to DPTP69D mutant embryos (Desai *et al.*, 1996). Only in the triple mutant the SNb axon does not progress beyond the first branch point, indicating that any of the three RPTPs is sufficient for progression beyond this point (Desai *et al.*, 1997). It is noteworthy that these three RPTPs are only required for pathway decisions of particular axons, since the SN root branches (SNa and SNc) and central nervous system axons are relatively normal in embryos lacking all three RPTPs (Desai *et al.*, 1997).

Although it is clear that RPTPs play an important role in axon pathfinding in *Drosophila*, the molecular mechanism underlying the involvement of these RPTPs remains to be determined. It has been demonstrated that the small GTP binding protein Rac is genetically downstream of DLAR (Kaufmann *et al.*, 1998). This interaction may be mediated by Trio, a large multi-domain protein that binds to human LAR and contains a Rac guanine nucleotide exchange factor domain (Debant *et al.*, 1996). It is noteworthy that *C. elegans* UNC-73, which is closely related to vertebrate Trio, specifically activates Rac and is required for proper cell and growth cone migration, presumably through regulation of cytoskeletal rearrangements (Steven *et al.*, 1998). Taken together, these genetic and biochemical data suggest that Trio couples LAR to Rac. However, it is unlikely that the function of the *Drosophila* RPTPs is limited to effects on Rac, since many other proteins have been identified to interact with the cytoplasmic domain of LAR, including LIP-1 and other Liprins (Pulido *et al.*, 1995; Serra-Pagès *et al.*, 1998). In conclusion, it is not clear how DLAR is involved in neuronal pathfinding, but intuitively, elucidation of the role of DLAR in axon guidance will require identification of specific ligands or binding proteins that interact with its extracellular domain.

Evidence that the extracellular domain of LAR is involved in axon pathfinding comes from the leech, *Hirudo medicinalis*. HmLAR2, the leech homologue of DLAR, is specifically expressed on the growth cones of neurons during rapid outgrowth of these cell processes. Injection of antibodies, directed at the extracellular domain of HmLAR2, leads to partial internalisation of HmLAR2 (Gershon *et al.*, 1998), similar to anti-LAR antibody induced LAR-internalisation in HeLa cells growing *in vitro* (O'Grady *et al.*, 1998). Antibody induced partial internalisation of HmLAR2 induces aberrant projections of neuronal cells over shorter distances than normal. The growth cones of anti-HmLAR2 antibody-injected leech embryos appear normal, even though the projections deviate from their highly regular trajectories, suggesting that the extracellular domain of HmLAR2 transduces extracellular signals that are required for normal growth cone pathfinding (Gershon *et al.*, 1998). The Laminin/Nidogen complex has been identified to bind to the extracellular domain of human LAR *in vitro* (O'Grady *et al.*, 1998). It will be interesting to see whether the Laminin/Nidogen complex, or similar complexes, are involved in axon pathfinding in the leech.

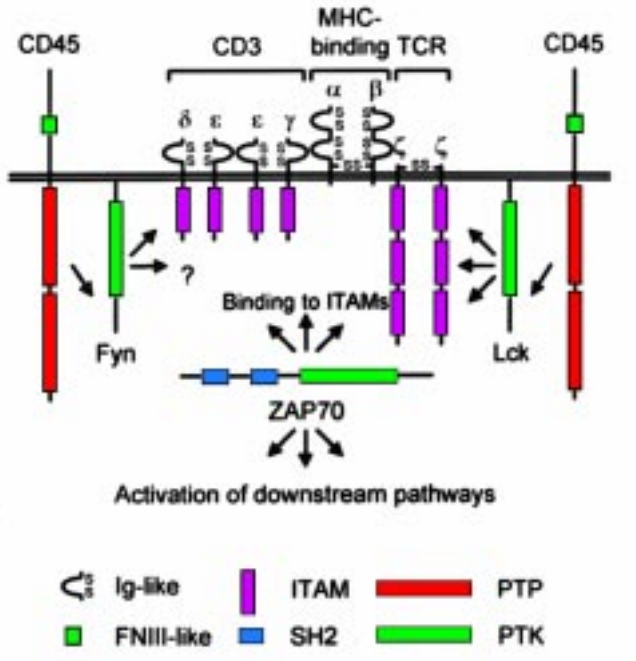

Fig. 4. CD45 potentiates T cell receptor signalling. *CD45 dephosphorylates the inhibitory P.Tyr in the C-terminus of the Src family PTKs, Lck and Fyn, thereby activating their PTK activity. The T cell receptor consists of eight different proteins. The alpha-chain/beta-chain heterodimer binds ligands, while the two CD3 heterodimers, CD3-gamma/CD3-epsilon and CD3-delta/CD3-epsilon, and the TCR-zeta homodimer are signalling components. Upon ligand binding, the signalling components are phosphorylated by active Lck and Fyn on Immunoreceptor Tyrosine-based Activation Motifs (ITAMs). Phosphorylation of ITAMs, in turn, provides binding sites for the SH2 domains of the PTK, ZAP-70. ZAP-70 translocates to the ITAMs, is phosphorylated on tyrosine (initially by Lck and subsequently through autophosphorylation). Tyrosine phosphorylated, active ZAP-70 phosphorylates substrates, thereby initiating downstream signalling. CD45 plays a crucial role in TCR signalling, since ligation of the TCR in cells that lack CD45 does not induce tyrosine phosphorylation of the ITAMs, nor any of the downstream events, due to the fact that Lck and Fyn are not activated in such cells. The identity of the different domains is explained in the key at the bottom: Ig-like, immunoglobulin-like; FNIII-like, fibronectin type III-like; ITAM, Immunoreceptor Tyrosine-based Activation Motif; SH2, Src Homology 2; PTP, protein-tyrosine phosphatase; PTK, protein-tyrosine kinase.*

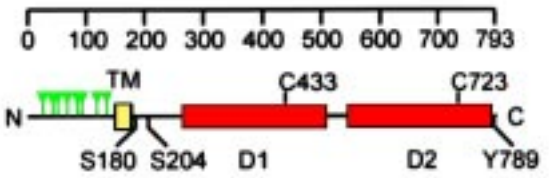

Fig. 5. Receptor Protein-Tyrosine Phosphatase alpha. *A schematic representation of murine RPTPalpha is depicted with the eight N-linked glycosylation sites in the extracellular domain in green, the transmembrane domain in yellow (TM) and the two catalytic domains in red (D1 and D2). The catalytic site cysteines in each PTP domain are depicted (C433 and C723), as well as the phosphorylation sites, Ser180 and Ser204 in the juxtamembrane domain and Tyr789 in the C-terminus. The scale bar indicates amino acid residue number.*

Definitive proof regarding the identity of the extracellular ligands that are involved in binding to the ectodomain of LAR, thereby directing axon pathfinding may be provided by genetic experiments in *Drosophila*.

As described above, it is clear that DLAR is involved in axon pathfinding in *Drosophila*. In contrast, it is not clear what the function of LAR is in vertebrates. The mammalian LAR subfamily consists of three members, LAR, RPTPdelta and RPTPsigma. Due to alternative splicing, many different isoforms exist of these three RPTPs that may have different functions, and that are differentially expressed (Pulido *et al.*, 1995; Schaapveld *et al.*, 1998 and references therein). It is noteworthy that none of the mammalian splice isoforms have exactly the same structure as DLAR, in that DLAR contains nine FNIII-like repeats, while all members of the mammalian subfamily maximally contain eight FNIII-like repeats (Van Vactor *et al.*, 1998). Nevertheless, the mammalian LAR subfamily is generally considered to be the counterpart of DLAR. RPTPdelta and RPTPsigma are highly expressed in the nervous system, like DLAR, which may suggest that these RPTPs are the functional homologues of DLAR, rather than mammalian LAR itself. Gene targeting of LAR does not induce an obvious neuronal phenotype (Skarnes *et al.*, 1995). However, close examination of mice with a gene trap insertion in the LAR gene, in which expression of full length LAR is greatly reduced but not completely abolished, indicated that the size of cholinergic neurons was significantly reduced and that cholinergic innervation of the dentate gyrus was markedly decreased, suggesting that LAR indeed plays a role in development of the mammalian nervous system (Yeo *et al.*, 1997). Schaapveld *et al.* (1997) generated mice lacking the sequences encoding both LAR PTP domains by gene targeting. These $LAR^{PTP-/-}$ mice are viable and do not display an obvious neuronal phenotype. In fact, these mice develop normally, without obvious histological abnormalities. However, $LAR^{PTP-/-}$ females have mammary gland defects during gestation, due to impaired terminal differentiation of alveoli at late pregnancy, resulting in defective milk production (Schaapveld *et al.*, 1997). Since LAR-null mutant mice have not been described yet –in one case low levels of LAR expression were detected (Skarnes *et al.*, 1995; Yeo *et al.*, 1997) and in the other the extracellular domain is still expressed (Schaapveld *et al.*, 1997)– the exact role of LAR in mouse development remains to be determined.

The penetrance of the phenotype of the mice with mutations in LAR varies, depending on the genetic background. Moreover, on a C57BL/6 genetic background, only 50% of the expected $LAR^{PTP-/-}$ mice are born (W. Hendriks, personal communication), suggesting that lethal defects occur early during development in the $LAR^{PTP-/-}$ embryos that are not born. In the $LAR^{-/-}$ mice that do not show obvious defects, the highly homologous LAR-family members, RPTPdelta and RPTPsigma, may take over LAR's function. Preliminary reports suggest that $RPTPdelta^{-/-}$ mice are viable and appear to display growth retardation, early mortality, abnormal posture and dramatic motor defects (Uetani *et al.*, 1997). $RPTPsigma^{-/-}$ mice display pituitary dysplasia, defects in the olfactory lobes and a reduction in the total central nervous system size and cell number (M. Tremblay, personal communication). It is likely that the highly homologous LAR family members have redundant functions. In fact, all three family members associate with the same proteins (e.g. the LAR-interacting protein, LIP-1; Pulido *et al.*, 1995), suggesting that indeed downstream signalling is similar. In order to dissect the functions of the different members of the LAR subfamily, double or even triple knockout mice will have to be generated and analysed.

Taken together, some of the components in the LAR signalling pathway have been identified by genetics, e.g. DRac in *Drosophila* and Trio (UNC-73) in *C. elegans*, and others have been identified biochemically by direct interactions, e.g. Trio. Therefore, it is tempting to speculate that LAR-signalling is similar in invertebrates and vertebrates. However, the model in Figure 3 is far from complete, since i) LAR not only interacts with Trio, but also with other proteins, and ii) it remains to be determined whether LAR (or a LAR subfamily member) is involved in axon pathfinding in mammals.

The function of CD45 in vivo and in cell signalling

CD45 was the first RPTP to be identified, based on sequence homology with PTP1B in the intracellular domain (Charbonneau *et al.*, 1988; Tonks *et al.*, 1988a). In fact, CD45 had been cloned well before CD45 was identified as an RPTP (Thomas *et al.*, 1985). Homologues of CD45 have not been identified in invertebrates. In mammals, many splice isoforms have been identified of CD45, all of which are expressed exclusively in the haematopoietic system. The ectodomain of CD45 does not resemble cell adhesion molecules, although a single FNIII-like domain and possibly spectrin-like repeats have been identified (Fig. 2). A lectin, CD22, binds to the glycosylated CD45 ectodomain (Stamenkovic *et al.*, 1991), but whether CD22 is a *bona fide* physiological ligand is not clear.

Gene targeting of exon 6 was aimed at deletion of a specific isoform of CD45, but instead abolished expression of all CD45 isoforms in B cells and most T cells. In $CD45^{exon6-/-}$ mice, development of B cells appeared normal, but T cell development was severely affected (Kishihara *et al.*, 1993). CD45-null mice were generated by targeting of the invariant exon 9, and here T cell development was disrupted even more severely than in the $CD45^{exon6-/-}$ mice at the $CD4^{-}CD8^{-}$ to $CD4^{+}CD8^{+}$ transition and later when $CD4^{+}CD8^{+}$ cells develop into $CD4^{+}$ or $CD8^{+}$ thymocytes (Byth *et al.*, 1996).

The molecular mechanism underlying the phenotype in $CD45^{-/-}$ mice is not clear. However, by now it is well established that CD45 potentiates T cell receptor (TCR) and B cell receptor (BCR) responses. The mechanism of CD45-mediated potentiation of T and B cell receptors has been the subject of numerous studies over the past decade, which has led to relatively good insight into CD45-mediated signalling (reviewed by Frearson and Alexander, 1997). CD45

dephosphorylates the inhibitory C-terminal phosphorylation site in Lck and Fyn, thereby activating these Src-family PTKs. Upon ligation of the TCR, activated Lck and Fyn phosphorylate components of the TCR, including CD3-epsilon and TCR-zeta on Immunoreceptor Tyrosine-based Activation Motifs (ITAMs), generating binding sites for SH2-containing signalling molecules, including the PTK, ZAP-70, thereby initiating subsequent intracellular signalling (Qian and Weiss, 1997) (Fig. 4). In CD45$^{-/-}$ thymocytes, Lck and Fyn are not active and engagement of the TCR does not induce tyrosine phosphorylation of the TCR, nor any of the down-stream events, demonstrating that CD45 is essential for TCR signalling.

Structure and function of RPTPalpha

RPTPalpha is a typical RPTP with a short extracellular domain and two cytoplasmic PTP domains (Figs. 2 and 5). RPTPalpha is highly conserved in vertebrates, and homologues have been identified in human, mouse, rat, chicken and frog (Krueger *et al.*, 1990; Matthews *et al.*, 1990; Sap *et al.*, 1990; den Hertog *et al.*, 1992; Shock *et al.*, 1995; Fang *et al.*, 1996; Yang and Friesel, 1998). In addition, we have recently identified RPTPalpha in the zebrafish (A.M. van der Sar and J. den Hertog, manuscript in preparation). However, RPTPalpha homologues have not been identified in invertebrates, suggesting that RPTPalpha may be a vertebrate-specific RPTP.

In vivo, RPTPalpha is highly expressed in the developing central and peripheral nervous system of the mouse. Especially dorsal root ganglia and cranial sensory ganglia show high expression of RPTPalpha mRNA and protein during mouse development. Furthermore, RPTPalpha expression is elevated in the adrenal gland, suggesting that RPTPalpha expression is specifically elevated in derivatives of the neural crest. It appears that RPTPalpha expression is transient *in vivo*, with optimal levels of RPTPalpha expression preceding terminal differentiation of neural crest derivatives, suggesting that RPTPalpha is involved in this process (den Hertog *et al.*, 1996). High levels of RPTPalpha expression were also detected in the developing brain of rat, chicken and frog (Shock *et al.*, 1995; Fang *et al.*, 1996; Yang and Friesel, 1998). During chicken development, RPTPalpha mRNA and protein are expressed in pre-migratory and migrating granule cells, as well as in Bergmann glia and their radial processes (Fang *et al.*, 1996). Detailed analysis of RPTPalpha expression in the chicken retinotectal system demonstrates that RPTPalpha expression is restricted to Muller glia cells and radial glia of the retina and the tectum (Ledig *et al.*, 1999). Taken together, RPTPalpha is highly expressed in the developing brain of various species. Apparently, RPTPalpha is most prominently expressed in glia, suggesting that RPTPalpha is involved in controlling neuronal migration. However, the function of RPTPalpha during brain development remains to be determined. Mutant mice with targeted RPTPalpha alleles do not display obvious brain development phenotypes (J. Sap, personal communication). However, RPTPalpha$^{-/-}$ mice may have subtle brain development defects, or redundancy may annihilate brain phenotypes in RPTPalpha$^{-/-}$ mice. RPTPepsilon is very closely related to RPTPalpha (Krueger *et al.*, 1990; Elson and Leder, 1995) and therefore RPTPepsilon may take over RPTPalpha's function in RPTPalpha$^{-/-}$ mice. Mutant mice in which both RPTPalpha and RPTPepsilon have been knocked out may provide insight into the function of the type IV subfamily of RPTPs.

Even though RPTPalpha$^{-/-}$ mice do not show an obvious brain development phenotype, there is evidence that RPTPalpha plays a role in neurogenesis. For instance, RPTPalpha is transiently expressed during neuronal differentiation of several cell lines growing *in vitro*, with maximal expression levels preceding neuronal differentiation, suggesting that RPTPalpha may be involved in this process (den Hertog *et al.*, 1993). Moreover, overexpression of RPTPalpha in pluripotent P19 embryonal carcinoma cells induces an alteration in differentiation fate in favour of neuronal differentiation, suggesting that RPTPalpha indeed is involved in neuronal differentiation (den Hertog *et al.*, 1993). Overexpression of RPTPalpha in embryonic stem cells enhances neuronal differentiation, again suggesting that RPTPalpha is involved in neuronal differentiation (van Inzen *et al.*, 1996).

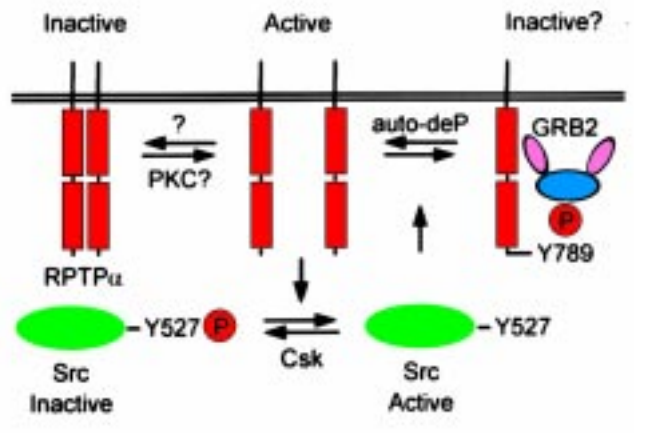

Fig. 6. Regulation of RPTPalpha. *RPTPalpha dimers are inactive (top left), and RPTPalpha is activated by monomerization through an unknown mechanism, possibly involving serine phosphorylation of RPTPalpha by protein kinase C (PKC). Even more appealing is the idea that there are ligands that induce monomerization of RPTPalpha. Presumably, somehow an equilibrium forms between monomeric and dimeric RPTPalpha. RPTPalpha monomers are active and mediate dephosphorylation of substrates. One of these substrates is Src and RPTPalpha dephosphorylates the inhibitory C-terminal P.Tyr527 in Src, thereby activating Src (Src is phosphorylated and inactivated by the C-terminal Src kinase, Csk). Active Src may, in turn, phosphorylate RPTPalpha on Tyr789, thereby providing a binding site for the SH2 domain of the SH3-SH2-SH3 adaptor protein GRB2. The GRB2 SH2 domain is blue and its flanking SH3 domains pink. The C-terminal SH3 domain of GRB2 binds (directly or indirectly) to a region close to the catalytic site in RPTPalpha-D1 and may therefore inactivate RPTPalpha catalytic activity. The process is reversible, since RPTPalpha has autodephosphorylation activity (auto-deP), rendering RPTPalpha monomeric and active again.*

The involvement of RPTPalpha in neuronal differentiation of P19 embryonal carcinoma cells is dependent on catalytic activity of RPTPalpha-D1, suggesting that RPTPalpha-D1-mediated dephosphorylation of one or more substrates accounts for the observed alteration in differentiation fate (den Hertog *et al.*, 1993). As described above, RPTPs may exert their activities through dephosphorylation of Src-family members. The PTK Src is involved in neuronal differentiation *in vivo* and *in vitro*, and we and others have identified the PTK Src as a substrate of RPTPalpha (Zheng *et al.*, 1992; den Hertog *et al.*, 1993). RPTPalpha dephosphorylates Src P.Tyr527 *in vivo* and *in vitro*, leading to activation of Src. Active Src in turn may be responsible for neuronal differentiation of P19 embryonal carcinoma cells (den Hertog *et al.*, 1993), or for transformation of Rat embryo fibroblasts (Zheng *et al.*, 1992). It is

noteworthy that Src activity is reduced in RPTPalpha$^{-/-}$ mouse embryo fibroblasts due to enhanced phosphorylation of Tyr527, strongly suggesting that RPTPalpha dephosphorylates and activates Src *in vivo* (J. Sap, personal communication).

Src-family PTKs are not the only targets of RPTPalpha. For instance, also the insulin receptor is a candidate substrate of RPTPalpha (Møller *et al.*, 1995). However, many other RPTPs may dephosphorylate the insulin receptor as well, and it remains to be determined which RPTPs dephosphorylate the insulin receptor under physiological conditions. Recently, the Kv1.2 potassium channel was identified as a substrate of RPTPalpha (Tsai *et al.*, 1999). M1 muscarinic acetylcholine receptor (m1 mAChR) activation induces tyrosine phosphorylation and suppression of Kv1.2 channel activity. RPTPalpha-mediated dephosphorylation of Kv1.2 channels, in turn, leads to desuppression and thus to an increase in the Kv1.2 resting current. It is noteworthy that overexpression of RPTPalpha does not induce massive changes in cellular P.Tyr levels in P19 embryonal carcinoma cells or NIH 3T3 cells (A. Buist and J. den Hertog, unpublished observations), suggesting that RPTPalpha specifically dephosphorylates substrates. Identification of additional physiological RPTPalpha substrates is a challenge, and will provide crucial insights into the function of RPTPalpha.

The fact that overexpression of RPTPalpha does not induce massive dephosphorylation of cellular P.Tyr-containing proteins may also be explained by tight negative regulation of RPTPalpha activity. Little is known about regulation of RPTPalpha activity. The extracellular domain of RPTPalpha is relatively short (123 residues in the mouse), suggesting that it may not act as a ligand binding domain. However, the extracellular domain is heavily glycosylated through N- and O-linked glycosylation (Fig. 5) (Daum *et al.*, 1994), suggesting that the extracellular domain of RPTPalpha may bind lectins. However, to date, such interactions have not been demonstrated yet.

Phorbol ester-treatment of cells transiently induces serine phosphorylation of RPTPalpha, which is concomitant with a transient increase in RPTPalpha activity. In fact, the increase in RPTPalpha activity is due to enhanced serine phosphorylation of RPTPalpha, since dephosphorylation of RPTPalpha reduces its activity to prestimulation levels (den Hertog *et al.*, 1995). The serine phosphorylation sites are localised at Ser180 and Ser204 in the juxtamembrane region of RPTPalpha (Fig. 5), and protein kinase C (PKC) phosphorylates these sites *in vitro* (Tracy *et al.*, 1995), suggesting that PKC is a direct regulator of RPTPalpha. Ser180 and especially Ser204 is located close to the helix-turn-helix wedge structure in RPTPalpha that may be involved in dimerisation. Phosphorylation of these sites introduces a negative charge that may prohibit dimerisation, thereby leading to activation of RPTPalpha (Bilwes *et al.*, 1996).

RPTPalpha from growing NIH 3T3 cells is not only constitutively phosphorylated on serine, but also on tyrosine. The tyrosine phosphorylation site was mapped to the C-terminus of RPTPalpha, at position 789 (Fig. 5) (den Hertog *et al.*, 1994). The kinase that is responsible for RPTPalpha Tyr789 phosphorylation may be Src. P.Tyr789 is a consensus binding site for the SH2 domain of the SH3-SH2-SH3 adaptor protein GRB2 and we and others have demonstrated that GRB2 binds to RPTPalpha *in vivo* and *in vitro* (den Hertog *et al.*, 1994; Su *et al.*, 1994). The guanine nucleotide exchange factor for Ras, Sos, which is bound to the N-terminal SH3 domain of GRB2, thereby linking receptor PTKs to the Ras pathway (reviewed by Schlessinger, 1993), is not present in the GRB2-RPTPalpha complex. Not only the GRB2 SH2 domain, but also the C-terminal SH3 domain is required for efficient binding to RPTPalpha (den Hertog and Hunter, 1996; Su *et al.*, 1996), suggesting that steric hindrance prohibits binding of Sos to the RPTPalpha-GRB2 complex. The region in RPTPalpha that is involved in GRB2-SH3 binding is localised in RPTPalpha-D1, close to the catalytic site, suggesting that GRB2-SH3 binding may inhibit RPTPalpha activity (den Hertog and Hunter, 1996). Overexpression of mutant RPTPalpha-Y789F, that does not bind GRB2, in P19 embryonal carcinoma cells altered the differentiation fate of these cells, like wild type RPTPalpha, demonstrating that the interaction between RPTPalpha and GRB2 is not required for this effect of RPTPalpha (J. den Hertog, unpublished observation). Taken together, the function of GRB2 binding to RPTPalpha remains to be determined.

In recent years, we have obtained insight into different mechanisms that may regulate RPTPalpha catalytic activity, as summarised in Figure 6. In addition, the expression pattern *in vivo* and the functional analyses in *in vitro* cell systems suggest that RPTPalpha may be involved in terminal differentiation of neural crest derivatives. However, elucidation of the biological function of RPTPalpha requires understanding of the regulation of RPTPalpha catalytic activity, and identification of specific, physiological substrates and effector proteins. Moreover, elucidation of the role of RPTPalpha in vertebrate development requires thorough analysis of vertebrate embryos that overexpress (mutant) RPTPalpha, and of homozygous RPTPalpha knock-out embryos, and presumably also double or multiple RPTP knock-out embryos. Current work in our lab is directed at these questions.

Conclusion

We are beginning to obtain insight into the function of RPTPs in development *in vivo*. Several RPTPs have been demonstrated to be essential for proper axon pathfinding in *Drosophila*. In addition, at least one RPTP (Clr-1) is essential for normal development of *C.elegans*. Whether the homologues of these RPTPs play similar roles in vertebrates remains to be determined. Analysis of the function of RPTPs in vertebrates in general may require the generation of mutant mice with multiple targeted RPTP genes, since many single knock-outs did not display a phenotype, presumably due to redundancy. The powerful combination of genetics and biochemistry has already led to important new insights into the role of RPTP-mediated signalling in development, (e.g. LAR-signalling, Fig. 3), and presumably will provide conclusive answers as to the function of other RPTPs in the near future as well.

Note added in proof: RPTPalpha and RPTPsigma knock-out mice have now been reported:

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