The Cell Surface Receptor Tartan Is a Potential In Vivo Substrate for the Receptor Tyrosine Phosphatase Ptp52F

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Receptor-linked protein-tyrosine phosphatases (RPTPs) are essential regulators of axon guidance and synaptogenesis in Drosophila, but the signaling pathways in which they function are poorly defined. We identified the cell surface receptor Tartan (Trn) as a candidate substrate for the neuronal RPTP Ptp52F by using a modified two-hybrid screen with a substrate-trapping mutant of Ptp52F as “bait.” Trn can bind to the Ptp52F substrate-trapping mutant in transfected Drosophila S2 cells if v-Src kinase, which phosphorylates Trn, is also expressed. Coexpression of wild-type Ptp52F causes dephosphorylation of v-Src-phosphorylated Trn. To examine the specificity of the interaction in vitro, we incubated Ptp52F-glutathione S-transferase (GST) fusion proteins with pervanadate-treated S2 cell lysates. Wild-type Ptp52F dephosphorylated Trn, as well as most other bands in the lysate. GST “pull-down” experiments demonstrated that the Ptp52F substrate-trapping mutant binds exclusively to phospho-Trn. Wild-type Ptp52F pulled down dephosphorylated Trn, suggesting that it forms a stable Ptp52F-Trn complex that persists after substrate dephosphorylation. To evaluate whether Trn and Ptp52F are part of the same pathway in vivo, we examined motor axon guidance in mutant embryos. trn and Ptp52F mutations produce identical phenotypes affecting the SNa motor neuron. The genes also display dosage-dependent interactions, suggesting that Ptp52F regulates Trn signaling in SNa motor neurons.

Receptor-linked protein-tyrosine phosphatases (RPTPs) are enzymes with extracellular (XC) domains, a single transmembrane domain, and one or two cytoplasmic protein tyrosine phosphatase (PTP) homology domains. Many RPTPs have XC sequences that resemble those of cell adhesion molecules (for a review, see reference 33). This sequence organization suggests that RPTPs can couple cell-cell recognition events to dephosphorylation of cytoplasmic substrates. Interestingly, while phosphotyrosine (PY) pathways involved in cell growth and differentiation typically involve receptor tyrosine kinases that bind to growth factors and are regulated by nontransmembrane PTPs, those that control axon guidance often use RPTPs and nontransmembrane TKs. This implies that the cues that affect PY signaling in axonal growth cones may interact with RPTPs rather than with receptor tyrosine kinases (reviewed in reference 14).

There are 17 active RPTPs encoded in the human genome, while Drosophila has six. Most of the mammalian RPTPs are expressed in nonneural tissues, but four of the six fly RPTPs are expressed only by central nervous system (CNS) neurons in late embryos. All published zygotic phenotypes produced by Rptp mutations are alterations in axon guidance or synaptogenesis. These results suggest that the major functions of the Drosophila RPTPs are in neural development (for a review, see reference 16). Analysis of axon guidance phenotypes in embryos bearing single or multiple Rptp mutations is consistent with the idea that RPTP interactions with ligands at growth cone choice points convey “information,” in the form of changes in substrate phosphorylation within growth cones, that is used to determine pathway decisions.

In the Drosophila neuromuscular system, 36 motor axons grow out within six nerve bundles in each abdominal hemisegment, and each axonal growth cone makes a series of genetically determined guidance decisions that direct it to the appropriate muscle fiber (for a review, see reference 27). Our work on Rptp mutant combinations suggests that each pathway decision uses a specific subset of the six RPTPs. RPTPs can exhibit functional redundancy, so that the loss of one does not produce a defect unless another RPTP is also absent, or competition, in which loss of one RPTP suppresses the phenotype produced by loss of another (5, 6, 31). Examination of RPTP expression patterns suggests that the RPTPs are expressed by most (or possibly all) CNS neurons, including motor neurons. If so, the requirements for individual RPTPs for execution of particular guidance decisions cannot be due to selective expression of these RPTPs on specific motor axons. These requirements might instead be determined by the expression patterns of RPTP ligands, so that only RPTPs whose ligands were localized to the vicinity of a growth cone choice point would participate in that pathway decision. Alternatively (or in addition), the necessity of a particular RPTP for a pathway decision might arise from selective expression of RPTP substrates, so that an RPTP would be important for guidance decisions made by a growth cone of a specific motor neuron only if that neuron expressed the relevant substrate(s). Evaluation of such models requires identification of specific XC ligands and intracellular substrates for the Drosophila RPTPs. Only one set of ligands has been identified thus far.
These are the heparan sulfate proteoglycans Syndecan (Sdc) and Dallylike (Dlp), which bind to the Lar RPTP with nanomolar affinity and contribute to its functions in axon guidance and synapse growth (9, 15). Similarly, little is known about substrate specificity in vivo. Lar can dephosphorylate the Enabled (Ena) protein, which regulates the growth cone cytoskeleton, and genetic interaction studies suggest that Ena may be an in vivo substrate for Lar (35). The transmembrane protein gp150 can be dephosphorylated by Ptp10D in cell culture and intact fly larvae, but genetics has not provided evidence that Ptp10D and gp150 are in the same signaling pathway in vivo (7).

The identification of in vivo substrates for RPTPs has been hampered by the fact that purified RPTP cytoplasmic domains often do not exhibit high selectivity in vitro when tested for dephosphorylation activity on peptides or proteins. The most fruitful method for finding substrates for both RPTPs and cytoplasmic PTPs has been the use of “substrate-trapping” mutants. The most effective substrate traps were devised by Tonks and coworkers, and are created by changing an invariant D to an A in these vectors. For Pts52F, this is D1258 (numbered in the context of the entire 1,433-amino-acid [aa] preprotein). The activated c-Src construct in BTM116 (bearing a TRP1 marker) was a gift from Kathy Keegan. The library was a 0 to 24 h CDNA library made in the ACT (LEU2) vector and was a gift from Stephen Elledge. The insert size range is 0.5 to 6 kb, with an average insert size of 1.8 kb. The strain used for selection are described in reference 13 and were a gift from P. James. Yeast cells were transformed by using standard lithium acetate protocols. For the screen, “bait” cells, bearing the substrate-trap PTP vector and the c-Src vector, and “prey” cells, bearing the library, were grown up in selection medium, and equal numbers of bait and prey cells were mixed for mating and grown up for a few hours. The mated cells were plated onto − Ade plates and incubated at 30°C for 7 days. Positive colonies were patched onto − Ade plates and then tested for activation of the HIS3 and LacZ reporters. Colonies that activated all of the reporters were tested for bait dependence by using 5-fluoroorotic acid to remove the URA3 bait plasmid. They were tested for kinase dependence by replica plating onto +Trp and − Trp plates, followed by examination of whether the colonies lacking the c-Src vector were still ADE+. Bait-dependent plasmids were sequenced and then retransformed into yeast, together with each substrate trap or wild-type PTP bait, with or without c-Src (see Table 1 for results).

Plasmid construction. We subcloned the Pts52F cytoplasmic domain into a modified version of the metallothionein (MT) promoter vector pRMHa3 (2) that includes an ATG and Src myristylation sequence, followed by restriction sites. We added a 9xMyc, His8 tag from the HTM53H plasmid (a gift from Ray Comer. The v-Src plasmid used in Fig. 2 and 3 was made by cloning the entire coding region of v-Src (from Tony Hunter) into the pRMHa3 vector. Details concerning cloning strategies (enzyme sites, primers, etc.) are available on request.

MATERIALS AND METHODS

Yeast two-hybrid screening. The cytoplasmic domains of the RPTPs were cloned in frame into a GAL4-DBD vector, pGBKDCU2, a gift from Philip James; this bears the URA3 selectable marker. We made point mutations to change the invariant D to an A in these vectors. For Pts52F, this is D1258 (numbered in the context of the entire 1,433-amino-acid [aa] preprotein). The activated c-Src construct in BTM116 (bearing a TRP1 marker) was a gift from Kathy Keegan. The library was a 0 to 24 h CDNA library made in the ACT (LEU2) vector and was a gift from Stephen Elledge. The insert size range is 0.5 to 6 kb, with an average insert size of 1.8 kb. The strain used for selection are described in reference 13 and were a gift from P. James. Yeast cells were transformed by using standard lithium acetate protocols. For the screen, “bait” cells, bearing the substrate-trap PTP vector and the c-Src vector, and “prey” cells, bearing the library, were grown up in selection medium, and equal numbers of bait and prey cells were mixed for mating and grown up for a few hours. The mated cells were plated onto − Ade plates and incubated at 30°C for 7 days. Positive colonies were patched onto − Ade plates and then tested for activation of the HIS3 and LacZ reporters. Colonies that activated all of the reporters were tested for bait dependence by using 5-fluoroorotic acid to remove the URA3 bait plasmid. They were tested for kinase dependence by replica plating onto +Trp and − Trp plates, followed by examination of whether the colonies lacking the c-Src vector were still ADE+. Bait-dependent plasmids were sequenced and then retransformed into yeast, together with each substrate trap or wild-type PTP bait, with or without c-Src (see Table 1 for results).

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S2 cell transfection. S2 cells were maintained in Schneider’s medium supplemented with 0.1% penicillin-streptomycin, 25 μg of amphotericin B/ml, and 10% heat-inactivated fetal bovine serum. Cells were grown at 25°C under standard conditions and were transiently transfected by calcium phosphate-mediated DNA transfer. Briefly, 3 × 10^6 cells per 60-mm plate were seeded in serum-only medium and expanded overnight till the cells reached 2 × 10^7 to 4 × 10^7 cells/ml. The cells were then transfected for 15 to 18 h by the addition of 600
µl of DNA-calcium phosphate coprecipitate mix (which included 3 µg of each plasmid DNA). For the findings shown in Fig. 3, experiments that examined the association of Trm with the Ptp52F trap, we cotransfected 3 µg each of Ptp52F-trap-Myc, the v-Src plasmid, and the Trm–FL, Trm–cyto-GFP, or Trm–FL–GFP plasmid. For the experiment to examine dephosphorylation of Trm–FL–GFP by Ptp52F, the Ptp52F–wild-type–Myc plasmid was substituted for the trap plasmid. For the experiment to examine dephosphorylation of Trm–FL–GFP by Ptp52F, the Ptp52F–wild-type–Myc plasmid was substituted for the trap plasmid.

**Cell lysis and immunoprecipitation.** Cells were washed with cold phosphate-buffered saline (PBS) and lysed in ice-cold lysis buffer (125 mM NaCl, 100 mM Tris–Cl [pH 7.4], 0.2% Triton X-100, 1 mM EDTA, 600 mM phenylmethylsulfonyl fluoride, 1 µg of protease inhibitor cocktail/ml, 10% glycerol). Lysates were centrifuged at 35,000 × g for 20 min and subjected to immuno precipitation. Lysates (200-µl volume) were used for the generation of the tagged proteins, and both proteins were phosphorylated in pervanadate-treated cells. Therefore, for Fig. 1, we discovered that phosphorylation was effective to be on the FLAG tag, which contains a Y, because a CG15028–FLAG phosphorylation is also due to the FLAG tag.

**RESULTS**

Identification of candidate RPTP substrates using a modified yeast two-hybrid screen. To search for substrates for the Drosophila RPTPs, we used methods similar to those devised by Keegan and Cooper (18) and by Noda and coworkers (10, 17). We cloned cytoplasmic domain sequences from four RPTPs into a yeast two-hybrid expression vector, inserting them in frame with the LexA DNA-binding domain (DBD). The four RPTPs are Ptp10D, Ptp52F, Ptp69D, and Ptp99A. We introduced substrate-trapping (DΔA) mutations into each of these RPTP sequences. Ptp10D and Ptp52F have only one PTP homology domain, while Ptp69D and Ptp99A have two. However, the second PTP homology domain in Ptp99A has a D in place of the essential C residue, so it is unlikely to have enzymatic activity. For Ptp69D, it is unknown whether the second PTP homology domain is active, so we made a construct with D→A mutations in both domains.

To perform the screen, we introduced a plasmid encoding a version of chicken c-Src bearing three activating mutations (18), together with the RPTP-DBD plasmids and a cDNA-activation domain (AD) fusion library, and selected for ADE+ yeast bearing all three types of plasmids (13). Constitutive expression of v-Src, which has additional activating mutations, is not tolerated well by yeast, while this version of c-Src can be expressed without adversely affecting growth.

To screen these candidates, we first tested them for His+ and LacZ−, the other two selectable markers in the strain (13). We removed the URA3 (RPTP-DBD) “bait” plasmid and discarded candidates that were still ADE+ in the absence of the bait. The bait-dependent clones we obtained are listed in Table 1. For each of these, we determined whether the ADE−
phenotype required c-Src expression. Four clones that passed this test were identified, each encoding a different protein. Three were identified by screening with Ptp52F/c-Src, and one was identified by screening with Ptp10D/c-Src.

Each of these putative substrate clones was then reintroduced into yeast, together with each RPTP bait plasmid, with or without the c-Src plasmid. We also tested the four classes of non-Src-dependent clones in the same manner. Table 1 shows that the four clones identified with Ptp52F/c-Src are ADE′ only with this bait, while the Xmas-2 clone identified with Ptp10D/cSrc also interacts with Ptp52F/c-Src. Figure 1 shows plates with streaked yeast colonies harboring one Ptp52F/c-Src-selected clone encoding a fragment of Trn. Yeast expressing Trn-AD grew on plates lacking Ade only when they also contained the Ptp52F-trap-DBD and c-Src plasmids.

Finally, we examined whether selection of these four putative Ptp52F substrate clones requires the substrate-trapping mutation by transforming them into yeast together with c-Src and a plasmid encoding the wild-type Ptp52F cytoplasmic domain fused to the DBD. None of the clones generated the ADE′ phenotype with wild-type Ptp52F. In summary, these data show that four Drosophila sequences, encoding portions of the Trn, Xmas-2, CG15022, and CG10283 proteins, selectively interact with the substrate-trapping mutant version of the RPTP in the presence of c-Src and therefore behave like substrates in this yeast assay.

Phosphorylation of Trn in cultured Drosophila cells. We then wanted to determine whether the candidates identified in the yeast screen could exhibit phosphorylation-dependent interactions with RPTP substrate-trapping mutants in transfected Drosophila S2 cells. Such a demonstration would provide a rigorous test of whether interactions between the candidate substrates and the substrate-trapping mutant can occur when both proteins are present within cells from the species of origin. In a recent review of PTP-substrate interactions, substrate-trapping within cotransfected cells is defined as the most selective criterion for establishing that a candidate protein is a bona fide substrate (32).

We present here the S2 transfection results for Trn. The data for the other three candidates are in Materials and Methods.

Phosphorylated Trn binds to the Ptp52F substrate-trapping mutant in S2 cells. Having demonstrated that the Trn cyto-
plasmic domain can be phosphorylated in S2 cells, we conducted cotransfection experiments to determine whether the Ptp52F substrate-trapping mutant could bind to v-Src-phosphorylated Trn. This experiment cannot be done in pervanadate-treated cells, because vanadate binds to the active sites of trap mutants and blocks their association with substrates.

Ptp52F is not endogenously expressed in S2 cells, so interactions between Trn and Ptp52F can only be studied in cells transfected with Ptp52F constructs. The predicted Ptp52F pre-protein is 1,433 aa in length, and its cytoplasmic domain is 369 aa. Full-length Ptp52F cDNAs have never been isolated, and we had to use reverse transcription-PCR to define the complete sequence of its XC domain (29). Because of this, we decided to express the Ptp52F cytoplasmic domain with a myristylation sequence in S2 cells, so that it would be likely to associate with the plasma membrane and might therefore come into contact with Trn. We made wild-type and substrate-trapping mutant constructs with N-terminal Src myristylation sequences and 9xMyc tags at the C terminus. When these were transfected into S2 cells, a single band of ~65 kDa was observed in lysates and anti-Myc IPs, which is consistent with the predicted molecular mass of the fusion protein (Fig. 3A).

Because we had demonstrated that Trn-cyto-GFP could be phosphorylated in S2 cells, we cotransfected Ptp52F-trap-Myc, Trn-cyto-GFP, and v-Src constructs in our initial experiments but did not find any evidence for association. This could have been due to insufficient localization of the Ptp52F and Trn proteins to the same compartment, to occlusion of trap binding to the cytoplasmic domain by GFP, or to insufficiently high expression of one of the proteins. To address these issues, we then made a construct (Trn-FL) with untagged full-length Trn. When transfected into S2 cells, this resulted in expression of wild-type Trn protein (~85 kDa) at levels severalfold greater than those seen for endogenous Trn in untransfected cells (data not shown). When we cotransfected Ptp52F-trap-Myc and Trn-FL constructs into S2 cells together with v-Src, we were able to detect Trn in Myc IPs from these cells (Fig. 3B, lane 2). This Trn band was much weaker in Myc IPs from Ptp52F-wild type-Myc-cotransfected cells (Fig. 3B, lane 5). It can be clearly visualized upon longer exposure, however, showing that Ptp52F-wild type-Myc can also form a complex with Trn, albeit with a lower efficiency than the substrate-trapping mutant.

To further examine association between Ptp52F and Trn, we needed to make a tagged Trn fusion protein that could bind to the Ptp52F substrate-trap, so that we could distinguish this protein from endogenous Trn. We thus made a construct in which GFP was attached to the C terminus of full-length Trn. When a plasmid bearing this construct, Trn-FL-GFP, was transfected, we observed a band of ~100 kDa that could be precipitated by either anti-GFP or anti-Trn antibodies (Fig. 3C). Interestingly, we also saw a second band of ~60 kDa that reacted with both antibodies. This appears to be a cleavage product, and given its size and the fact that it must contain GFP, we infer that the XC domain can be cleaved near the plasma membrane.

To study dephosphorylation of Trn-FL-GFP, we cotransfected it together with Ptp52F-wild type-Myc or Ptp52F-trap-
Myc and v-Src. Trn-FL-GFP and the cleavage product were both tyrosine phosphorylated when cotransfected with the Ptp52F trap and Src (Fig. 3D, lane 1). This was not due to protection from endogenous PTP activity by trap binding, because phosphorylation was detected at the same levels when Trn-FL-GFP and v-Src were cotransfected without the trap (data not shown). This is consistent with the observation that only a small fraction of Trn is bound by the trap (Fig. 3B). When wild-type Ptp52F was expressed together with Trn-FL-GFP and the kinase, tyrosine-phosphorylated Trn and its cleavage product were barely detectable (Fig. 3D, lane 2). These data show that Ptp52F can cause dephosphorylation of the Trn cytoplasmic domain.

To examine whether Ptp52F-trap-Myc could bind to Trn-FL-GFP, we immunoprecipitated the Trn protein with anti-GFP and then detected the Ptp52F protein by immunoblotting with anti-Myc. We observed a clear signal when Ptp52F-trap-Myc was cotransfected with both v-Src and Trn-FL-GFP (Fig. 3E, lane 5), but no association was detected when the kinase construct was omitted. These data show that the substrate-trapping mutant binds to Trn in a phosphorylation-dependent manner. With wild-type Ptp52F, a very faint band was observed in Myc immunoblots of anti-GFP IPs (Fig. 3E, lane 11), indicating that there was much less association between the wild-type RPTP and Trn. This band is clearly visible with longer exposure, however, demonstrating that the wild-type RPTP does form a complex with Trn-FL-GFP in cotransfected cells.

When lysates were immunoprecipitated with anti-Myc, we were able to observe the association of Trn-FL-GFP with Ptp52F-trap-Myc by immunoblotting with anti-PY (Fig. 3F), and detected the Ptp52F protein by immunoblotting with anti-Myc. We observed a clear signal when Ptp52F-trap-Myc was cotransfected with both v-Src and Trn-FL-GFP (Fig. 3E, lane 5), but no association was detected when the kinase construct was omitted. These data show that the substrate-trapping mutant binds to Trn in a phosphorylation-dependent manner. With wild-type Ptp52F, a very faint band was observed in Myc immunoblots of anti-GFP IPs (Fig. 3E, lane 11), indicating that there was much less association between the wild-type RPTP and Trn. This band is clearly visible with longer exposure, however, demonstrating that the wild-type RPTP does form a complex with Trn-FL-GFP in cotransfected cells.

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Selective in vitro binding of Ptp52F and Trn. The results described above demonstrate that the Ptp52F substrate-trapping mutant can bind to Trn in S2 cells and that wild-type Ptp52F can cause dephosphorylation of Trn. However, these data do not show that Trn is the only protein in S2 cells that binds to the Ptp52F trap. To address selectivity, it is necessary to examine binding to the entire spectrum of proteins that are tyrosine phosphorylated when cells are treated with pervanadate. This cannot be done by cotransfection, since vanadate occludes binding by PTP substrate-trapping mutants.

Many other investigators (see, for example, reference 11) have performed experiments in which they assessed binding of PTP substrate-trapping mutants purified as GST fusion proteins from *Escherichia coli* to proteins in a lysate from cells that were treated with pervanadate, followed by inactivation of the pervanadate with iodoacetate and dithiothreitol. We used this approach for Ptp52F, purifying substrate-trapping mutant and wild-type GST fusion proteins from *E. coli* lysates.

We first examined dephosphorylation by the wild-type Ptp52F fusion protein. To do this, we added various amounts of Ptp52F-wild type-GST to pervanadate-treated S2 cell lysates, followed by incubation for 1 h at 20°C before fractionating the samples on a gel and immunoblotting them with anti-PY. Figure 4A shows that all of the bands in the lysate were reduced in intensity as the amount of Ptp52F-wild type-GST was increased. It is not obvious that any bands were reduced in intensity when 150 nM). This amount of Ptp52F-wild type-GST produces no detectable effect when the entire lysate is analyzed. However, since there are so many PY bands in a pervanadate-treated S2 cell lysate that individual bands cannot be resolved (e.g., lane 1 of Fig. 4A, and lanes 1 and 4 of Fig. 4D), we would not be able to detect 50% dephosphorylation of other proteins (data not shown).

To address the selectivity of Ptp52F trap binding, we incubated various amounts of Ptp52F-trap-GST with pervanadate-treated S2 cell lysates and precipitated protein bound to the trap using glutathione-agarose beads. We then analyzed bead-bound proteins by blotting with anti-PY as in Fig. 4A. This produced a remarkable result: only a single prominent phosphoprotein band, of ~85 kDa, was precipitated by the substrate-trapping mutant (Fig. 4C). The migration of this band was consistent with that of endogenous Trn, and it was recognized by anti-Trn antibody (Fig. 4E, lane 1).

Although the cells used for the experiment of Fig. 4C were transfected with Trn-cyto-GFP plasmid, no band corresponding to Trn-cyto-GFP was detectable on the blot. We also could not detect Trn-cyto-GFP or Trn-FL-GFP binding to Ptp52F-trap-GST when glutathione-agarose precipitates were analyzed by immunoblotting with anti-GFP. Perhaps the GFP and GST domains of the fusion proteins sterically interfere with each other to prevent binding.

To further evaluate the interaction between Ptp52F-GST and Trn, we then incubated 10 μg of Ptp52F-trap-GST or Ptp52F-wild type-GST with pervanadate-treated lysates from cells transfected with untreated Trn-FL plasmids (or left untransfected) and detected bound phosphoproteins by blotting the cells with anti-PY. Lysates from these cells are shown in lanes 1 and 4 of Fig. 4D. As in Fig. 4C, we observed that Ptp52F-trap-GST precipitated only a single ~85-kDa band from the lysates and that the intensity of this band was increased when the cells had been transfected with Trn-FL, a finding consistent with the identification of this band as phospho-Trn (Fig. 4D, lanes 2 and 5).

When Ptp52F-wild type-GST was used for the pulldown, no band was detected on the anti-PY blot. Interestingly, however, when pulldowns from Trn-FL-transfected cells were blotted with anti-Trn, equal amounts of Trn were observed to be precipitated by the wild-type and substrate-trapping mutant proteins (Fig. 4E). These data show that the wild-type Ptp52F protein can also form a stable complex with Trn that persists after dephosphorylation. The stability of this complex, or the affinity of Trn and Ptp52F for each other, may be lower for the wild-type than for the substrate-trapping mutant, since much more Trn associated with the substrate-trapping mutant in cotransfected S2 cells (Fig. 3B, lane 2 versus lane 5; Fig. 3E, lane 5 versus lane 11). However, the vast excess of wild-type Ptp52F used in pulldown experiments is apparently capable of bringing down the same amount of Trn even if its affinity for dephosphorylated Trn is lower than that of the substrate-trapping mutant for phosphorylated Trn.

Genetic interactions between Ptp52F and *tartan*. The results described above show that Trn is a preferred substrate for Ptp52F in S2 cells. It would be difficult to use biochemical techniques to determine whether this is also the case in vivo, because Trn is not an abundant protein, and it is unlikely to be heavily tyrosine phosphorylated during normal development. However, we can examine whether Ptp52F and Trn are involved in the same developmental processes by using genetics.

Ptp52F, like other *Drosophila* RPTPs, is selectively expressed in CNS neurons and is a regulator of axon guidance. When we identified the Ptp52F gene and isolated mutations in it, we discovered that Ptp52F mutant embryos have two strong axon guidance phenotypes. The longitudinal tracts in the CNS
are disorganized and incomplete, and one branch of the motor axon network, the SNa nerve, exhibits guidance errors (29).

During embryonic development, the 36 motor axons in each abdominal hemisegment grow out from the CNS and segregate into six major branches, called SNa, SNc, ISN, ISNb, ISNd, and TN, which innervate the 30 body wall muscle fibers. Motor axons can be selectively visualized by staining dissected embryos with MAb 1D4 (34). Single mutations or combinations of mutations in the six Rptp genes affect the guidance of all motor axon branches (see reference 14 for a recent summary). Ptp52F is the only Rptp gene for which single mutations affect the SNa branch. In Ptp52F-null mutants, >40% of SNa nerves exhibit guidance errors (Fig. 5F), while 22 to 27% have phenotypes in hypomorphic (weaker) mutants. These are the only high-penetrance motor axon phenotypes seen in Ptp52F single mutants. There are also some ISN phenotypes (16%), but only rare ISNb defects (7%) (29).

The SNa grows out from the CNS in the SN root and bifurcates dorsal to muscle 12. Its posterior (or ventral) branch innervates muscles 5 and 8, while its anterior (or dorsal) branch innervates muscles 22 to 24 (Fig. 5A and E). The most common SNa guidance error observed in Ptp52F mutants is a failure to bifurcate, so
FIG. 5. *Ptp52F* and *tartan* mutants have the same motor axon guidance phenotypes, and the genes display a dosage-dependent interaction. Motor axons (brown) in late-stage 16/early-stage 17 embryo “fillets” were stained with MAb 1D4, using HRP immunohistochemistry for visualization, and photographed using differential interference contrast optics. (A) Two hemisegments in a wild-type (wt) control embryo. SNa, the branch that exhibits phenotypes in both of these mutants, is labeled. Its bifurcation point is indicated by white arrows in both segments. The ISN and ISNb (out of focus) are also labeled. Muscle fibers are labeled by number (compare to diagram of panel E). The anterior SNa branch normally extends dorsally along muscle 22 and then across muscle 23 to reach muscle 24, and the posterior branch extends across muscle 5 to reach muscle 8. (B) Two hemisegments in a *Ptp52F/+ , trn/+* embryo (lacking one wild-type copy of each gene). The anterior branch of the SNa is missing in both segments. The approximate points at which bifurcation would have occurred if the anterior branches were present are indicated by asterisks. (C) Three hemisegments in a *Ptp52F/Ptp52F* embryo. The anterior branch of the SNa is missing, truncated, or misguided in all three. In the left-hand hemisegment the branch is missing, while in the middle the branch extends slightly beyond the bifurcation point (asterisk) but then grows posteriorly and rejoin the posterior SNa branch, forming a loop (arrowhead). In the right-hand hemisegment a single axon appears to have extended partway along the normal anterior branch pathway (arrowhead). (D) Two hemisegments in a *trn/trn* embryo. In the right-hand segment, the anterior branch is missing, while in the left-hand hemisegment a single axon appears to have extended partway along the normal anterior branch pathway (arrowhead). (E) Diagram of the SNa and adjacent muscle fibers in wild-type. The muscles are indicated as semitransparent to show their layering. The deepest (most external) muscles are 21 to 24, and they are overlaid by muscles 5, 12, 13, and 8. SNa extends underneath (external to) 12 and 13. (F) Bar graph of phenotypic penetrance for SNa guidance errors, in control (*balancer/+); *trn/+; Ptp52F/+; Ptp52F/+ , trn/+; trn/trn*; and *Ptp52F/Ptp52F* cells. The numbers of hemisegments examined and the distribution among phenotypic classes are shown in Table 2.
that one branch is missing. The anterior branch is lost more frequently than the posterior branch (Fig. 5C).

Trn is expressed in CNS neurons but also in a variety of other cell types (3). Motor axon phenotypes had not been previously analyzed in trn mutants. When we examined trn mutant embryos by staining with MAb 1D4, we made the remarkable discovery that they have SNa phenotypes that are identical to those of remarkable discovery that they have SNa phenotypes that are mutant embryos by staining with MAb 1D4, we made the

### TABLE 2. Quantitation of SNa phenotypes

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<th>Genotype</th>
<th>No. of hemisegments (A2 to A6) scored</th>
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<td>Ptp52F/+</td>
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<td>Ptp52F/+; trn/+</td>
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<tr>
<td>trn/trn</td>
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<td>30</td>
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<td>Ptp52F/Ptp52F</td>
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<td>42</td>
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</table>

DISCUSSION

In this study, we present evidence that the cell surface receptor Trn is a substrate for Ptp52F. Trn was identified in a yeast screen for phosphoproteins that bind selectively to a Ptp52F substrate-trapping mutant (Table 1 and Fig. 1). We showed that Trn can be phosphorylated on tyrosine in S2 cells (Fig. 2) and that phosphorylated Trn binds selectively to the substrate-trapping mutant of Ptp52F when it is coexpressed with Trn and the v-Src kinase (Fig. 3B, E, and F). Wild-type Ptp52F causes dephosphorylation of v-Src-phosphorylated Trn in S2 cells (Fig. 3D). A purified Ptp52F–wild-type–GST fusion protein can dephosphorylate Trn in a pervanadate-treated S2 cell lysate, but it also dephosphorylates many other proteins in the lysate. However, a Ptp52F-trap-GST fusion protein binds to only one phosphoprotein in pervanadate-treated S2 lysates, and we identified this protein as Trn (Fig. 4C to E). Ptp52F–wild-type–GST forms a complex with Trn that persists after dephosphorylation (Fig. 4D and E). These data demonstrate that the Ptp52F substrate-trapping mutant has a strong specificity for Trn binding. Our results fulfill all three of the criteria listed by Tiganis and Bennett (32) as necessary for the rigorous definition of a protein as a PTP substrate: (i) direct interaction with the PTP substrate-trapping mutant in transfected cells, (ii) modulation of cellular substrate tyrosine phosphorylation by the PTP in transfected cells, and (iii) in vitro dephosphorylation of substrate by the PTP.

Our genetic results (Fig. 5) are consistent with a model in which Trn signaling in SNa motor neurons is necessary for correct axon pathfinding at the SNa bifurcation point, and Ptp52F regulates Trn via dephosphorylation. In trn and Ptp52F mutants, SNa axons destined for muscles 22 to 24 sometimes fail to separate from those destined for muscles 5 and 8. This result in a phenotype in which the anterior branch of the SNa is missing. In SNa growth cones, signaling through Trn and dephosphorylation of Trn by Ptp52F might be regulated by the interactions of these two receptors with unknown ligands on cells near the SNa bifurcation point. Activation of Ptp52F might involve a secreted protein called Folded gastrulation (Fog), which is expressed in this vicinity. Fog is a positive regulator of Ptp52F function in SNa neurons (26).

Trn is also important for ISNb axon guidance (22) and is involved in many other developmental processes, including tracheal development and the sorting of cells within imaginal discs and developing appendages (21, 23, 24, 28). Trn signaling in ISNb neurons and trachea may be independent of Ptp52F, since trn and Ptp52F do not share ISNb or tracheal phenotypes.

How does Ptp52F regulate Trn signaling in neurons? One might have expected that Ptp52F would be a negative regulator of Trn, because dephosphorylation of Trn would prevent it...
from binding to SH2-domain downstream signaling proteins. Several Y residues in the Trn cytoplasmic domain are in sequence contexts that suggest that they could bind to SH2 domains if they were phosphorylated. In this model, however, mutation of Ptp52F should lead to an increase rather than a decrease in Trn signaling. We do not know what consequences might result from increasing Trn signaling, since overexpression of Trn in neurons does not produce phenotypes (data not shown). However, the fact that the trn and Ptp52F loss-of-function phenotypes are the same and that the two genes interact in a dosage-dependent manner suggests that Trn signaling is reduced in the absence of Ptp52F and thus that Ptp52F is actually a positive regulator of Trn.

Interestingly, these relationships between the Trn receptor, the unknown TK that phosphorylates Trn in vivo, and the RPTP resemble those described for the Robo receptor, which controls axon guidance across the CNS midline. Robo signaling is antagonized by the Abl TK (1), which can phosphorylate its cytoplasmic domain, and is facilitated by the Ptp10D and Ptp69D RPTPs (30). There are several models that could explain this apparent positive regulation by dephosphorylation. Tyrosine-phosphorylated Trn or Robo might be downregulated by endocytosis. It is known that Robo can be regulated in this manner: the transmembrane protein Commissureless, which downregulates Robo function in vivo, can remove it from the cell surface by diverting it into an endocytic pathway (12, 19). In another model, an SH2-domain protein might bind to a phosphorylated tyrosine residue in Trn or Robo and occlude binding of a positive regulator that normally binds to an adjacent site in a phosphorylation-independent manner.

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