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Transmembrane Glycoprotein gp150 Is a Substrate for Receptor Tyrosine Phosphatase DPTP10D in *Drosophila* Cells

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We have begun to explore the downstream signaling pathways of receptor protein tyrosine phosphatases (RPTPs) that control axon guidance decisions in the *Drosophila* central nervous system. We have focused our studies on the adhesion molecule-like gp150 protein, which binds directly to and is an in vitro substrate for the RPTP DPTP10D. Here we show that gp150 and DPTP10D form stable complexes in *Drosophila* Schneider 2 (S2) cells and in wild-type larval tissue. We also demonstrate that the DPTP10D cytoplasmic domain is sufficient to confer binding to gp150. gp150 has a short cytoplasmic domain containing four tyrosines, all found within sequences similar to immunoreceptor family tyrosine-based activation motifs (ITAMs). We demonstrate that gp150 is tyrosine phosphorylated in wild-type larvae. In S2 cells, gp150 becomes tyrosine phosphorylated following incubation with PTP inhibitors or upon coexpression of the Dsrc tyrosine kinase. Phosphorylated Dsrc and an unknown 40-kDa phosphoprotein form stable complexes with gp150, thereby implicating them in a putative gp150 signaling pathway. When coexpressed with gp150, either full-length DPTP10D or its cytoplasmic domain mediates gp150 dephosphorylation whereas a catalytically inactive DPTP10D cytoplasmic domain does not. The neural RPTP DPTP99A can also induce gp150 dephosphorylation but does not coimmunoprecipitate with gp150. Taken together, the results suggest that gp150 transduces signals via phosphorylation of its ITAM-like elements. Phosphotyrosines on gp150 might function as binding sites for downstream signaling molecules, thereby initiating a signaling cascade that could be modulated in vivo by RPTPs such as DPTP10D.

Protein phosphorylation on tyrosine residues is regulated by the activities of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Like PTKs, PTPs are a large family of multidomain proteins that exist in both cytoplasmic and transmembrane (receptor) forms. Many receptor PTPs (RPTPs) have extracellular regions that include tandemly arranged fibronectin type III repeats alone or in conjunction with immunoglobulin-like domains. This organization resembles that of some cell adhesion molecules, suggesting that RPTPs could participate in intercellular communication. Of note, the RPTPs PTPμ and PTPκ mediate homophilic adhesion in aggregation assays (3, 35, 50) and RPTPβ (PTPζ) interacts with several neural cell adhesion molecules (1a, 27, 31, 32). Although the significance of these interactions on catalytic activity is not known, engagement of their extracellular domains could modulate phosphatase activity and/or colocalize RPTPs with substrates (reviewed in reference 12).

Of the five identified *Drosophila* RPTPs, four (DPTP10D, DPTP69D, DPTP99A, and DLAR) are selectively expressed on central nervous system (CNS) axons (9, 18, 44, 47) and contribute to growth cone guidance during nervous system development. Genetic evidence shows that DPTP69D, DPTP99A, and DLAR control specific motoneuron growth cone guidance decisions in the embryonic neuromuscular system (8, 22). These RPTPs control defasciculation of axons from common pathways and can also regulate the entry of these axons into synaptic target regions (7). Genetic evidence also implicates DPTP10D and DPTP69D in the guidance of interneuronal axons within the CNS (43a). RPTPs are also expressed on neuronal growth cones and processes in vertebrates (2, 41, 42), suggesting that their functions in controlling axon guidance might be conserved between vertebrates and arthropods.

To investigate the signaling pathway(s) through which *Drosophila* neural RPTPs transmit intracellular messages, we initially designed an in vitro system to identify proteins that interact with the cytoplasmic domain of DPTP10D. The DPTP10D cytoplasmic region, which includes a single PTP catalytic domain, was used as an affinity matrix to isolate putative substrates and/or downstream signaling proteins from *Drosophila* primary neuronal and Schneider 2 (S2) cell lysates. A transmembrane glycoprotein (gp150) which binds to the DPTP10D catalytic domain was identified by this assay and characterized as an in vitro substrate for DPTP10D (45). Of note, the expression pattern of gp150 mRNA overlaps temporally with that of DPTP10D mRNA during embryogenesis. Furthermore, gp150 mRNA is expressed throughout the embryo, with the highest levels at segmental boundaries and along the CNS midline. Since DPTP10D mRNA and protein are expressed in most CNS neurons (44, 47), it is likely that gp150 and DPTP10D are coexpressed in the embryonic CNS.

The extracellular domain of gp150 contains 18 leucine-rich repeat (LRR) sequences. The presence of LRRs in gp150 is suggestive in that a number of *Drosophila* adhesion/signaling molecules, including chaoptin, connectin, and Toll, also contain multiple LRRs in their extracellular domains (19, 29, 33). The short (62-amino-acid) predicted cytoplasmic domain of gp150 includes four tyrosine residues arranged in motifs similar to vertebrate immunoreceptor family tyrosine-based activation motifs (ITAMs) (5). ITAMs such as those of the T-cell receptor (TCR) ζ chain are rapidly phosphorylated following receptor ligation, and these phosphorylated ITAMS bind to downstream effector proteins that contain Src homology 2 (SH2) domains (20). Phosphorylated ITAMs are also in vitro substrates for the RPTP CD45, which is required for normal TCR signaling (14).

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Our laboratory has previously shown that the ITAM-like elements of gp150 can be phosphorylated in vitro and that these phosphorylated tyrosine residues are in vitro substrates for the bacterially expressed DPTP10D catalytic domain (45). These results suggested that gp150, like vertebrate immunoreceptors, might become phosphorylated in vivo and consequently interact with phosphotyrosine-binding downstream signaling molecules. Moreover, DPTP10D and perhaps other PTPs could modulate the levels of gp150 phosphorylation, thereby affecting potential gp150-mediated signaling pathways.

In this study, we investigated interactions between DPTP10D and gp150 in lysates derived from larval tissue and from transfected *Drosophila* S2 cells. We show that gp150 and DPTP10D form stable complexes in these cellular contexts and that the cytoplasmic domain is sufficient to confer gp150 binding. We also show that gp150 derived from larval tissue is tyrosine phosphorylated and that gp150 can be inducibly phosphorylated in S2 cells. In addition to forming stable complexes with gp150, DPTP10D dephosphorylates gp150 in a concentration-dependent manner in S2 cells. In summary, these results support the hypothesis that DPTP10D regulates gp150 signaling in vivo.

MATERIALS AND METHODS

Plasmid construction. An epitope-tagged full-length gp150 was generated by a "bridge PCR" procedure in which the 3′ terminus of gp150 DNA was fused in frame to six tandem *myc* epitope tags. The bridge PCR product was sequenced by the Caltech Sequencing Facility to confirm accurate amplification of the two fused DNAs. The gp150myc DNA was then subcloned into the metallothionein promoter vector pRMHa3 (4), and this construct was designated gp150myc-pRM. Full-length, untagged gp150, DPTP10D, DPTP99A, and DPTP69D cDNAs were also subcloned into pRMHa3 and denoted gp150-pRM, DPTP10D-pRM, DPTP99A-pRM, and DPTP69D-pRM, respectively. The 10Dwt-pRM construct includes the DPTP10D cytoplasmic domain which has been modified to include a *Src* myristylation sequence (34) at its N terminus (this sequence should confer membrane association) and a single *myc* epitope at the 3′ terminus. 10D(C-S)-pRM is a derivative of 10Dwt-pRM, in which the essential cysteine (DPTP10D residue 1468) has been mutated to serine by standard procedures. The *Drosophila* tyrosine kinase constructs pPac/Dsrc, pPac/Dabl, and pPac/Dfer have been described previously (16). Enzymes were obtained from Boehringer Mannheim Biochemical Co. and New England Biolabs.

gp150 MAb generation. A *Bal*I fragment encoding amino acids 250 to 746 of gp150 was subcloned into a glutatione *S*-transferase vector, and the resulting fusion was protein purified with glutathione-Sepharose. Monoclonal antibodies (MAbs) against this fusion protein were generated at the Caltech Monoclonal Antibody Facility and screened based on their ability to recognize gp150 on Western blots and to immunoprecipitate native gp150 from cell lysates. The MAb 8C1.1B4, which worked well for both immunoprecipitations and immunoblotting, was used in this study.

Larval CNS and imaginal disc dissections and lysis. Wild-type third-instar larvae were Oregon R. In the experiment of Fig. 2B, lane 1, the larvae were from a cross between females bearing an X-linked GAL4 source (denoted C155; the insertion is probably in the *elav* gene [25] that is selectively expressed in differentiated neurons) and males homozygous for an autosomal insertion of a P element vector containing an upstream activation sequence-driven cDNA encoding the "long" spliced form of DPTP10D (47). This line was generated and characterized by Bahri et al. (1). It overexpresses DPTP10D in embryonic and larval neurons. DPTP10D overexpression does not cause any detectable phenotypes in embryos or adults. The CNS and imaginal discs were dissected away from the gut, salivary glands, and other tissues and washed three times in ice-cold 1× phosphate-buffered saline (PBS). For each sample, CNS or disc isolates from 30 larvae were lysed in 500 μl of ice-cold lysis buffer (125 mM NaCl, 100 mM Tris-Cl [pH 7.5], 0.2% Triton X-100) supplemented with 600 μM phenylmethylsulfonyl fluoride (PMSF), 2 μg of aprotinin per ml, 10 μg of leupeptin per ml, and 1 mM benzamidine. The tissue was then homogenized in microcentrifuge tubes by ~10 strokes with a plastic pestle, and the macerated tissue was incubated on ice for an additional 10 min. Nuclei and insoluble membrane components were then removed by 10 min of centrifugation (Eppendorf microcentrifuge 5415 rotor) at 13,200 rpm and 4°C. For immunoprecipitations, either 800 μl of 8C1.1B4 anti-gp150 MAb supernatant or an equivalent amount of purified mouse immunoglobulin G (IgG) (Sigma Immunochemicals) was incubated with the clarified lysate for >2 h and immune complexes were isolated and analyzed as described below.

S2 cell transfection, induction, activation treatments, and lysis. S2 cells (36) were maintained in Schneider's medium supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.25 μg of amphotericin B (Life Technologies) per ml, and 10% heat inactivated fetal calf serum (Gemini, Inc.). Cells were grown at 25°C under standard atmospheric conditions. S2 cells were transiently transfected by calcium phosphate-mediated DNA transfer (38). Briefly, 10^7 cells in 7 ml of growth medium were seeded in 10-cm plates and expanded overnight. The cells were then transfected for 15 to 18 h by the addition of 2 ml of DNA-calcium phosphate coprecipitate mix (which included 0.5 to 20 μg of plasmid DNA), washed in 1× PBS, and resuspended in growth medium. $CuSO_4$ (0.3 mM) was added to the cells for ~24 h to induce the MT promoter to drive expression from the transfected plasmids. To induce phosphorylation of gp150myc, transfected cells were treated as follows (incubation times listed in parentheses): 2 mM Na_3VO_4 and/or 3 mM H_2O_2 (35 min), 10 μg of insulin per ml (Collaborative Research; 5 min), or 200 nM calcium ionophore A23187 (Sigma Immunochemicals; 5 min). The cells were then harvested, washed with 1× PBS, and lysed in 450 μl of ice-cold lysis buffer (125 mM NaCl, 100 mM Tris-Cl [pH 7.5], 0.2% Triton X-100, 2 mM Na_3VO_4, 25 mM NaF, 600 μM phenylmethylfulfonyl fluoride, 2 μg of aprotinin per ml). Nuclei and insoluble membrane components were then removed by centrifugation.

Immunoprecipitations and Western blot analysis. For immunoprecipitations, lysates (450 μl) were incubated with 1 to 3 μg of anti-myc mouse MAbs (Oncogene Science, Inc.), 600 μl of 8C1.1B4 anti-gp150 MAb supernatants, or 2.4 μg of affinity-purified anti-Enabled (Ena) rabbit antiserum for >2 h at 4°C. The purified anti-Ena antiserum, which was generated against a carboxyl-terminal Ena fusion protein (16), was generously provided by A. R. Comer and F. M. Hoffmann (McArdle Laboratory for Cancer Research and Laboratory of Genetics, University of Wisconsin Medical School). Immune complexes were isolated by binding to protein G plus protein A-agarose beads (Oncogene Science, Inc.) followed by two cycles of centrifugation and lysis buffer washes. The washed immunoprecipitates were then boiled in sodium dodecyl sulfate sample buffer, resolved on sodium dodecyl sulfate–9% polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp.). The filters were blocked with either 3% bovine serum albumin (for anti-phosphotyrosine immunoblots) or 1 to 5% Blotto in TBST (25 mM Tris-Cl [pH 7.4], 137 mM NaCl, 0.2% Tween 20). Immunoblots were performed with anti-myc MAbs (0.1 μg/ml), anti-DPTP10D mouse MAbs 45E10 and 37F5 (1:5 dilution), anti-DPTP69D mouse MAb 3F11 (1:5 dilution), 4G10 anti-phosphotyrosine mouse MAbs (1 μg/ml; Upstate Biotechnology Inc.), or anti-gp150 MAb 8C1.1B4 (1:5 dilution). The filters were then washed with TBST, incubated with alkaline phosphatase-conjugated goat anti-mouse antibodies (Jackson Immunoresearch), and developed to detect phosphatase activity. The immunoblots were scanned to quantitate the intensity of different protein bands by using NIH 1.52 software. For Dsrc immunoblots, the filters were incubated sequentially with anti-Dsrc rat polyclonal antiserum (1:1,000 dilution [23]) and horseradish peroxidase conjugated goat anti-rat antibodies (Santa Cruz Biotechnology) and developed by enhanced chemiluminescence (Amersham).

RESULTS

gp150 forms complexes with DPTP10D in S2 cells. To study endogenous gp150, we first generated MAbs against the gp150 extracellular domain. MAb 8C1.1B4, which worked well for both immunoprecipitations and immunoblotting, was used in this study. Immunoblot analysis of anti-gp150 immunoprecipitated with MAb 8C1.1B4 revealed that endogenous gp150 was expressed in two forms in S2 cells, a 150-kDa species and a 180-kDa species (Fig. 1A, lane 1). Upon transfection of an expression plasmid containing a full-length gp150 cDNA into S2 cells, ~20-fold increases in the levels of both the 150- and 180-kDa species were observed (Fig. 1A, compare lanes 1 and 2), thereby confirming that both species were encoded by the gp150 cDNA. Differential posttranslational modification is a likely explanation for the presence of these two gp150 species.

Since gp150 was originally identified as a protein that bound in vitro to the DPTP10D cytoplasmic domain (45), we sought to determine if gp150 and DPTP10D could interact in cells. To this end, we transfected S2 cells with an expression plasmid encoding the "long" form of DPTP10D (47), which migrates as a tight doublet or a single band at ~210 kDa. We detected a faint DPTP10D doublet in anti-gp150 immunoprecipitates derived from cells expressing endogenous gp150 and high levels of exogenous DPTP10D (Fig. 1A, lane 1), demonstrating that overexpression of gp150 was not required to visualize this interaction. The amount of coimmunoprecipitating DPTP10D was greatly increased, however, in cells expressing high levels of both exogenous gp150 and DPTP10D (lane 2). As shown in lanes 3 and 4, equal amounts of DPTP10D protein were ex-

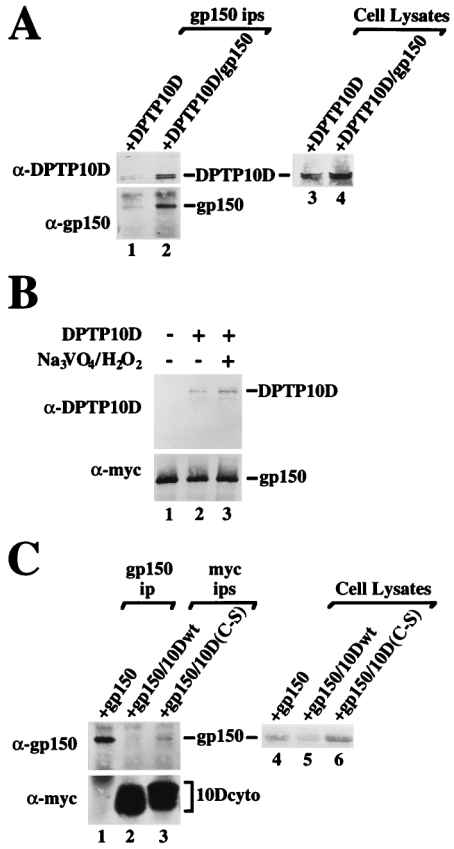

FIG. 1. gp150 forms complexes with DPTP10D in S2 cells. (A) Lysates from transfected S2 cells expressing DPTP10D alone (lane 1) or with untagged gp150 (lane 2) were immunoprecipitated with anti-gp150 (α-gp150) antibodies and processed for immunoblotting with either anti-DPTP10D (α-DPTP10D) (top) or anti-gp150 (bottom) antibodies. Note that endogenous gp150 was expressed at detectable levels in S2 cells (lane 1) and gp150 expression was augmented following transfection of gp150-pRM (lane 2). The fuzzy upper band in each anti-gp150 panel is the 180-kDa gp150 form. DPTP10D was observed in gp150 immunoprecipitates (ip) from lysates expressing endogenous (lane 1) and exogenous (lane 2) levels of untagged gp150. Total-cell lysates from transfected S2 cells expressing DPTP10D alone (lane 3) or with untagged gp150 (lane 4) were processed for immunoblotting with anti-DPTP10D antibodies. DPTP10D expression levels were similar between these lysates and were therefore not affected by coexpression of gp150. (B) Lysates from transfected S2 cells expressing *myc*-tagged gp150 (gp150myc) alone (lane 1) or with DPTP10D (lanes 2 and 3) were immunoprecipitated with anti-myc (α-myc) antibodies and processed for immunoblotting with either anti-DPTP10D (top) or anti-myc (bottom) antibodies. DPTP10D was observed in gp150myc immunoprecipitates from cells that were untreated (lane 2) or treated with Na_3VO_4 and H_2O_2 (lane 3). Note the increase in the amount of DPTP10D coimmunoprecipitating with gp150myc in Na_3VO_4- and H_2O_2-treated cells (compare lanes 2 and 3). gp150myc expression levels were not altered by coexpression of DPTP10D. (C) Lysates from transfected S2 cells expressing untagged gp150 alone (lane 1) or with *myc*-tagged forms of either the wild-type (lane 2) or mutant (lane 3) DPTP10D cytoplasmic domain were immunoprecipitated with either anti-gp150 (lane 1) or anti-myc (lanes 2 and 3) antibodies and processed for immunoblotting with either anti-gp150 (top) or anti-myc (bottom) antibodies. Note that untagged gp150 coimmunoprecipitated with mutant [10D(C-S); lane 3] but not wild-type (10Dwt; lane 2) protein. 10Dwt and 10D(C-S) were expressed at similarly high levels (bottom). Although this panel is overdeveloped to emphasize the lack of *myc* immunoreactivity in cells that were not transfected with 10Dmyc constructs, other experiments done with shorter development times reveal that the 10Dwt and 10D(C-S) bands are of equivalent intensities. Total-cell lysates from transfected S2 cells expressing untagged gp150 alone (lane 4) or with *myc*-tagged forms of either the wild-type (lane 5) or mutant (lane 6) DPTP10D cytoplasmic domain were processed for immunoblotting with anti-gp150 antibodies.

pressed in the S2 cell lysates from which these gp150 immunoprecipitates (lanes 1 and 2) were isolated. These data demonstrate that gp150 and DPTP10D form stable complexes in *Drosophila* cells, thereby confirming and extending the results of the original in vitro binding studies (45).

We also expressed a gp150 fusion protein (gp150myc) which bears a *myc* epitope tag at its carboxyl terminus. gp150myc can be efficiently immunoprecipitated with anti-myc antibodies and distinguished from endogenous (untagged) gp150. The 210-kDa DPTP10D band was also present in anti-myc immunoprecipitates derived from S2 cells coexpressing gp150myc and DPTP10D (Fig. 1B, lanes 2 and 3). Interestingly, when we induced tyrosine phosphorylation of gp150myc by treatment of transfected cells with PTP inhibitors (see Fig. 3), we noted a modest increase in the amount of coimmunoprecipitating DPTP10D (Fig. 1B, compare lanes 2 and 3). The ability to associate stably with gp150 is not a common feature of all RPTPs, however, since coexpression of high levels of either DPTP99A or DPTP69D did not result in the appearance of detectable amounts of these RPTPs in anti-gp150 immunoprecipitates (unpublished results). Taken together, our data underscore the selective ability of DPTP10D to form complexes with gp150 in vivo.

We also evaluated the ability of the isolated DPTP10D cytoplasmic domain to form complexes with gp150 in S2 cells. To this end, we coexpressed untagged gp150 together with *myc*-tagged versions of either the wild-type cytoplasmic domain (10Dwt) or the derivative cytoplasmic domain 10D(C-S) in which the essential catalytic cysteine residue was mutated to a serine. This mutation renders PTPs catalytically inactive, and analogous mutations in the catalytic domains of other PTPs have been shown to stabilize phosphatase-substrate interactions (for a review, see reference 28). Immunoblot analysis of anti-myc immunoprecipitates showed that the *myc*-tagged DPTP10D cytoplasmic proteins 10Dwt and 10D(C-S) were expressed at high levels in S2 cells (Fig. 1C, lanes 2 and 3). The lower electrophoretic mobility of the 10D(C-S) protein than that of 10Dwt is probably due to the presence of phosphorylated tyrosine residues in the mutant protein (unpublished results).

Immunoblots of anti-myc immunoprecipitates probed with anti-gp150 MAbs revealed that untagged gp150 formed complexes with the 10D(C-S) mutant protein (Fig. 1C, lane 3). The levels of coprecipitated gp150 were quite low, however, suggesting that the extracellular domain of DPTP10D may strengthen and/or stabilize the interaction. We could not detect gp150 in anti-myc immunoprecipitates derived from cells coexpressing 10Dwt and gp150 (lane 2). Although there was some variability in the amount of gp150 expressed in the lysates (lanes 4 to 6) from which these immunoprecipitates were derived, anti-myc immunoprecipitates of the 10D(C-S) mutant protein consistently included gp150 whereas those of 10Dwt did not. These findings may reflect the recently described phenomenon of PTP substrate trapping, in which mutations in the catalytic domain of other PTPs stabilize their association with substrates by inhibiting dissociation (13, 15; reviewed in reference 28). Taken together, our results demonstrate that the cytoplasmic domain of DPTP10D is sufficient to confer stable gp150 binding.

gp150 forms complexes with DPTP10D in larvae. To examine whether endogenous gp150 is associated with DPTP10D in vivo, we analyzed gp150 immunoprecipitates derived from wild-type third-instar larval CNS and imaginal disc lysates. As shown in Fig. 2A (lane 2), immunoblotting gp150 immunoprecipitates with anti-gp150 MAbs revealed that a single 180-kDa band was observed in larval tissue. This species of gp150 comi-

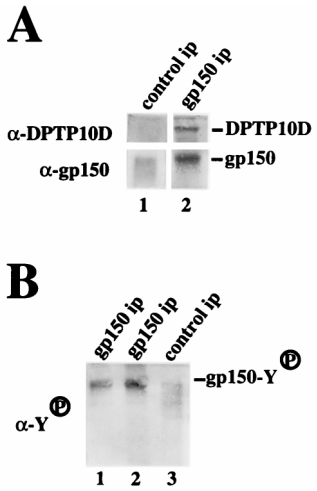

FIG. 2. gp150 is tyrosine phosphorylated and forms complexes with DPTP10D in vivo. (A) Lysates from larval CNS and imaginal disc tissue were immunoprecipitated with either anti-gp150 (α-gp150) (lane 2) or control IgG (lane 1) antibodies and processed for immunoblotting with either anti-DPTP10D (α-DPTP10D) (top) or α-gp150 (bottom) antibodies. Note that larval DPTP10D was observed in anti-gp150 immunoprecipitates (ip) (lane 2) but not in control IgG immunoprecipitates (lane 1). (B) Lysates from larval CNS or imaginal disc tissue derived from wild-type larvae (lane 2) or from larvae in which DPTP10D is overexpressed in neurons (lane 1) were immunoprecipitated with anti-gp150 antibodies and processed for immunoblotting with anti-phosphotyrosine (α-$Y^{(P)}$) antibodies. Larval gp150 was phosphorylated on tyrosine residues (gp150-$Y^{(P)}$) in vivo (lanes 1 and 2), and gp150 phosphotyrosine levels were reduced in DPTP10D overexpression larvae (compare lanes 1 and 2). Phosphorylated gp150 was not observed in control IgG immunoprecipitates derived from wild-type larval lysates (lane 3).

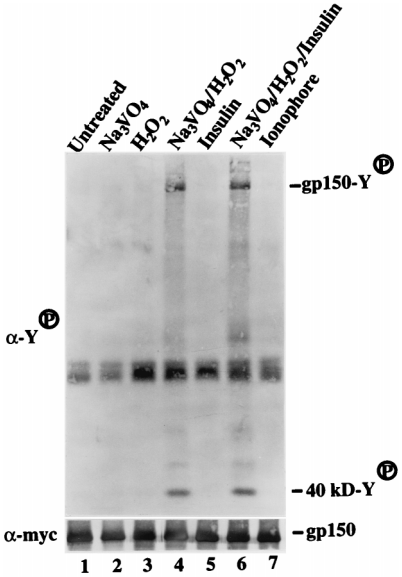

FIG. 3. gp150 can be tyrosine phosphorylated in S2 cells. Lysates from transfected S2 cells expressing gp150myc were immunoprecipitated with anti-myc (α-myc) antibodies and processed for immunoblotting with either anti-phosphotyrosine (α-$Y^{(P)}$) (top) or anti-myc (bottom) antibodies. Prior to lysis, cells were either left untreated (lane 1) or treated with the indicated agents (lanes 2 to 7). Note that a dramatic increase in gp150myc tyrosine phosphorylation was induced following combined treatments with Na_3VO_4 and H_2O_2 (lane 4) and Na_3VO_4, H_2O_2, and insulin (lane 6). Prominent 40-kDa (lanes 4 and 6) and faint 60-kDa (lane 6) tyrosine-phosphorylated proteins coimmunoprecipitated with phosphorylated gp150myc. The 60-kDa band is located just above the prominent immunoreactive band observed in all lanes, which is the ~50-kDa Ig heavy chain of the immunoprecipitating anti-myc antibodies. gp150myc expression levels were not affected by these cell treatments (bottom).

grates with the higher-molecular-mass form seen in S2 cells (Fig. 1). When the gp150 immunoprecipitates were probed with anti-DPTP10D MAbs, a strong 210-kDa DPTP10D band was observed (lane 2). Control mouse IgG did not immunoprecipitate either gp150 or DPTP10D from CNS or disc lysates (lane 1). These data confirm our results with S2 cells and demonstrate that gp150 and DPTP10D form complexes in a native context in which neither the distribution nor the cellular concentration of the proteins has been manipulated.

Since the cytoplasmic domain of gp150 includes four tyrosine residues, all within ITAM-like sequences, we next evaluated the phosphotyrosine content of native gp150 derived from larval CNS and imaginal disc tissue. We probed immunoblots of gp150 immunoprecipitates with anti-phosphotyrosine MAbs and found that gp150 was phosphorylated in lysates derived from wild-type larval CNS and imaginal disc tissue (Fig. 2B, lane 2). Interestingly, gp150 tyrosine phosphorylation levels were somewhat reduced in anti-gp150 immunoprecipitates derived from fly larvae in which DPTP10D was overexpressed in neurons (Fig. 2B, lane 1; see Materials and Methods for details). Overexpression of DPTP10D in this line did not alter gp150 expression levels or cause a global dephosphorylation of tyrosine-phosphorylated proteins in larval lysates (data not shown). Control mouse IgG did not immunoprecipitate phosphorylated gp150 from larval lysates (lane 3). These data demonstrate that gp150 is tyrosine phosphorylated in vivo and support the potential involvement of the gp150 ITAM-like sequences in downstream signaling pathways that can be modulated by DPTP10D activity.

gp150 is inducibly phosphorylated in pervanadate-treated S2 cells. To examine further the regulation of gp150 phosphorylation levels, we next examined the phosphotyrosine content of immunoprecipitated gp150myc isolated from transfected S2 cells that were exposed to reagents that are known to alter cellular protein tyrosine phosphorylation levels. gp150myc was not phosphorylated to a detectable level in untreated cells (Fig. 3, lane 1). Treating intact cells with sodium orthovanadate (Na_3VO_4), H_2O_2, insulin, or a Ca^{2+} ionophore had no effect on gp150myc tyrosine phosphorylation (lanes 2, 3, 5, and 7). When the potent PTP inhibitor combination of Na_3VO_4 and H_2O_2 (pervanadate) (11, 49) was added, however, phosphorylation of gp150myc on tyrosine was observed (lanes 4 and 6). The inducible phosphorylation of gp150myc in the presence of pervanadate suggests that gp150 is a substrate for endogenous S2 cell PTKs. The balance of PTK and PTP activities may be shifted by the presence of pervanadate, allowing tyrosine-phosphorylated gp150myc to accumulate.

Two additional tyrosine-phosphorylated proteins with molecular masses of 40 and 60 kDa are seen on phosphotyrosine blots of anti-gp150 immunoprecipitates (Fig. 3, lanes 4 and 6). The 40-kDa phosphoprotein was detected in anti-myc immunoprecipitates derived from lysates of pervanadate-treated cells (lanes 4 and 6). gp150 immunocomplexes also included a faint 60-kDa phosphorylated band following treatment of the cells with a combination of pervanadate and insulin (lane 6). Neither the 40-kDa nor the 60-kDa phosphoprotein was recognized by anti-myc antibodies, indicating that they were not carboxy-terminal gp150myc breakdown products (data not shown). One interpretation of these data is that upon gp150 tyrosine phosphorylation, the 40- and 60-kDa proteins bind to the phosphorylated ITAM-like motifs.

Dsrc mediates phosphorylation of gp150. We next evaluated three *Drosophila* tyrosine kinases, Dsrc, Dabl, and Dfer, for

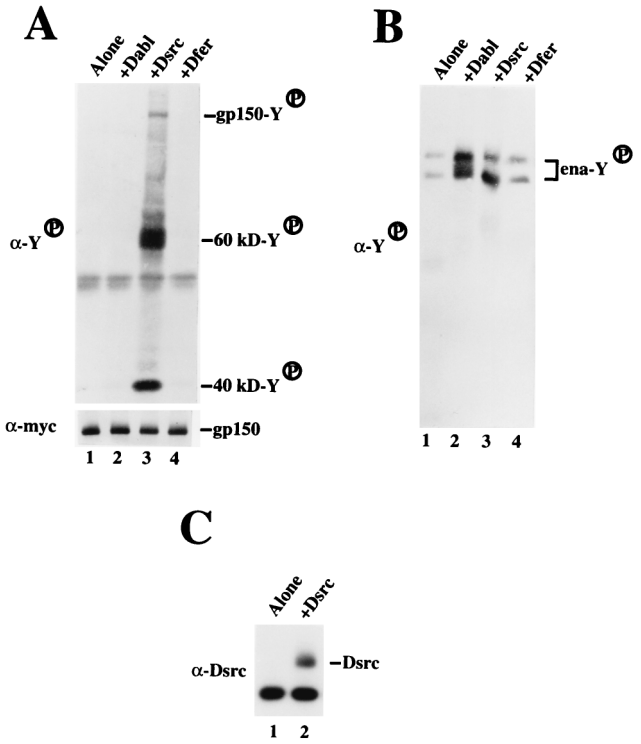

FIG. 4. The tyrosine kinase Dsrc can phosphorylate and bind gp150myc. (A) Lysates from transfected S2 cells expressing gp150myc alone (lane 1) or together with Dabl (lane 2), Dsrc (lane 3), or Dfer (lane 4) were immunoprecipitated with anti-myc (α-myc) antibodies and processed for immunoblotting with anti-phosphotyrosine (α-Y(P)) (top) or anti-myc (bottom) antibodies. Note that only Dsrc phosphorylates gp150myc (compare lanes 2 to 4). Significantly, gp150myc-associated 40- and 60-kDa phosphoproteins were also evident in the presence of Dsrc (lane 3). gp150myc expression levels were not affected by coexpression of the kinases (bottom). (B) Lysates from transfected S2 cells expressing Ena alone (lane 1) or with Dabl (lane 2), Dsrc (lane 3), or Dfer (lane 4) were immunoprecipitated with anti-Ena antibodies and processed for immunoblotting with anti-phosphotyrosine antibodies. As expected, Dabl mediated a dramatic increase in Ena phosphotyrosine levels (lane 2). Both Dsrc (lane 3) and Dfer (lane 4) induced Ena phosphorylation, although to a lesser degree. (C) Lysates from transfected S2 cells expressing gp150myc alone (lane 1) or with Dsrc (lane 2) were immunoprecipitated with anti-myc antibodies and processed for immunoblotting with anti-Dsrc (α-Dsrc) antibodies. The 60-kDa Dsrc kinase was observed in gp150myc immunoprecipitates upon Dsrc coexpression (lane 2).

their ability to induce gp150 phosphorylation by cotransfecting S2 cells with expression plasmids encoding gp150myc and each of these kinases (16). Anti-myc immunoprecipitates from cells coexpressing gp150myc and each of these tyrosine kinases were analyzed by probing immunoblots with anti-phosphotyrosine MAbs. We found that only Dsrc kinase induced tyrosine phosphorylation of gp150myc (Fig. 4A, lane 3). Coexpression of either Dabl or Dfer did not lead to phosphorylation of gp150myc (compare lanes 1, 2, and 4). To demonstrate that the three kinases were active in this system, we performed parallel experiments with the known Dabl substrate Enabled (Ena [16]). As previously shown (16), Ena immunoprecipitated from such transfectants was heavily phosphorylated when Dabl plasmids were cotransfected, demonstrating that Dabl was expressed and active (Fig. 4B, lane 2). Coexpression of the Dsrc or Dfer kinases also caused modest increases in Ena phosphotyrosine content (lanes 3 and 4). Thus, all three kinases were catalytically active but only Dsrc could mediate gp150 phosphorylation.

Tyrosine-phosphorylated 60- and 40-kDa proteins were consistently observed in anti-myc immunoprecipitates from cells coexpressing gp150myc and Dsrc (Fig. 4A, lane 3), paralleling the result observed with gp150 immunocomplexes derived from cells treated with pervanadate and insulin (Fig. 3). The intensity of the 60-kDa phosphorylated protein, however, was much greater in Dsrc transfectants. This finding, together with its electrophoretic mobility and the known ability of Dsrc to autophosphorylate (23), suggested that the coprecipitating 60-kDa phosphoprotein might be Dsrc. This suggestion is also consistent with the presence of three potential *src* family SH2 domain recognition sites (YXXL/I [39]) in the cytoplasmic domain of gp150. To address this issue, we probed immunoblots of anti-myc immunoprecipitates with rat antisera generated against Dsrc. As shown in Fig. 4C (lane 2), Dsrc coimmunoprecipitated with gp150myc when the two proteins were coexpressed in S2 cells. Dabl, which could be detected in immunoprecipitates by virtue of its tyrosine phosphorylation in S2 cells (17), was not observed in anti-myc immunoprecipitates from cells coexpressing gp150myc and Dabl (Fig. 4A, lane 2). These data demonstrate that phosphorylation of and coimmunoprecipitation with gp150 are not common features of overexpressed kinases in S2 cells. Furthermore, the presence of Dsrc and 40-kDa phosphoproteins in gp150 complexes suggests that these proteins may be components of a gp150 signaling pathway.

DPTP10D and DPTP99A mediate dephosphorylation of gp150myc in S2 cells. To determine whether phosphorylated gp150 might function as a substrate for DPTP10D in intact cells, we expressed gp150myc and Dsrc together with DPTP10D in transfected S2 cells. To generate cells expressing different levels of DPTP10D, we cotransfected different amounts of DPTP10D expression vector together with fixed amounts of both gp150myc and Dsrc expression vectors. As determined by densitometric analysis of immunoblots probed with anti-DPTP10D MAbs, when 0.05 to 5 μg of DPTP10D expression vector was used per transfection, there was an approximately linear relationship between the amount of DNA transfected and the levels of DPTP10D protein expressed in total-cell lysates (data not shown).

We found that the phosphotyrosine levels of immunoprecipitated gp150myc were reduced in a dose-dependent manner as the amount of cotransfected DPTP10D plasmid increased (Fig. 5A, compare lane 2 with lanes 3 to 5). Maximal dephosphorylation of gp150myc was achieved in S2 cells transfected with >0.25 μg of DPTP10D plasmid (lane 5 to 7). The decrease in the level of phosphorylated gp150myc was not due to a decrease in expression of the gp150myc protein, since anti-myc immunoprecipitates contained equal amounts of gp150myc (Fig. 5A, bottom panel). These data, combined with earlier results demonstrating that gp150 is an in vitro substrate for DPTP10D (45), are consistent with the hypothesis that gp150 is a direct target of DPTP10D catalysis. The intensities of the coimmunoprecipitated Dsrc and 40-kDa phosphoprotein bands were also reduced when DPTP10D was coexpressed, suggesting that these proteins either were dephosphorylated by DPTP10D or had dissociated from gp150myc in a phosphotyrosine-dependent manner (lanes 5 to 7).

We also investigated whether coexpression of the neural RPTP DPTP99A, which does not stably associate with gp150, could mediate the dephosphorylation of gp150myc and the disappearance of Dsrc and the 40-kDa protein from the immunoprecipitates. Again, we transfected fixed amounts of gp150myc and Dsrc expression vectors together with different amounts of DPTP99A expression plasmid. As described above for DPTP10D, when 0.5 to 10 μg of DPTP99A expression vector was used per transfection, there was an approximately

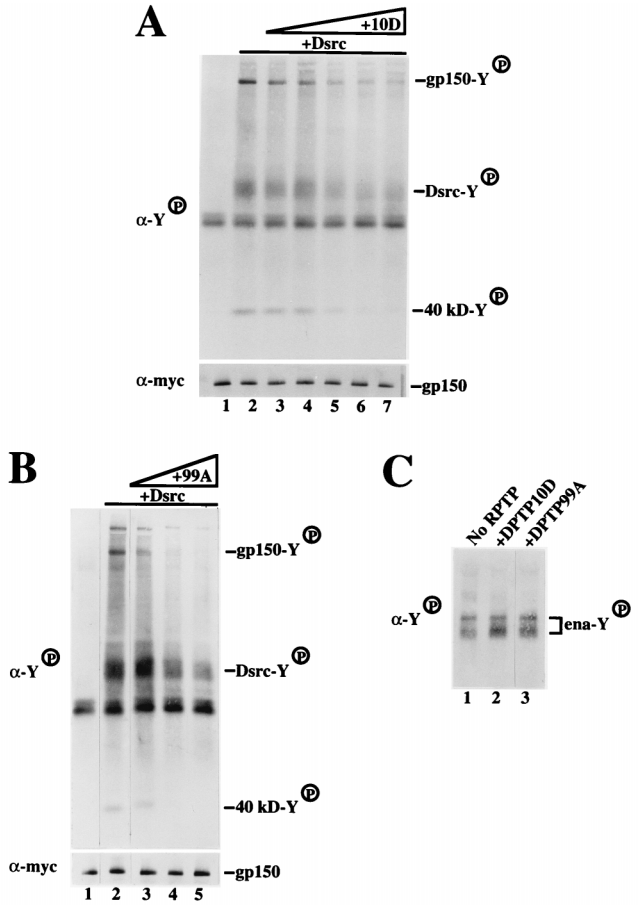

FIG. 5. DPTP10D and DPTP99A mediate dephosphorylation of gp150 in S2 cells. (A and B) Lysates from transfected S2 cells expressing gp150myc alone (lane 1) or with Dsrc (lanes 2 to 7 in panel A; lanes 2 to 5 in panel B) were immunoprecipitated with anti-myc (α-myc) antibodies and processed for immunoblotting with either anti-phosphotyrosine (α-Y^{P}) (A and B, top) or anti-myc (A and B, bottom) antibodies. Note that the phosphotyrosine content of gp150myc was reduced in a dose-dependent manner as the amount of cotransfected 10D-pRM (0.05 μg [lane 3], 0.25 μg [lane 4], 0.5 μg [lane 5], 5 μg [lane 6], or 10 μg [lane 7]) (A) or 99A-pRM (0.5 μg [lane 3], 5 μg [lane 4], or 10 μg [lane 5]) (B) increased. gp150myc expression levels were not affected by coexpression of either DPTP10D or DPTP99A (A and B, bottom). (C) Lysates from transfected S2 cells expressing Ena and Dabl alone (lane 1) or with either DPTP10D (lane 2) or DPTP99A (lane 3) were immunoprecipitated with anti-Ena antibodies and processed for immunoblotting with anti-phosphotyrosine antibodies. Expression of either RPTP did not appreciably alter Ena phosphotyrosine levels.

linear relationship between the amount of DNA transfected and the level of DPTP99A protein expressed (data not shown). DPTP99A coexpression caused a reduction in the phosphotyrosine content of gp150myc and the coordinate disappearance of the 40-kDa phosphoprotein band (Fig. 5B, lanes 3 to 5). Phosphorylated Dsrc was more resistant than gp150 to DPTP99A-mediated dephosphorylation, since higher levels of DPTP99A driven by increasing amounts of transfected DPTP99A plasmid were required to reduce its phosphotyrosine content substantially (Fig. 5B, compare Dsrc and gp150 bands in lanes 2, 3, and 5).

We also examined the effects of DPTP10D and DPTP99A overexpression on the phosphotyrosine content of cellular proteins in stably transfected S2 cell lines in which all cells express the RPTP. Immunoblots of total-cell lysates from such transfectants revealed that neither RPTP produced a global reduction in cellular phosphotyrosine levels (data not shown). An example of a specific phosphorylated protein that was not dephosphorylated by these RPTPs is the Dabl substrate Ena. As shown in Fig. 5C (lanes 1 to 3), Ena phosphotyrosine levels were not affected by coexpression of either DPTP10D or DPTP99A. In summary, our data support the conclusions that DPTP10D and DPTP99A display some specificity in their catalytic activities and that gp150 can apparently function as a substrate for DPTP10D and DPTP99A.

Like DPTP10D and DPTP99A, DPTP69D (43) is also neuron specific in late embryos (9). Unlike the other two RPTPs, however, DPTP69D is constitutively expressed in S2 cells. DPTP69D does not stably associate with gp150 (data not shown). To determine whether DPTP69D overexpression might also mediate gp150 dephosphorylation, we transfected fixed amounts of gp150myc and Dsrc expression vectors alone or together with a DPTP69D expression plasmid. We were able to achieve modest (three- to fivefold) overexpression of DPTP69D when more than 10 μg of DPTP69D plasmid was used for transfection (Fig. 6B). DPTP69D is proteolytically cleaved to different extents in S2 cells and larvae (reference 9 and unpublished results), and the two major forms detected are 160 and 116 kDa.

DPTP69D overexpression did not significantly reduce the phosphotyrosine content of gp150 or alter the amount of phosphorylated Dsrc present in anti-myc immunoprecipitates (Fig. 6A, compare lanes 2 and 3). Overexpression of DPTP69D did, however, produce dephosphorylation and/or dissociation of the 40-kDa phosphoprotein (compare lanes 2 and 3). These data suggest that gp150 phosphotyrosine residues are resistant to DPTP69D activity even under conditions in which this RPTP is overexpressed.

Differential effects of wild-type and mutant DPTP10D catalytic domains on gp150 phosphorylation. To confirm that the effect of DPTP10D coexpression on gp150 phosphorylation requires catalytic activity, we evaluated the effect of coexpressing the myc-tagged cytoplasmic domain (10Dwt) or the catalytically inactive cytoplasmic domain [10D(C-S)] on gp150 phosphotyrosine levels. As shown in Fig. 7 (compare lanes 4 and 2), coexpression of 10Dwt eliminated phosphorylation of gp150myc and caused the disappearance of phosphorylated Dsrc and the 40-kDa protein from the complex. The effect of 10Dwt coexpression was comparable to that seen for full-length DPTP10D (lanes 3 and 4), suggesting that the cytoplasmic domain was necessary and sufficient for the enzymatic interaction. In contrast, coexpression of the catalytically inactive 10D(C-S) mutant had only a small effect on gp150myc phosphorylation levels and no effect on the associated phosphoproteins (lane 5). The small reduction in gp150myc phosphorylation levels observed upon coexpression of 10D(C-S) could be due to substrate masking (for a review, see reference 28). Since 10D(C-S) binds well to gp150 (Fig. 1C), this interaction might mask gp150 sites normally phosphorylated by PTKs, thereby reducing the overall levels of phosphorylated gp150. These results demonstrate that a catalytically active PTP domain is required for gp150 dephosphorylation by DPTP10D.

DISCUSSION

We have begun to explore the downstream signaling pathways of *Drosophila* neural RPTPs by a biochemical approach. In this study, we focus on the adhesion molecule-like gp150 protein, which binds to the cytoplasmic domain of DPTP10D and is an in vitro substrate for its catalytic activity (45). There are two species of gp150, with apparent molecular masses of

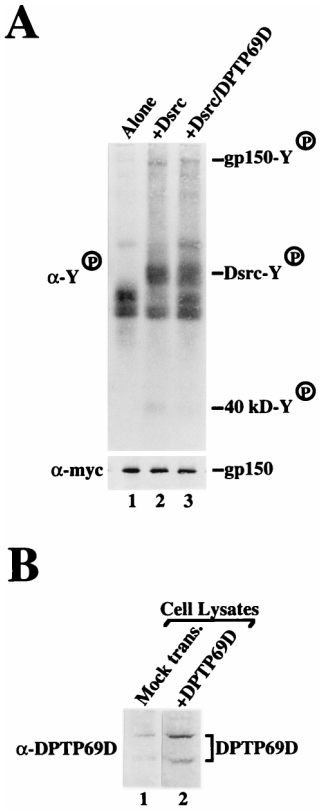

FIG. 6. DPTP69D does not mediate gp150 dephosphorylation in S2 cells. (A) Lysates from transfected S2 cells expressing gp150myc alone (lane 1) or with Dsrc (lanes 2 and 3) were immunoprecipitated with anti-myc (α-myc) antibodies and processed for immunoblotting with either anti-phosphotyrosine (α-Y(P)) (top) or anti-myc (bottom) antibodies. Note that the phosphotyrosine content of gp150myc was not appreciably affected by cotransfection of 69D-pRM (10 μg; lane 3). gp150myc expression levels were also not affected by coexpression of DPTP69D (bottom). (B) Lysates from mock-transfected S2 cells (lane 1) or S2 cells transfected with DPTP69D plasmids (lane 2) were processed for immunoblotting with anti-DPTP69D (α-DPTP69D) antibodies. After transfection with 69D-pRM (10 μg), a three- to fivefold increase in the amount of DPTP69D in cell lysates was observed.

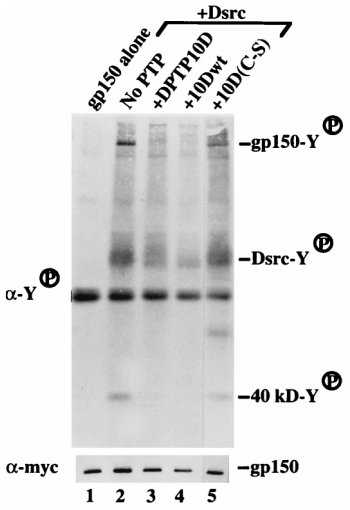

FIG. 7. The catalytic domain of DPTP10D mediates dephosphorylation of gp150. Lysates from transfected S2 cells expressing gp150myc alone (lane 1) or with Dsrc (lanes 2 to 5) were immunoprecipitated with anti-myc (α-myc) antibodies and then processed for immunoblotting with either anti-phosphotyrosine (α-Y(P)) (top) or anti-myc (bottom) antibodies. The expression of either the DPTP10D cytoplasmic domain 10Dwt (lane 4) or DPTP10D (lane 3) caused dephosphorylation of gp150myc and also led to the disappearance of the associated Dsrc and 40-kDa phosphoprotein bands (top). In contrast, expression of the catalytically inactive DPTP10D cytoplasmic domain 10D(C-S) led to only a small decrease in gp150myc levels and no appreciable decrease in the phosphorylation levels and/or association of Dsrc and the 40-kDa protein (lane 5). gp150myc expression levels were not appreciably altered by coexpression of the other proteins (bottom).

150 and 180 kDa, that are expressed in *Drosophila* S2 cells (Fig. 1) and larvae (Fig. 2). The molecular mass of the larger species (180 kDa) may reflect the presence of additional posttranslational modifications that are absent from the smaller species. In addition to a large extracellular domain which resembles those of known adhesion molecules (19, 29, 33), gp150 has a short cytoplasmic domain containing tyrosine motifs that resemble the SH2 domain-binding ITAM elements found in vertebrate immunoreceptors. The presence of these ITAM-like motifs suggests that gp150 could transmit signal(s) via interactions between phosphotyrosine residues and signaling molecules with SH2 domains. DPTP10D and other RPTPs could modulate such a signaling pathway by dephosphorylating gp150.

In this study, we have analyzed the physical and enzymatic interactions of gp150 with other proteins, including RPTPs and PTKs, to explore their intracellular signaling pathways. We show that gp150 exists in a complex with DPTP10D in S2 cells (Fig. 1) and in larval CNS and imaginal disc tissue (Fig. 2). Moreover, the ability of the mutant 10D(C-S) protein to bind stably to gp150 in S2 cells (Fig. 1) demonstrates that the cytoplasmic domain of DPTP10D is sufficient to confer binding. The stable association of 10D(C-S) with gp150 is consistent with results examining the binding properties of other PTPs in which critical residues in their catalytic domains, including the essential cysteine residue, have been mutated (for a review, see reference 28). For example, analogously mutated PTP-PEST(C231S) forms stable complexes with the PTP-PEST substrate p130^{cas} whereas wild-type PTP-PEST does not (15).

We have also investigated whether the ITAM-like motifs in the cytoplasmic domain of gp150 could function analogously to those of immunoreceptors. gp150 derived from wild-type larval tissue contains phosphotyrosine (Fig. 2). In S2 cells, gp150 phosphorylation can be induced by treating the cells with pervanadate (Fig. 3) or upon coexpression of the Dsrc PTK (Fig. 4A). Coexpression of two other catalytically active PTKs, Dabl and Dfer, did not mediate gp150 phosphorylation, demonstrating that gp150 is not indiscrimately phosphorylated by overexpressed tyrosine kinases (Fig. 4A). The phosphorylation of gp150 following Dsrc coexpression is consistent with the presence of a sequence motif (I/V-Y-hydrophilic-hydrophilic-hydrophobic) in the gp150 cytoplasmic domain that matches sites recognized and phosphorylated by *src* family tyrosine kinases (39).

In addition to inducing gp150 phosphorylation, Dsrc coimmunoprecipitates with gp150 (Fig. 4C). This is consistent with the fact that three of the four tyrosine motifs in the gp150 cytoplasmic tail match the consensus recognition site (YXXL/I) characterized for mammalian *src* family SH2 domains (40). It is possible that the 60-kDa phosphoprotein observed in gp150 immunoprecipitates derived from pervanadate- and insulin-treated cells is also Dsrc. We could not, however, detect endogenous Dsrc in gp150 immunoprecipitates under these conditions (unpublished results), perhaps due to the low level of endogenous Dsrc expression in S2 cells. Although Dsrc can phosphorylate a variety of cellular proteins

(23), the ability of Dsrc to complex with and phosphorylate gp150 supports the hypothesis that the catalytic interaction of these proteins is specific.

Dephosphorylation of gp150 is induced in a concentration-dependent manner by the neural RPTP DPTP10D (Fig. 5A). The DPTP10D cytoplasmic domain protein 10Dwt, but not its catalytically inactive derivative 10D(C-S), also facilitates the dephosphorylation of gp150 (Fig. 7). Dephosphorylation of gp150 can also be induced by another neural RPTP, DPTP99A (Fig. 5B). Overexpression of either of these RPTPs does not, however, facilitate a global reduction in cellular protein phosphotyrosine levels (data not shown) or mediate the dephosphorylation of the Ena phosphoprotein (Fig. 5B). Moreover, overexpression of another neural RPTP, DPTP69D, did not mediate a decrease in gp150 phosphotyrosine levels or alter the amount of phosphorylated and/or associated Dsrc (Fig. 6), suggesting that DPTP69D may recognize a different spectrum of substrates. In total, our data support the conclusions that DPTP10D and DPTP99A discriminate among potential substrates and that gp150 is not dephosphorylated by all RPTPs. These results, together with earlier results from our laboratory showing that DPTP10D can dephosphorylate gp150 in vitro (45), support the hypothesis that gp150 is a direct target for DPTP10D catalytic activity in S2 cells.

In addition to dephosphorylating gp150, both full-length DPTP10D and its cytoplasmic domain 10Dwt induce the dephosphorylation and/or dissociation of the gp150-associated phosphoproteins Dsrc and the unidentified 40-kDa protein (Fig. 5A and 7). DPTP99A also mediates the disappearance of the 40-kDa phosphoprotein and, to a lesser degree, that of Dsrc (Fig. 5B). Conclusions about the differing substrate specificities of DPTP10D and DPTP99A cannot be drawn, however, since data on relative protein concentrations and substrate kinetics of these proteins in S2 transfectants are lacking. Several possible models, however, could explain the apparent dephosphorylation of the three proteins that are detected in gp150 immunoprecipitates. In the first model, all three phosphoproteins might be direct targets for DPTP10D and/or DPTP99A. In the second model, gp150 might be the primary target, and its dephosphorylation would facilitate the dissociation of Dsrc and the 40-kDa protein from the complex. In the third model, the activating tyrosine residue on Dsrc (23) could be the primary target, and its dephosphorylation would lead to Dsrc inactivation. The activity of DPTP10D, DPTP99A, and/or endogenous S2 cell PTPs would then lead to the dephosphorylation of gp150 and/or the 40-kDa protein. Our results do not unequivocally distinguish among these models. For DPTP99A, however, the second and third models are less likely, because concentrations of DPTP99A that are sufficient to mediate complete gp150 dephosphorylation do not dramatically reduce Dsrc phosphorylation (Fig. 5B).

Many studies have been performed to evaluate the substrate specificities of PTPs in vitro and in vivo. "Classical" (tyrosine-specific) PTPs often appear to be fairly promiscuous with respect to catalytic substrates, but exceptions to this generalization have been characterized, some of which are mentioned below. Of note, the hematopoietic cell RPTP CD45 is known to act on a limited spectrum of physiological substrates, including members of the *src* family PTKs and the TCR ζ chain (reviewed in reference 46). RPTPα also exhibits substrate specificity in that it specifically dephosphorylates the negative regulatory site of mammalian $pp60^{c\text{-}src}$, thereby activating the kinase (6, 10, 48). PTP-PEST exhibits a very high degree of substrate specificity, selectively dephosphorylating the $p130^{cas}$ protein (15). Substrate specificity may be imparted by several characteristics of phosphatases, including the primary structure of the catalytic domain, intrinsic localization signals such as SH2 domains, and intracellular colocalization with potential substrates (reviewed in references 12 and 26).

In summary, we have initiated the biochemical dissection of the downstream signaling pathways of gp150 and of the *Drosophila* neural RPTPs. Under some circumstances, DPTP10D and/or DPTP99A may regulate gp150 signaling by modifying the interactions of gp150 with downstream signaling molecules such as Dsrc and the 40-kDa phosphoprotein. Of note, in addition to Dsrc and the 40-kDa phosphoprotein, gp150 immunocomplexes include the SH2 domain-containing signaling molecules Dshc (21, 24) and Drk (30, 37; our unpublished results). These findings suggest that these signaling proteins may also be components of a putative gp150 signal transduction pathway(s) whose interactions may be modulated by RPTP activity. Since the RPTPs appear to function primarily in the control of axon guidance, gp150 might also play a role in growth cone path-finding decisions.

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