A Drosophila Protein-tyrosine Phosphatase Associates with an Adapter Protein Required for Axonal Guidance*

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We have used the yeast two-hybrid system to isolate a novel Drosophila adapter protein, which interacts with the Drosophila protein-tyrosine phosphatase (PTP) dPTP61F. Absence of this protein in Drosophila causes the mutant photoreceptor axon phenotype dreadlocks (dock) (Garrity, P. A., Rao, Y., Salecker, I., and Zipursky, S. L. (1996) Cell 85, 639–650). Dock is similar to the mammalian oncoprotein Nck and contains three Src homology 3 (SH3) domains and one Src homology 2 (SH2) domain. The interaction of dPTP61F with Dock was confirmed in vivo by immune precipitation experiments. A sequence containing five PXXP motifs from the non-catalytic domain of the PTP is sufficient for interaction with Dock. This suggests that binding to the PTP is mediated by one or more of the SH3 domains of Dock. Immune precipitations of Dock also co-precipitate two tyrosine-phosphorylated proteins having molecular masses of 190 and 145 kDa. Interactions between Dock and these tyrosine-phosphorylated proteins are likely mediated by the Dock SH2 domain. These findings identify potential signal-transducing partners of Dock and propose a role for dPTP61F and the unidentified phosphoproteins in axonal guidance.

The Drosophila PTP, dPTP61F, undergoes differential splicing resulting in proteins that are targeted to either the nucleus or a reticular structure in the cytoplasm (2). The PTP is comprised of an N-terminal catalytic domain followed by a differently spliced C-terminal targeting domain (see Fig. 1A). The PXXP motifs share varying degrees of similarity with the consensus sequence of the Src SH3 binding site (3, 4). In order to gain insights into the biological role of dPTP61F, we initiated a two-hybrid screen to isolate interacting proteins. These experiments identified an adapter protein containing three SH3 domains and a single SH2 domain, which is similar to the mammalian oncoprotein Nck (5). This adapter protein was independently isolated in a genetic screen for genes important in photoreceptor axon guidance (1), where it was referred to as Dreadlocks (Dock), which describes the appearance of photoreceptor axons in Drosophila dock loss-of-function mutants. Adapter proteins often link receptor tyrosine kinases (RTKs) with downstream signaling proteins (6–8). There is a growing body of evidence that suggests that tyrosine phosphorylation plays a critical role in axonal targeting during Drosophila nervous system development. Derailed is a RTK that is necessary for pathfinding in a subset of neurons in the embryonic ventral nerve cord (9). Genetic and biochemical studies also have shown that the receptor PTPs DLAR (10), DPTP69D, and DPTP99A (11) are required for motor axon guidance. In vertebrates, experiments suggest that members of the large Eph family of RTKs and their ligands are involved in sensing and providing positional cues for axons in the retinotectal system (12).

Our two-hybrid results were confirmed by in vitro binding experiments and by immune precipitation experiments that demonstrate that Dock and dPTP61F are associated in vivo. In situ hybridization experiments reveal overlapping expression patterns of Dock and the PTP in the developing Drosophila brain and ventral nerve cord. Finally, we have discovered that the Dock protein is associated in vivo with two additional tyrosine-phosphorylated proteins (190 and 145 kDa). These interactions suggest a working model in which the tyrosine-phosphorylated proteins interact with Dock via its SH2 domain, while one or more of the PXXP motifs found in the PTP binds to one or more SH3 domains found in Dock. This complex likely plays an important role in photoreceptor axon guidance.

MATERIALS AND METHODS

Plasmid Constructions—A 1.6-kilobase PCR product encoding dPTP61F was ligated into pT7-7 and pAS2 (13) to produce dPTP61Fn/pT7-7 for bacterial expression and dPTP61Fn/pAS2 for the yeast two-hybrid system. The cDNA encoding Dock was amplified by PCR and ligated into pAS2 or pGEX-KG (14). The expression construct for the C-terminal truncated form of dPTP61Fn was also generated by PCR and inserted into pT7-7.

Two-hybrid Screens—Y190 cells (13) containing dPTP61Fn/pAS2 or Dock/pAS2 were transformed with a pACT Drosophila third instar larva cDNA library (Stephen J. Elledge, Baylor College of Medicine), and positive colonies were isolated as described (15). pAS2 plasmids were selectively removed by cycloheximide treatment (13), and pACT plasmid DNA was isolated and sequenced.

Antibodies—Polyclonal antibodies to Dock were generated in rabbits (Cocalico Biologicals, Reamstown, PA) by injection of GST-Dock. Anti-Dock antibodies were isolated by preadsorption using a GST/Esherichia coli acetone powder followed by affinity purification (16) on a GST-Dock-linked Affi-Gel-10 (Bio-Rad) column. Polyclonal antibodies to dPTP61F (2) were purified by binding to dPTP61Fn-linked Affi-Gel-10. IgG2b, (Upstate Biotechnology Inc., Lake Placid, NY) was used to detect tyrosine phosphorylation.

In Vitro Binding Assays—GST and GST-Dock were expressed in BL21 cells (Novagen). pT7-7-based dPTP61Fn expression constructs were introduced into BL21(DE3) (Novagen) cells. Recombinant proteins were expressed at 25 °C in the presence of 100 μg/ml ampicillin (Boehringer Mannheim) and 400 μg/ml isopropyl β-D-thiogalactopyranoside (Boehringer Mannheim). The cells were collected by centrifugation, suspended in 400 μl of 4 °C binding buffer (10 mM Tris, pH 7.4, 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 1 mM 1,4-dithiothreitol), and

1 The abbreviations used are: PTP, protein-tyrosine phosphatase; RTK, receptor tyrosine kinase; PCR, polymerase chain reaction; GST, glutathione S-transferase.

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lysed by sonication.

50 μl of GST or GST-Dock supernatant was diluted in 1 ml of binding buffer, added to a 50-μl bed volume of glutathione-agarose (Sigma), and rocked for 1 h at 4 °C. The resins were collected by centrifugation and washed with binding buffer. 50 μl of full-length or truncated dPTP61Fn supernatant was diluted in 1 ml of binding buffer and then added to the resins. Incubation was continued at 4 °C for 3 h. The beads were collected, washed with binding buffer, and boiled in 50 μl of 5 × Laemmli loading buffer. 5 μl of the sample was subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose (Schleicher & Schuell). dPTP61Fn was detected using anti-dPTP61Fn in conjunction with the HRPL kit (National Diagnostics).

In Situ Hybridization—Probe DNA was prepared following the digoxigenin RNA labeling reaction protocol provided by Boehringer Mannheim and modified by use of only 20 units of either T3 or T7 RNA polymerase (Stratagene or Pharmacia Biotech Inc.) and the addition of 40 units of RNase inhibitor (Boehringer Mannheim) per reaction. After transcription, the RNA was hydrolyzed in carbonate buffer (60 mM Na2CO3, 40 mM NaHCO3) at 65°C for 40 min. Alternatively, 10 μl of non-staged dechorionated Drosophila embryos were lysed per 40 μl of RIPA buffer using 10 strokes of a tight 40-ml Daunce homogenizer. Insoluble debris was removed in both cases by centrifugation at 100,000 × g for 30 min. Three μg of affinity-purified anti-Dock or anti-dPTP61Fn was used to immune precipitate from 1 ml of Schneider II extract or 7 ml of embryonic extract as described (16). Five μl of preimmune serum was also used for immune precipitation and served as a nonspecific control. Precipitated proteins were analyzed by immunoblotting with anti-dPTP61Fn or IgG2b. Immunoreactive bands were detected using horseradish peroxidase-conjugated Protein A (Amersham Corp.).

In Situ Hybridization—RNA probes were prepared following the digoxigenin RNA labeling reaction protocol provided by Boehringer Mannheim and modified by use of only 20 units of either T3 or T7 RNA polymerase (Stratagene or Pharmacia Biotech Inc.) and the addition of 40 units of RNase inhibitor (Boehringer Mannheim) per reaction. After transcription, the RNA was hydrolyzed in carbonate buffer (60 mM Na2CO3, 40 mM NaHCO3) at 65°C for 40 min. In situ hybridization experiments were performed on 0–24-h embryos using sense and antisense digoxigenin-labeled RNA probes from Dock and dPTP61Fn cDNA clones. The whole mount in situ hybridization protocol is based on that described by Tautz and Pfeifle (18).

RESULTS AND DISCUSSION

The yeast two-hybrid system was employed to identify proteins that interact with dPTP61F. The cDNA encoding the nuclear form of dPTP61F (dPTP61Fn) was placed downstream of the GAL4 DNA binding domain of pAS2 (13) and used to screen a Drosophila third instar larva two-hybrid library. An estimated 4 × 107 transfectants were analyzed, resulting in 79 colonies that were positive for β-galactosidase activity. Thirty-nine plasmids were partially sequenced, seven of which contained overlapping sequences that activate the two-hybrid reporter genes only in the presence of the dPTP61Fn hybrid. The longest of the seven open reading frames was completely sequenced. During the course of our studies we learned that Garrity et al. (1) had determined that the protein encoded by this clone was essential for photoreceptor axon guidance in the fly. Both groups have opted to use the name Dreadlocks (Dock) for the protein that describes the mutant photoreceptor pathfinding phenotype. Data base comparisons (19) indicate that Dock is similar to the mammalian Nck oncprotein (5) (Fig. 1B). Both molecules consist of three SH3 domains and one SH2 domain and are similar in length and domain order. The most highly conserved sequences shared by these molecules are within the SH3 and SH2 domains (55–72% identity). Comparison of the SH3 domains from Dock with SH3 domains from proteins other than Nck yield percent identities averaging 38%, suggesting that Dock and Nck must be either homologues or two members of a family of Nck-like molecules.

To further define the nature of the interaction between dPTP61F and Dock in vitro binding assays were performed. GST and GST fused to Dock were expressed independently in E. coli. Both proteins were immobilized on glutathione-agarose and allowed to bind bacterially expressed full-length dPTP61Fn protein. Also tested for binding was the C-terminal half of dPTP61Fn containing the proline-rich domain (amino acids 298–535, Fig. 1A). Bound dPTP61Fn was detected by Western analysis using an affinity-purified polyclonal antibody. Immunoreactive proteins corresponding to the full-length and truncated version of dPTP61Fn were retained on the GST-Dock column but were not retained on the GST column (Fig. 2A). As expected for interactions mediated by the Dock SH3 domains and the PXXP motifs of dPTP61F, the C-terminal domain by itself was sufficient for binding to Dock.

In order to demonstrate that Dock and dPTP61Fn interact in vivo, polyclonal antibodies recognizing Dock and dPTP61F were produced and affinity-purified. These antibodies detected a 50-kDa Dock protein and a 72-kDa dPTP61F protein by Western analysis present in Schneider II and Drosophila embryos (data not shown). Both affinity-purified antibodies were used separately to immune precipitate Dock or dPTP61Fn from Schneider II cell and Drosophila embryonic soluble protein extracts. Proteins present in immune complexes were analyzed by immunoblotting with antibodies recognizing dPTP61F or phosphotyrosine (Fig. 2, B–D). dPTP61F was detected when precipitated by anti-dPTP61F and is co-precipitated with Dock by anti-Dock. No dPTP61F was detected in preimmune precipitations. These results were
observed in immune complexes from both Schneider II cell and Drosophila embryo extracts and provide conclusive evidence that dPTP61F and Dock associate in vivo. Precipitated proteins were also analyzed by immunoblotting with anti-phosphotyrosine. No tyrosine-phosphorylated bands corresponding to the molecular weights of Dock or dPTP61F were observed. However, two tyrosine-phosphorylated proteins of 190 kDa (p190) and 145 kDa (p145) co-precipitated with Dock (Fig. 2).

Dock was also employed in a two-hybrid screen to identify interacting proteins. Approximately $3.8 \times 10^6$ Drosophila third instar larval library transformants were screened resulting in the identification of 128 $\beta$-galactosidase-positive colonies. Plasmid DNA was isolated from 37 positives, and their insert cDNAs were sequenced. Translation of these sequences indicated that 29 of these clones were rich in proline. Most of these encoded known proteins, which were translated in altered reading frames resulting in artificial proteins that were rich in proline. Notably, three of the eight non-proline-rich clones encoded dPTP61F. Two of the three clones encoded the full-length PTP while one encoded the C-terminal half only (amino acids 269–535). The latter result indicates that sequences in the C-terminal half of the PTP are sufficient for interaction with Dock. The additional five interacting clones are currently being characterized.

In situ hybridization experiments were carried out to compare the expression patterns of dPTP61F and Dock. Drosophila embryos were hybridized with antisense digoxigenin-incorporated RNA probes. The dPTP61F RNA probe was generated from sequences common to both splice variants. Dock mRNA expression localizes to the developing brain and ventral nerve cord of these embryos (Fig. 3A). dPTP61F mRNA also localizes to the central nervous system (Fig. 3B) but requires increased staining times due to lower abundance of message. The signal
observed in the central nervous system is contributed by expression of the membrane-associated splice variant (dPTP61Fm) while staining outside of the central nervous system is due to background and low level staining of dPTP61Fn in the gut.\(^2\) Comparison of the expression patterns of dPTP61F and Dock transcripts by Northern analysis indicates that these messages are expressed in a similar manner throughout development (Fig. 3, C and D). The overlapping expression patterns of dPTP61Fm and Dock in the Drosophila embryo and the similarly regulated mRNA expression patterns during development are in complete agreement with the in vivo association experiments.

We have demonstrated that Dock and dPTP61F interact both in vitro and in vivo and that both proteins are coexpressed in the fly nervous system. It is unlikely that Dock is a substrate of dPTP61F since immune precipitated Dock is not tyrosine-phosphorylated. The possibility that the Dock SH2 domain binds to the PTP is ruled out by the absence of tyrosine phosphorylation of dPTP61F. The interaction between Dock and dPTP61F is likely mediated by one or more SH3 domain/PXXP motif binding events as demonstrated by two-hybrid and in vitro binding data. Since every Dock two-hybrid positive contained all three SH3 domains (data not shown), high affinity binding may be dependent on interactions involving more than one SH3 domain. This hypothesis is attractive since none of our two-hybrid screens with dPTP61F identified Drosophila proteins with two or fewer SH3 domains (i.e. Src, Drk, and SOS). Immune precipitation of Dock followed by Western analysis using anti-phosphotyrosine antibodies detected two tyrosine-phosphorylated proteins of 190 and 145 kDa associated with Dock. Although possible mechanisms for the association of Dock with p190 and p145 have not been explored in detail, a working hypothesis is that these proteins associate via the SH2 domain of Dock and the phosphotyrosine present on p190 and p145. The molecular characterization of p190 and p145 is currently in progress. The association of dPTP61F with Dock and its interaction with p190 and p145 provide initial insights into the complex of proteins likely to play a significant role in the Drosophila nervous system guidance mechanism.

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