The Sorting Nexin, DSH3PX1, Connects the Axonal Guidance Receptor, Dscam, to the Actin Cytoskeleton*

Dock, an adaptor protein that functions in Drosophila axonal guidance, consists of three tandem Src homology 3 (SH3) domains preceding an SH2 domain. To develop a better understanding of axonal guidance at the molecular level, we used the SH2 domain of Dock to purify a protein complex from fly S2 cells. Five proteins were obtained in pure form from this protein complex. The largest protein in the complex was identified as Dscam (Down syndrome cell adhesion molecule), which was subsequently shown to play a key role in directing neurons of the fly embryo to correct positions within the nervous system (Schmucker, D., Clemens, J. C., Shu, H., Worby, C. A., Xiao, J., Muda, M., Dixon, J. E., and Zipursky, S. L. (2000) Cell 101, 671–684). The smallest protein in this complex (p63) has now been identified. We have named p63 DSH3PX1 because it appears to be the Drosophila orthologue of the human protein known as SH3PX1. DSH3PX1 is comprised of an NH2-terminal SH3 domain, an internal PHOX homology (PX) domain, and a carboxyl-terminal coiled-coil region. Because of its PX domain, an internal PHOX homology (PX) domain, and a carboxyl-terminal coiled-coil region. Because of its PX domain, DSH3PX1 is considered to be a member of a domain known as Dock that was essential for the guidance of photoreceptor axons in third instar larva (5). Dock contains three tandem SH3 domains and a single SH2 domain and is the fly orthologue of the mammalian protein, Nck. Using an epitope-tagged SH2 domain of Dock, we recently isolated a novel receptor-like molecule known as Dscam, the Drosophila orthologue of the human Down’s syndrome cell adhesion molecule (DSCAM), which is essential for guidance of embryonic axons within Bolwig’s nerve and elsewhere in the developing nervous system of the fly (6). Dscam was one of five proteins found in the complex isolated using the Dock epitope-tagged SH2 domain. We have now identified and characterized the smallest protein in this complex, p63. We have named p63, DSH3PX1, because it appears to be the Drosophila orthologue of the human protein known as SH3PX1. DSH3PX1 contains an SH3 domain, a PHOX homology (PX) domain, and a coiled-coil domain. The PX and coiled-coil domains are found in a number of main proteins containing proteins. One of the earliest identified sorting nexins, SNX1, was isolated based on its ability to bind to the cytoplasmic domain of the epidermal growth factor receptor and to enhance degradation of ligand-activated receptors (8). The PX domain is a conserved stretch of 130 amino acids of unknown function, first identified in the p40phox and p47phox subunits of the NADPH oxidase complex (10). PX domain-containing proteins belong to a large family of hydrophilic molecules, most of which are found partially associated with cellular membranes. Recently, the NMR solution structure for the PX domain of p47phox was determined (11). In addition, the p47phox PX domain and others, including the PX domain of sorting nexin SNX3, were also shown to mediate binding to phosphoinositides, thereby targeting these proteins to cellular membranes (12, 13).

Axon guidance is a form of cell movement in which the axon cell body remains stationary while a specialized structure at the tip of the axon, known as the growth cone, receives signals from the environment and translates these signals into directed neurite outgrowth (reviewed in Ref. 1). Growth cone movement is accomplished via adhesion molecules that modulate attachment to the extracellular matrix and neighboring cells (2, 3). In addition, growth cones express guidance receptors responsive to extracellular ligands that convey attractive or repulsive signals, thereby specifying the direction of growth (4). Although a number of guidance receptors have been reported, the mechanisms by which these receptors coordinate changes in the actin cytoskeleton of the growth cone are not well understood.

Several years ago, a genetic screen designed to find genes important in axonal guidance identified a Drosophila protein known as Dock that was essential for the guidance of photoreceptor axons in third instar larva (5). Dock contains three tandem SH3 domains and a single SH2 domain and is the fly orthologue of the mammalian protein, Nck. Using an epitope-tagged SH2 domain of Dock, we recently isolated a novel receptor-like molecule known as Dscam, the Drosophila orthologue of the human Down’s syndrome cell adhesion molecule (DSCAM), which is essential for guidance of embryonic axons within Bolwig’s nerve and elsewhere in the developing nervous system of the fly (6). Dscam was one of five proteins found in the complex isolated using theDock epitope-tagged SH2 domain. We have now identified and characterized the smallest protein in this complex, p63. We have named p63, DSH3PX1, because it appears to be the Drosophila orthologue of the human protein known as SH3PX1. DSH3PX1 contains an SH3 domain, a PHOX homology (PX) domain, and a coiled-coil domain. The PX and coiled-coil domains are found in a number of proteins involved in vesicular trafficking including members of the sorting nexin family (7–9). One of the earliest identified sorting nexins, SNX1, was isolated based on its ability to bind to the cytoplasmic domain of the epidermal growth factor receptor and to enhance degradation of ligand-activated receptors (8). The PX domain is a conserved stretch of 130 amino acids of unknown function, first identified in the p40phox and p47phox subunits of the NADPH oxidase complex (10). PX domain-containing proteins belong to a large family of hydrophilic molecules, most of which are found partially associated with cellular membranes. Recently, the NMR solution structure for the PX domain of p47phox was determined (11). In addition, the p47phox PX domain and others, including the PX domain of sorting nexin SNX3, were also shown to mediate binding to phosphoinositides, thereby targeting these proteins to cellular membranes (12, 13).

Using a two-hybrid screen employing DSH3PX1, we demonstrate that this putative sorting nexin interacts directly with

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‡ Present address: Howard Hughes Medical Institute, Dept. of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA 90095.

¶ To whom correspondence should be addressed. Tel.: 734-647-3998; Fax: 734-763-4581; E-mail: jedixon@umich.edu.

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Carolyn A. Worby, Nancy Simonson-Leff, James C. Clemens‡, Robert P. Kruger, Marco Mudaš, and Jack E. Dixon†

From the Life Sciences Institute and the Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109-0606

From the Life Sciences Institute and the Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan 48109-0606.
the Wiskott-Aldrich syndrome protein (Wasp). Members of the Wasp family are multidomain proteins that serve as scaffolds, bringing together components of signal transduction pathways with cellular machinery that promote actin polymerization and microfilament reorganization (14–16). Execution of this program then leads to formation of protrusive, actin-based membrane structures at the cell surface. When activated by Cdc42 and phosphatidylinositol 4,5-bisphosphate, the Wasp homologue, N-Wasp, induces long actin microspike formation (17). Recently Nck and phosphatidylinositol 4,5-bisphosphate have been shown to synergistically activate actin polymerization through N-Wasp in an in vitro actin preyne assay (18). N-Wasp has also been shown to be essential in nerve growth factor–stimulated neurite extension in PC12 cells, presumably through Arp2/3 complex-induced actin polymerization (19). Wasp’s ability to promote the polymerization of actin has also been implicated in endocytosis (20). Ligand-activated epidermal growth factor receptor was found associated with N-Wasp (21), and lymphocytes from Wasp knockout mice exhibited both a reduction in actin polymerization and defects in T cell receptor endocytosis (22). Endocytosis of cell surface receptors often involves clathrin-coated pits, which are composed of a clathrin lattice and the AP2 protein complex (23, 24). Interestingly, we demonstrated that DSHP3PX1 co-immunoprecipitated with AP-50, one of the clathrin coat adaptor proteins also known as \( \mu_2 \) (25). Collectively, our results suggest that DSHP3PX1 links Dscam signaling to Wasp, a protein capable of modulating the actin cytoskeleton, and that this activity may serve to regulate the intracellular trafficking of Dscam.

**MATERIALS AND METHODS**

**S2 Cell Culture, Transfections, and RNA interference (RNAi)**—

Two-hybrid Screens—

Full-length DSHP3PX1 was fused in-frame into pLexAde and transfected into the L40 yeast strain. These cells were then transformed with a pAct *Drosophila* third instar larva cDNA library (Stephen J. Elledge, Baylor College of Medicine (29)), and positive colonies were isolated as described (30). Briefly, 11 million transformants were assayed for a positive two-hybrid interaction on histidine-deficient plates, followed by confirmation with a \( \beta \)-galactosidase filter assay. The bait plasmid was selectively removed, aided by ADE selection (30), and pACT fusions were isolated and sequenced. The \( \beta \)-galactosidase assay was scored as follows: blue color apparent by 15 min (\( ++ \)), blue color apparent by 30 min (\( ++ \)), blue color apparent by 45 min (\( + \)), and either no blue color apparent or blue color apparent after 1 h (\( - \)).

**Results**

**DSH3PX1 interacts with Dock**—To identify tyrosine-phosphorylated proteins that are capable of interacting with Dock, a hexahistidine-tagged Dock-SH2 domain was stably expressed in S2 cells (S2HisSH2). The overexpression of Dock-SH2 domain in these cells leads to the accumulation of specific tyrosine-phosphorylated proteins. This is presumably because the SH2 domain protects these phosphorylated proteins from endogenous tyrosine phosphatases. Proteins co-purified with the epitope-tagged SH2 domain of Dock were purified by nickel–agarose affinity chromatography and visualized by Western analysis using anti-phosphotyrosine antibodies (4G10) (Fig. 1A, left panel). The eluate from the nickel column was further purified by 4G10 affinity chromatography (6). Elution of the proteins from this column, subsequent separation by SDS-PAGE, and visualization by Coomassie staining resulted in the recovery of five protein bands (Fig. 1A, right panel). The band designated p63 was excised from the gel and digested with trypsin, and the peptides were separated by high pressure liquid chromatography. Sequence analysis of the tryptic peptides provided five support (Bio-Rad) according to the protocol supplied by the manufacturer. Eluted fractions containing antibody were combined and concentrated in 10\% phosphate-buffered saline using a Centricon Plus 20 centrifugal filtration device (Millipore Corp.).

**Immunoprecipitations**

Cell extracts were made from \( 1 \times 10^7 \) S2 cells. Cells were collected by centrifugation and lysed by repeated passage through a 25-gauge needle in 1 ml of RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.2 mM sodium vanadate, 10 mM NaF, 0.4 mM EDTA, 10% glycerol) containing protease inhibitors. Extracts were cleared by ultra centrifugation at 107,000 \( \times g \) for 30 min at 4 °C. 3 ml of affinity-purified antibody were added to 450 ml of extract supernatant and incubated at 4 °C on a rocker platform. After 1 h, 50 ml of Protein A–agarose (Life Technologies, Inc.) were added to bind immune complexes. Following an additional 1 h of incubation on the rocker platform at 4 °C, samples were washed four times with RIPA buffer, resuspended in 50 ml of Laemmli loading buffer, and stored frozen at –20 °C. For Western analysis, 10–15 ml samples were electrophoresed on SDS-polyacrylamide gels and transferred to Immobilon-P (Millipore) at 100–105 V for 1 h in transfer buffer (12 mM Tris base, 96 mM glycine, 20% methanol) cooled to –20 °C. Affinity-purified antibodies were used at the following dilutions: anti-DSH3PX1 (1:5000), anti-Dock (1:1000), anti-Dscam (1:1000), anti-Tyr(\( P \)) (1:5000), and anti-V5 (1:1000). All antibodies were diluted in blotting solution consisting of Tris-buffered saline with 5% dried milk and 0.1% Tween or consisting of 4% ovalbumin, 0.05% Tween for 46F1 anti-c-Myc (26) or goat anti-mouse IgG horseradish peroxidase-linked secondary antibody (1:3000) from Bio-Rad was used where appropriate. HRPL reagents (National Diagnostics) were used to detect immunoreactive proteins by chemiluminescence. Blots were exposed to Biomax MR film (Eastman Kodak Co.).

**GST-Dock in Vitro Pull-down Assay**—S2 cell extract was prepared by lysing \( 1 \times 10^7 \) cells in 4 ml of RIPA buffer as described above. GST-tagged proteins for each of Dock’s 3 SH3 domains and its SH2 domain were expressed in bacteria and purified on glutathione agarose (28). 1 ml of S2 extract was mixed with 10 \( \mu \)g of protein attached to beads, incubated for 1 h at 4 °C with rocking, washed three times with RIPA (1 ml each), and resuspended in 50 ml of Laemmli loading buffer. 5 \( \mu \)l were analyzed on a 10% SDS-polyacrylamide gel and Western blotted using antibodies directed against DSHP3PX1.

**Two-hybrid Screens**—Full-length DSHP3PX1 was fused in-frame into pLexAde and transfected into the L40 yeast strain. These cells were then transfected with a pAct *Drosophila* third instar larva cDNA library (Stephen J. Elledge, Baylor College of Medicine (29)), and positive colonies were isolated as described (30). Briefly, 11 million transformants were assayed for a positive two-hybrid interaction on histidine-deficient plates, followed by confirmation with a \( \beta \)-galactosidase filter assay. The bait plasmid was selectively removed, aided by ADE selection (30), and pACT fusions were isolated and sequenced. The \( \beta \)-galactosidase assay was scored as follows: blue color apparent by 15 min (\( ++ \)), blue color apparent by 30 min (\( ++ \)), blue color apparent by 45 min (\( + \)), and either no blue color apparent or blue color apparent after 1 h (\( - \)).
unambiguous amino acid sequences (underlined in Fig. 1B) that were used to search the Berkeley Drosophila Genome data base for corresponding expressed sequence tags. One expressed sequence tag, LD17205, was sequenced in its entirety, and it encoded the full-length protein sequence shown in Fig. 1B. All of the predicted peptides obtained for p63 were encoded in expressed sequence tag LD17205. p63 contained two well conserved domains, an NH2-terminal SH3 domain (gray shading) and an internal PX domain (black shading). In addition, the last 20 amino acids are predicted to form a coiled-coil (clear box) (31). p63 contains two proline-rich (pxxp) motifs which could serve as binding sites for SH3 domains. The first is present at amino acid 86 and the second is contained within the PX domain at amino acid 274 (Fig. 1B, gray boxes) (10). The presence of a PX domain places p63 in the growing family of hydrophilic membrane-associated sorting nexins believed to be involved in vesicular trafficking and targeting (8, 9, 32). The presence of the SH3 domain is uncommon for sorting nexins, but it is found in human sorting nexin 9 (SNX9), also known as SH3PX1 (33). Sequence alignment of p63 with human and Caenorhabditis elegans orthologues shows a high degree of identity, especially in the SH3 and PX domains (Fig. 2). Furthermore, the proline-rich sequence found in the PX domain is conserved among these species, while the PXXP motif present at amino acid 86 is not conserved in the human orthologue (Fig. 2). Based on the similar domain arrangement and high degree of identity, we named Drosophila p63 DSH3PX1.

Since DSH3PX1 had been purified using the SH2 domain of Dock, we thought it was important to demonstrate that the two molecules associate with one another in vivo. Fig. 3A shows that DSH3PX1 co-immunoprecipitates with Dock from S2 cell extracts. However, only a fraction of the endogenous DSH3PX1 is tyrosine-phosphorylated, and it is this form of the protein that is preferentially co-immunoprecipitated by Dock-specific antibodies, as is evident from Fig. 3B. The most obvious conclusion to draw from these results is that Dock's SH2 domain mediates the association with DSH3PX1-pY. However, since DSH3PX1 also contains two PXXP motifs, it is conceivable that the Dock SH3 domains could also participate in the observed protein-protein interaction. To better define the binding of DSH3PX1 and Dock, GST fusions of the three SH3 domains and the SH2 domain of Dock were isolated from bacteria and used in an in vitro pull-down assay. Protein extracts were prepared from S2 cells, and pull-down assays using the GST fusion proteins are shown in Fig. 3C. In S2 cell extracts, the SH3.3 domain is able to interact weakly with DSH3PX1 either alone or in the context of SH3 domains 1–3. However, the principal interaction between DSH3PX1 and Dock appears to be through Dock's SH2 domain.

DSH3PX1 Is Present in a Complex with Dscam and the Clathrin Coat Adaptor Protein, AP-50—Since DSH3PX1 resembles a sorting nexin, we sought to determine if it is capable of interacting with Dscam and, if so, whether the interaction is dependent upon the presence of Dock. Other sorting nexins have been shown to be associated with specific subsets of cell...
In untreated S2 cell extract (UT), DSH3PX1 immunoprecipitates with Dscam antiserum (Fig. 4A). Using RNAi to abrogate the expression of DSH3PX1 (DSH3PX1i) verifies that the protein with an apparent molecular mass of 63 is indeed DSH3PX1 (26). Using double-stranded RNA to eliminate Dock expression (Docki) had no effect on the association between DSH3PX1 and Dscam (Fig. 4A). These results suggest that Dock is not a bridging molecule required for Dscam’s association with DSH3PX1.

Cell surface receptors are also commonly endocytosed in clathrin-coated vesicles and targeted for either degradation or recycling to the plasma membrane (23, 34). The clathrin adaptor protein, AP-50, is a member of the AP-2 complex that recognizes proteins destined for internalization in clathrin-coated vesicles (35). We therefore sought to determine if DSH3PX1 is in a complex with AP-50. Stably introducing a V5 epitope-tagged AP-50 construct into S2 cells, followed by immunoprecipitation with V5 antiserum, verifies the association of DSH3PX1 with AP-50 (Fig. 4B). To date, we have been unable to demonstrate that Dscam will co-immunoprecipitate with antibodies directed against AP-50. This suggests that DSH3PX1 could play an important role in bridging cell surface receptors with proteins in clathrin-coated vesicles.

**DSH3PX1 Interacts with Proteins Involved in Cytoskeletal Rearrangements**—We are interested in the mechanisms by which Dscam could transduce signals leading to cytoskeletal rearrangements necessary for axonal extension along a distinct trajectory. Since Dscam exists in a complex with DSH3PX1, we reasoned that studying the protein-protein interactions of DSH3PX1 would lend insight into the downstream effectors of Dscam’s signal transduction. To identify proteins that interact with DSH3PX1, we conducted a yeast two-hybrid screen of a third instar *Drosophila* library using full-length DSH3PX1 as the bait. 12 of the 34 interacting clones represented DSH3PX1 itself. This interaction was not dependent upon its SH3 or PX domains, suggesting that DSH3PX1 contains a dimerization domain in its COOH terminus. Analysis of DSH3PX1 sequence using the Coils program indicates a high probability of the COOH-terminal 20 amino acids forming a coiled-coil structure that could promote dimerization or the formation of higher order multimers (31). Other two-hybrid positives included Wasp, a known regulator of the actin cytoskeleton that is also involved in the process of endocytosis (36).

Our two-hybrid library plasmid for Wasp contained sequences coding for the COOH-terminal two-thirds of the protein, which includes the GBD/CRIB, proline-rich, verpolin homology, and cofilin homology domains. To obtain the full-length sequence for *Drosophila* Wasp, two additional expressed sequence tags, LP11964 and GH10436, were sequenced in their entirety. During the preparation of this manuscript, a sequence identical to ours for *Drosophila* Wasp was reported (37). The protein is 527 amino acids in length and contains the same domain organization as its mammalian counterpart (Fig. 6), namely an amino-terminal pleckstrin homology domain that binds phospholipids, an IQ domain that mediates calcium binding, a CRIB domain that binds Rac and Cdc42, a proline-rich region, two verpolin homology domains, and a cofilin homology domain (14). The proline-rich sequences of mammalian Wasp
Coomassie-stained Dock GST fusion proteins present in each reaction is indicated in the affinity-purified antibodies directed against DSH3PX1. The amount of proteins to bind DSH3PX1 was assessed by Western analysis using extracts prepared from S2 cells. The ability of the GST fusion proteins of the indicated Dock domains prepared in bacteria were mixed and monitored by Western blotting using DSH3PX1 antibodies. An arrow indicates DSH3PX1. In cells that lack Dock (Docki), Dock immunoprecipitates concentrate the tyrosine-phosphorylated form of DSH3PX1. Dock and DSH3PX1 immunoprecipitates (IP) from S2 cell extracts were analyzed by Western blotting with Tyr(P) antibodies. An arrow indicates DSH3PX1.

In vivo and in vitro analyses of Dock’s association with DSH3PX1. A, Dock associates with DSH3PX1 in S2 cells. Dock and DSH3PX1 immunoprecipitates (IP) from S2 cell extracts were analyzed by Western blotting using DSH3PX1 antibodies. An arrow indicates DSH3PX1. B, Dock immunoprecipitates concentrate the tyrosine-phosphorylated form of DSH3PX1. Dock and DSH3PX1 immunoprecipitates from S2 cell extracts were analyzed by Western blotting with Tyr(P) (pTyr) antibodies. An arrow indicates DSH3PX1. C, in vitro analysis of the ability of Dock GST fusions to interact with DSH3PX1. GST fusion proteins of the indicated Dock domains prepared in bacteria were mixed and monitored by Western blotting using DSH3PX1 antibodies. An arrow indicates DSH3PX1 was assessed by Western analysis using affinity-purified antibodies directed against DSH3PX1. The amount of Dock GST fusion proteins present in each reaction is indicated in the Coomassie-stained panel.

have been shown to bind a number of different SH3 domain-containing proteins including Nck (38). Our two-hybrid results indicate that DSH3PX1 interacts with Wasp via its SH3 domain (Table I). To verify our two-hybrid result, we demonstrated that in S2 cells stably expressing Wasp engineered to contain a COOH-terminal V5-tag, DSH3PX1 co-immunoprecipitates with Wasp (Fig. 5, left panel). As expected, this co-immunoprecipitation is abolished in extracts prepared from cells exposed to double-stranded RNA for DSH3PX1 (DSH3PX1i). In cells that lack Dock (Docki), DSH3PX1 co-immunoprecipitates with Wasp as effectively as in untreated cells. Dock also immunoprecipitates the same amount of DSH3PX1 in the presence (UT) or absence of Wasp (Waspi) in these cell extracts (Fig. 5, right panel). Therefore, there is no competition for DSH3PX1 between these two binding partners, suggesting that Dock is interacting with DSH3PX1 via a phosphorylated tyrosine residue, while Wasp is interacting with DSH3PX1 via its SH3 domain. Anti-Tyr(P) Western analysis of Wasp immunoprecipitates indicates that DSH3PX1 is not tyrosine-phosphorylated when associated with Wasp (data not shown).

Protein-Protein Interactions among Dock, DSH3PX1, Wasp, and Dscam—We are interested in deciphering the protein-protein interactions among DSH3PX1, Dock, Wasp, and Dscam to further understand how these proteins contribute to directed growth cone motility. The results of a directed two-hybrid screen to address these interactions are shown in Table I. A full-length Dock bait is able to interact with Wasp and Dscam (Table I). This is consistent with previously published results demonstrating the interaction between Dock-SH3.1–3 domains and proline-rich sequences in Decam (6) and with the data demonstrating that mammalian Nck will interact with mammalian Wasp (18, 38). In agreement with Rohatgi et al. (18), in vitro pull-down assays using GST fusions of Dock’s SH3 domains indicate that Drosophila Wasp does not interact with the Dock-SH3.1 or SH3.3 domains but does interact weakly with the Dock-SH3.2 domain and strongly with the fusion protein containing all three of Dock’s SH3 domains (data not shown).

In addition, Dock can bind to truncated forms of DSH3PX1 (Δ136) that do not contain the SH3 domain or the first PX domain. However, further truncation of the sequence to amino acid 361 (Δ361) abolishes binding, suggesting that one or more of Dock’s SH3 domains can interact with sequences spanning amino acids 136–361. This region includes the PX domain that contains DSH3PX1’s second PX domain. It is interesting that Dock cannot interact with full-length DSH3PX1, suggesting that DSH3PX1 may be in a conformation in which the second PX domain is unavailable for interaction with other SH3 domains. Full-length DSH3PX1 interacts with Wasp via its SH3 domain as well as with all truncated versions of itself presumably via the CC region. As indicated by the qualitative assessment of β-galactosidase activity, DSH3PX1’s SH3 domain interacts more strongly with Wasp’s proline-rich sequences than with Dscam’s proline-rich sequences or with its own sequences spanning amino acids 136–361. As expected, the COOH-terminal bait construct for DSH3PX1 interacts with versions of itself, all of which contain the CC region. Taken together, these results suggest that DSH3PX1 exists as a dimer or multimer whose SH3 domain strongly interacts with Wasp. There is also a growing body of evidence that suggests that PX domains bind phosphatidylinositol 3-phosphate and phosphatidylinositol 3,4-bisphosphate (12, 39). Likewise, recent studies reviewed by Wishart et al. (13) speculate that the availability of the PX domain to bind lipid can be controlled by an intramolecular...
interaction between the SH3 domain and a PXXP motif found within the PX domain. This raises the intriguing possibility that DSH3PX1’s protein-protein interactions could be regulated by an intramolecular interaction between its SH3 domain and the PXXP motif present in its PX domain. Finally, it is clear that an unspecified tyrosine kinase can phosphorylate DSH3PX1 and that modification modulates its interaction with Dock. Possible DSH3PX1 protein and lipid interactions are summarized in Fig. 6.

DISCUSSION

The most compelling evidence for the involvement of sorting nexins in vesicular trafficking has been reported in yeast. For example, Vps5p, the S. cerevisiae orthologue of SNX1, is a subunit of the retromer complex and is involved in recycling the carboxypeptidase Y receptor from endosomes to the trans-Golgi network (40). The yeast orthologue of SNX3, Grd19, maintains the localization of two late Golgi enzymes, dipeptidyl amino peptidase A and Kex2, by retrieving molecules from prevacuolar endosomes (41). Additionally, Mvp1p is believed to participate in the formation of vesicles that facilitate vacuolar protein targeting (42). In mammalian studies, SNX1 is involved in regulating the endocytosis of ligand-activated epidermal growth factor receptor (8), while SNX15 is involved in the internalization and degradation of the platelet-derived growth factor receptor as well as affecting the post-translational processing of the proreceptors for insulin and hepatocyte growth factor (32). SNX2 and SNX4 have been shown to associate with the platelet-derived growth factor receptor, the insulin receptor, and the long isoform of the leptin receptor as well as epidermal growth factor receptor but have yet to be associated with increased receptor turnover (9). Therefore, there is a strong correlation between PX domain-containing proteins and vesicular trafficking.

In this study, we have identified a novel Drosophila sorting nexin, DSH3PX1, and determined that tyrosine-phosphorylated DSH3PX1 interacts with the SH2 domain of the axon guidance adaptor protein Dock. We have further characterized its protein-protein interactions by co-immunoprecipitation, in vitro pull-down, and two-hybrid analyses. Our results link the axon guidance receptor Dscam to DSH3PX1, AP-50, and Wasp proteins that may form part of the machinery necessary for receptor internalization and sorting (Fig. 6).

As discussed previously, DSH3PX1 is abundantly expressed in S2 cells, but only a small fraction of the protein is tyrosine-phosphorylated. Increased levels of DSH3PX1 tyrosine phosphorylation can be generated in response to overexpression of the Dock-SH2 domain or vanadate treatment (data not shown). Moreover, most of the tyrosine-phosphorylated DSH3PX1 can be immunoprecipitated with Dock antibodies, indicating that tyrosine phosphorylation may target DSH3PX1 to a new cellular program involving the Dock-Dscam receptor complex. Presumably, the remaining pool of unphosphorylated DSH3PX1 is involved in other cellular activities. Given the association of DSH3PX1 with Dscam and Dock, a potential role for DSH3PX1 in axon guidance was examined in Drosophila embryos using RNAi. Unfortunately, pleiotropic effects were observed in knockout embryos that disrupted the development of head structures and made assessment of axon guidance defects

### Table I

| TABLE I | DSH3PX1 Protein Interactions |
|---------|-----------------------------|
| LIBRARY | DSH3PX1 Protein Interactions |
| Dock   | SH3 (Δ136) | SH3 (Δ361) | Wasp | Dscam | VP16 | Raf |
| Dock   | 1            | 1            | 1    | 1    | 1    | 1    |
| DSH3PX1| 1            | 1            | 1    | 1    | 1    | 1    |
| DSH3PX1| 1            | 1            | 1    | 1    | 1    | 1    |
| DSH3PX1| 1            | 1            | 1    | 1    | 1    | 1    |
| Lamin  | 1            | 1            | 1    | 1    | 1    | 1    |
| Ras    | 1            | 1            | 1    | 1    | 1    | 1    |

**Fig. 5.** Different pools of DSH3PX1 associate with Dock and Wasp. Extracts were prepared from S2 cells stably expressing Wasp with a COOH-terminal V5 tag. Untreated extracts (UT) or extracts depleted of the indicated proteins (Wasp, DSH3PX1, or Dock) were immunoprecipitated (IP) with antibodies against Dock or V5 (Wasp). The presence of DSH3PX1 was detected by Western analysis using affinity-purified DSH3PX1 antibody.
Fig. 6. DSH3PX1 protein and lipid interactions. In the schematic representation of Dscam, blackened ovals represent IgG repeats, while blackened rectangles represent fibronectin repeats. The DSH3PX1 schematic consists of an SH3 domain (shaded gray) and a PX domain (shaded black). The penultimate 20 amino acids are predicted to form a coiled-coil (CC, clear box). The schematic of Dock contains three SH3 domains, as indicated, and an SH2 domain represented by a blackened oval. In the schematic of Drosophila Wasp, the phospholipid-binding domain (PH) is represented by a stippled oval, the Ca\textsuperscript{2+} binding domain (IQ) is represented as a blackened box, and the Cdc42-binding domain (GBD/CRIB) is depicted by a clear oval. The proline-rich sequence is represented by a box, and the verpolin (VH) and cofilin (CH) binding domains are depicted by stippled hexagons and a blackened oval, respectively. All domain predictions were made using the SMART program (52). The arrows indicate interactions between protein and lipids present in this complex, and arrows with question marks indicate interactions that may not be direct. In the case of the Dock-Dscam interaction, all three Dock SH3 domains are known to interact with several PX/PH motifs present in Dscam (6). In addition, Dock’s SH2 domain is capable of interacting with tyrosine-phosphorylated Dscam (interaction not indicated). Similarly, all three SH3 domains of Dock are required for efficient interaction with Wasp as discussed in Protein-Protein Interactions among Dock, DSH3PX1, Wasp, and Dscam in "Results.”

possible to score. Therefore, any role for DSH3PX1 in vesicular trafficking is likely to be more global than merely regulating the presentation of the Dscam receptor on the plasma membrane of growth cones to control axon guidance.

Since DSH3PX1 can be co-immunoprecipitated with Dscam and AP-50, it is tempting to speculate that this molecule is involved in sorting Dscam from the plasma membrane via clathrin-coated pits to other cellular compartments that direct it either for recycling or degradation. Since neither Dscam nor AP-50 was represented as positive in our two-hybrid screen, it is possible that these interactions are not direct but rely on bridging molecules (Fig. 6). We have started to address this question by the removal of potential bridging partners from the cells using RNAi technology (26). By the removal of Dock from the S2 cells, we can show that the co-immunoprecipitation of DSH3PX1 with Dscam does not depend upon the presence of this protein. Additional experiments of this type will have to be performed using combinations of double-stranded RNAs to decipher the important protein-protein interactions maintaining this complex of proteins surrounding the Dscam receptor.

It seems reasonable that one of the ways a growth cone could direct its growth would be to cycle guidance receptors to and from its plasma membrane to sample the surrounding environment for directional cues. In this scenario, the appearance of guidance receptors on the plasma membrane and their timely removal after ligand stimulation would be of critical importance to directing growth. Wasp, one of our two-hybrid positives, could be instrumental in effecting the cytoskeletal changes necessary for endocytosis or growth cone remodeling. Wasp is a multidomain protein whose COOH terminus contains verpolin and cofilin homology domains (14). These domains act in concert with the Arp2/3 complex to polymerize globular actin into filaments (43). Wasp can be activated by binding GTP-Cdc42 or by interactions of specific SH3 domains with its proline-rich sequences. The proline-rich sequences in mammalian Wasp have been shown to bind a large number of SH3 domain-containing proteins including Nck (18). In S2 cells, Dock’s SH3 domains are known to interact with Dscam, DPTP61F, and Pak (6, 44, 45). All three SH3 domains interact with Dscam, while the SH3.2 domain interacts with DPTP61F or Pak. In in vitro pull-down assays, all three SH3 domains interact with Wasp (data not shown), while SH3.3 interacts with DSH3PX1. It is interesting to speculate that depending upon the intracellular signaling environment Wasp, Dock, and SH3PX1 may be involved in different protein-protein interactions and therefore in different aspects of actin cytoskeletal regulation.

Until recently, Wasp’s role in endocytosis was not appreciated. In mammalian cells, it was reported that actin-binding proteins such as DNase I and thymosin B4 or drugs such as latrunculin A, which selectively sequester actin monomers, inhibit the formation of clathrin-coated vesicles at the plasma membrane (46). Furthermore, it has been suggested that N-Wasp participates in vesicle transport at nerve endings by transmitting signals from tyrosine kinases to cortical actin filaments (17). This view is supported by immunostaining and tissue fractionation studies, showing that N-Wasp is concen-
trated at nerve endings (17). Taken together, it seems likely that Wasp in conjunction with a sorting nexin could play a variety of roles in the growth cone depending upon the signaling milieu.

The sorting nexin family is defined by the presence of a PX domain, a domain that was originally discovered in proteins involved in the regulation of NADPH oxidase and is also found in a subset of phosphatidylinositol 3-kinases, Saccharomyces cerevisiae Bem1p, and Schizosaccharomyces pombe Scd2 (10). The PX domain commonly contains a proline-rich sequence that could potentially bind an SH3 domain. In the case of the PX domain-containing proteins involved in the regulation of NADPH oxidase, p47phox and p67phox, the SH3 domain-mediated interactions are regulated. p47phox contains two SH3 domains and an SH3 domain target sequence in addition to a PX domain. In both cases, a proposed conversion from intramolecular interactions to intermolecular SH3-polyproline interactions occurs in response to extracellular signals to up-regulate NADPH oxidase activity (47). The SH3 domain of DSH3PX1 is also capable of interactions involving sequences that span its PX domain. It is intriguing that Dock cannot interact with full-length DSH3PX1, raising the possibility that DSH3PX1’s protein-protein interactions may be hindered by an intramolecular interaction.

Mammalian SH3PX1 (also known as SNX9) has been identified through a two-hybrid screen using the intracellular portions of the metalloprotease disintegrins, MDC9 or MDC15, as the baits (33). These metalloproteases are part of a family of membrane-anchored glycoproteins (ADAMS) that function among other things in neurogenesis, myogenesis, and ectoderm processing of cytokines and other proteins (48–51). These proteases also interact with endophilin I, which is thought to play a role in synaptic vesicle endocytosis (33). The fact that DSH3PX1 interacts with Dscam as well as MDC9 and MDC15 could imply that the sorting nexins has the ability to interact with numerous cell surface receptors.

In summary, we have characterized the intra- and intermolecular interactions of DSH3PX1. This is the first report of a tyrosine-phosphorylated sorting nexin, a modification that most likely transforms DSH3PX1 into a binding partner for Dock. We extend this complex to contain Dscam, AP-50, and Wasp. DSH3PX1’s ability to interact with Wasp and AP-50 links clathrin-coated pits to the actin cytoskeleton, suggesting that one of DSH3PX1’s roles may be to serve as a sorting nexin for the Dscam axon guidance receptor.

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Carolyn A. Worby, Nancy Simonson-Leff, James C. Clemens, Robert P. Kruger, Marco Muda and Jack E. Dixon

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