DAck targets its substrate, the sorting nexin, DSH3PX1, to a protein complex involved in axonal guidance

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Summary

Dock, the *Drosophila* orthologue of Nck, is an adaptor protein that is known to function in axonal guidance paradigms in the fly including proper development of neuronal connections in photoreceptor cells and axonal tracking in Bolwig’s organ. To develop a better understanding of axonal guidance at the molecular level, we purified proteins in a complex with the SH2 domain of Dock from fly S2 cells. A protein designated p145 was identified and shown to be a tyrosine kinase with sequence similarity to mammalian ACKs. We demonstrate that *Drosophila* Ack can be co-immunoprecipitated with Dock and DSH3PX1 from fly cell extracts. The domains responsible for the *in vitro* interaction between DAck and Dock were identified and direct protein-protein interactions between complex members were established. We conclude that DSH3PX1 is a substrate for DAck in vivo and in vitro and define one of the major in vitro sites of DSH3PX1 phosphorylation to be Tyr 56. Tyr 56 is located within the SH3 domain of DSH3PX1 placing it in an important position for regulating the binding of proline-rich targets. We demonstrate that Tyr 56 phosphorylation by DAck diminishes the DSH3PX1 SH3 domain interaction with the Wiskott-Aldrich Syndrome protein (WASP) while enabling DSH3PX1 to associate with Dock. Furthermore, when Tyr 56 is mutated to aspartate or glutamate, binding to WASP is abrogated. These results suggest that phosphorylation of DSH3PX1 by DAck targets this sorting nexin to a protein complex that includes Dock, an adaptor protein important for axonal guidance.
Introduction

To form precise patterns of connections between cells, guidance receptors expressed on neuronal growth cones respond to extracellular signals provided by the developing nervous system (1). Interestingly, a particular guidance signal may act as either a repellent or an attractant dependent solely upon the activity of the neuron’s intracellular signal transduction pathways (2). Thus, understanding how individual neurons translate extracellular signals into the cytoskeletal rearrangements necessary for the directed movement of their growth cones requires the molecular identification of protein-protein interactions within the guidance receptor complex.

Previous reports have proposed that Dock, the *Drosophila* orthologue of the mammalian SH3/SH2 adaptor protein, Nck, links guidance signals to changes in the actin cytoskeleton in photoreceptor growth cones (3). Recently, using the epitope-tagged SH2 domain of Dock, we biochemically purified five proteins (molecular weights 270, 145, 74, 69, and 63 kDa), which are present in a putative complex with the SH2 domain of Dock. Further characterization revealed that p270 is a novel receptor-like protein that is a member of the immunoglobulin superfamily and shares extensive sequence similarity to the human Down’s Syndrome Cell Adhesion Molecule (Dscam). Additionally, we demonstrated that Dscam lies upstream of Dock signaling and is important for normal axonal pathfinding in the developing nervous system in the fly (4).

The second protein that was characterized from the collection of Dock SH2 domain interacting proteins was the 63 kDa protein identified as the SH3 and PX domain containing protein, DSH3PX1 (5). DSH3PX1 is a member of the sorting nexin family and is able to interact with both Dock and Dscam (5). Importantly, we also demonstrated that the SH3 domain of DSH3PX1 interacts with Wiskott-Aldrich syndrome protein (WASP), a protein involved in regulating the actin cytoskeleton (5). This suggests that DSH3PX1 may link the Dscam receptor complex to cytoskeletal modification machinery.
Here we describe the identification of the 145 kDa protein, which we have named Drosophila Ack (DAck). Mammalian ACKs are tyrosine kinases that are specifically activated by Cdc42, but not by Rac or Rho (6,7). ACK1 is activated by stimulation of M3 muscarinic receptors via a mechanism that requires the Src-like kinase, Fyn (8). Association of ACK1 with Cdc42 may result in sustained activation of Cdc42 (6). In addition, ACK1 has recently been shown to interact with Nck via its proline-rich regions and with clathrin via a clathrin-binding motif (9). ACK2, a smaller splice variant of ACK1, is stimulated by bradykinin, epidermal growth factor (EGF) or integrin β1-mediated cell adhesion (10). Similar to ACK1, ACK2 activity is regulated by Src and it also interacts with clathrin (11,12). Recently, ARK-1, the Caenorhabditis elegans orthologue of ACK1, was shown to negatively regulate ERF receptor (EGFR) signaling in a Grb 2 dependent manner (13).

This report describes the isolation, identification and characterization of DAck. We demonstrate that DAck co-immunoprecipitates in a cellular complex with Dock and DSH3PX1 and confirm these observations with a systematic yeast two-hybrid analysis. The specific domains by which Dock directly interacts with DAck are identified in vitro by GST pull down assays employing various GST-Dock fusion proteins. We also demonstrate that DSH3PX1 is a substrate for DAck in vivo and in vitro and determine that a major site of DSH3PX1 phosphorylation is a conserved tyrosine residue (Y56) present in its SH3 domain. The phosphorylation of this tyrosine residue reduces the binding of DSH3PX1 with WASP, an interaction previously shown to require the DSH3PX1 SH3 domain (5). Instead, tyrosine phosphorylated DSH3PX1 interacts with Dock via its SH2 domain. This suggests that phosphorylation of DSH3PX1 by DAck changes its preference for protein interaction partners.

Materials and Methods
S2 cell culture, transfections and RNA interference (RNAi)- Schneider 2 (S2) cells were grown in Schneider Drosophila Medium (GIBCO) supplemented with 10% fetal bovine serum (FBS), 50
units/ml penicillin, and 50 µg/ml streptomycin in 75-cm² cell culture flasks (Corning) at room temperature.

S2 cells were transfected with the *Drosophila* expression vector pMT/V5-His (Invitrogen) containing wild type or mutant DAck coding sequences for the generation of stable transformants using Fugene (Roche) as previously described (5). To induce protein synthesis, CuSO₄ was added to the MT/V5-His stable transformants at a final concentration of 500 µM 48 h prior to harvest.

RNAi experiments were conducted as described previously (14). Primers used to create the PCR template for dsRNA production incorporate the T7 promoter 5’ to the gene specific sequences. Primer sequences used to make PCR templates are as follows: DSH3PX1, GenBank accession no. AF223381, sense-primer 1124-1139, antisense-primer 1749-1765; DAck, GenBank accession no. AF181642, sense-primer 2197-2213, anti-sense 2807-2824; Src42, accession no. D42125, sense-primer 977-997, anti-sense 1710-1733; Src64, accession no. M11917, sense-primer 688-710, anti-sense 1393-1415; Shark, accession no. U37773, sense-primer 990-1014, anti-sense 1713-1732; Abl, accession no. M19692, sense-primer 2318-2338, anti-sense 3497-3513.

**Two-hybrid screen** – The selected two-hybrid screen was run as previously described (5). The β-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 hr. (-).

**Antibody affinity purification, Co-Immunoprecipitation and Western analysis - Polyclonal antisera** were raised in rabbits against recombinant fusion proteins for Dock, DSH3PX1, and DAck (Cocalico, Reasmtown, PA). The pTyr antibody (4G10) and V5-epitope antibody were purchased from Upstate Biotechnology and Invitrogen, respectively. Affinity columns for purification of antisera were produced by coupling the appropriate recombinant fusion protein to
an Affigel support (Biorad) according to the protocol supplied by the manufacturer. Eluted
fractions containing antibody were combined and concentrated in 1X PBS using a Centricon
plus-20 centrifugal filtration device (Millipore). Affinity purified antibodies were stored in
aliquotes at –20°C.

Cell extracts for immunoprecipitations were made from ~1*10⁷ Schneider 2 cells. Cells
were collected by centrifugation and lysed by repeated passage through a 25G needle in 1 ml
RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 0.2
mM sodium vanadate, 10 mM NaF, 0.4 mM EDTA, 10 % glycerol) containing protease
inhibitors. Immunoprecipitations and Western analyses were carried out as previously described
(5).

GST-Dock/GST-SH3PX1 in vitro pull-down assay - S2 cell extract was prepared by lysing 2*10⁷
cells per ml Ripa buffer as described above. GST-tagged proteins for each of Dock’s SH3
domains, its SH2 domain, and the SH3 domain of DSH3PX1 were expressed in bacteria and
purified on glutathione agarose (15). 1 ml of S2 cell extract (~2X10⁷ cells/ml) prepared in Lysis
buffer (50 mM Tris, pH 8.0, 100 mM Nacl, and 1% NP40) was mixed with ~10 µg of protein
attached to beads, incubated for 1 h at 4°C with rocking, washed 3 times with Lysis buffer (1 ml
each), and resuspended in 50 µl Laemmli loading buffer. 5-10 µl were analyzed on a 10% SDS-
polyacrylamide gel and Western blotted using antibodies directed against DAck.

In vitro kinase assay - S2 extracts used for DAck kinase reactions were made using RIPA buffer
minus deoxycholate and SDS (RIPA kinase). Immunoprecipitations were as follows: lysates
were cleared by centrifugation at 14,000 x g at 4°C for 20 minutes and incubated at 4°C mixing
for 1 hour with 25 µl of FLAG beads (Sigma). Beads were washed 3 times with 1 ml RIPA
kinase, 4 times with 1ml RIPA kinase containing 1M NaCl and 2 times with kinase buffer (25
mM Tris, pH 7.4, 3 mM MgCl₂, 3 mM MnCl₂, 0.5 mM EGTA, 0.1 mM NaVO₄, 0.5 mM DTT).
10 µl of FLAG beads were mixed with 10 µg of bacterially expressed wild type or mutant
DSH3PX1, incubated for 30 min. at 25 °C in the presence of 100 µM ATP spiked with 10 µCi of \([\gamma-^{32}P]\) ATP (6000Ci/mmol). Mutants were made in GST-DSH3PX1 using the QuikChange Site-Directed mutagenesis kit (Stratagene). The reaction was terminated by adding Laemmli loading buffer and analyzed by SDS gel electrophoresis followed by autoradiography.

Pull down analysis using recombinant Dock and immunoprecipitated WASP – The in vitro kinase reaction was performed as previously described except approximately 1-2 µg of recombinant GST-DSH3PX1 was used in the kinase reaction along with DACK (released from the FLAG beads by the addition of 50 µg of FLAG peptide) in a final volume of 100 µl for 2-3h at 25°C. Under these conditions the stoichiometry of phosphorylation of DSH3PX1 ranges between 30-50%. His-tagged Dock was purified from bacteria using Ni-agarose as described by the manufacturer (Qiagen). V5-tagged WASP stably expressed in S2 cells was immunoprecipitated from cells treated with dsRNA directed against DSH3PX1 (DSH3PX1 RNAi) as previously described (5). Approximately 0.5-1 µg of His-tagged Dock and immunoprecipitated WASP were used per binding reaction as analyzed by silver staining.

Binding reactions (200 µl) containing 20 µl of the protein affinity resins and 20 µl of the kinase reaction (+/- kinase) were rocked for 1h at 4°C followed by 3 washes using RIPA kinase buffer. Beads were resuspended in 50 µl of Laemmli sample buffer and analyzed by SDS-PAGE followed by Western analysis using anti-DSH3PX1 (1:5000).

Results and Discussion

Dock was first described by Garrity et al. (3), as an adaptor protein playing a critical role in axonal pathfinding. Since tyrosine phosphorylation is known to play an important role in the development of the fly nervous system (16-18), we were interested in the interactions directed by the SH2 domain of Dock. In an effort to identify tyrosine phosphorylated proteins that interact with Dock, the SH2 domain of Dock was over-expressed as a His-tagged protein in Drosophila S2 cells (4, 5 and JCC, manuscript in preparation). Protein lysates were prepared from S2 cells...
and were subjected to Ni-agarose affinity chromatography followed by purification on a p-Tyr-specific antibody (4G10) affinity column. Proteins eluted from the anti-p-Tyr affinity column were separated by SDS-PAGE electrophoresis and visualized by Coomassie staining. A protein of 145 kDa was excised from the gel, digested with trypsin and the peptides purified by HPLC for sequence analysis.

Sequence determination of the tryptic peptides obtained from p145 resulted in seven unambiguous amino acid sequences (underlined in Fig. 1) that were used to search the Berkeley Drosophila Genome database for corresponding expressed sequence tags (ESTs). Four overlapping ests, GH10884, GH07655, GH11561, and LD18821, in conjunction with 5’ RACE products were completely sequenced in order to obtain a full length nucleotide sequence encoding p145. The cDNA contains an open reading frame of 3219 nucleotides encoding a tyrosine kinase (light gray box) with sequence similarity to mammalian kinases of the ACK family. Immediately following the kinase domain is an SH3 domain (dark gray box) followed by a COOH-terminal ubiquitin associated (UBA) domain (black box) (Fig. 1). The UBA domain is a motif found in UV excision repair proteins, certain protein kinases and proteins playing roles in the ubiquitination pathway (19). Although the role of this domain is not known, it has been suggested that UBA domains are involved in conferring target specificity to enzymes of the ubiquitination system (19). To date, there have been no reports of ACK ubiquitination, but we have observed that DAck is rapidly processed into smaller fragments in S2 cell extracts (data not shown).

Between the SH3 domain and the UBA domain, DAck’s COOH-terminal sequence contains four proline-rich (PXXP) motifs (clear boxes) which could be involved in interactions with SH3 domains. There are also six YXXφ motifs (bold print) which are thought to be important in vesicle trafficking particularly with regard to interactions with the adaptor proteins in clathrin coated vesicles (Fig. 1) (20). Moreover, a putative clathrin binding core motif, LIDIS,
is also found in this region of DAck (Fig. 1, gray box with white lettering). Mammalian ACK1 and ACK2 have recently been shown to interact directly with clathrin using this conserved motif (9,11). The most distinctive differences between mammalian ACKs and DAck are the apparent absence of a Cdc42 binding domain (CRIB domain) and the presence of a UBA domain in the Drosophila enzyme. The apparent absence of a CRIB domain in DAck raises the intriguing possibility that this enzyme may be activated by a different mechanism than mammalian ACKs.

Since DAck and DSH3PX1 were purified from S2 cell extracts using the SH2 domain of Dock, we thought it was important to determine whether these molecules associate with one another in vivo. DSH3PX1 can be co-immunoprecipitated with both DAck and Dock from S2 cell extracts (Fig. 2A) (5). Furthermore, DAck can also be co-immunoprecipitated with Dock (Fig. 2B). Since DAck contains several proline-rich motifs, we wanted to know if Dock’s SH3 domains and/or its SH2 domain interacts with DAck. GST-fusions of Dock’s SH3 domains and SH2 domain were produced in bacteria and were used in a pull down assay in conjunction with S2 cell extracts as the source of interacting proteins. Under these conditions, Dock interacts with DAck via its SH2 domain (Fig. 2C). In the case of mammalian Nck and ACK1, it has been reported that Nck’s SH3 domains are able to bind to ACK1 in an overlay assay (9). The discrepancy between our results and Teo et al. (9) most likely results from the type of assay employed. It is possible that DAck isolated from S2 cell extract is conformationally unavailable for binding to Dock’s SH3 domains. However, we also tested the ability of the DSH3PX1 SH3 domain to interact with DAck. As can be seen in Figure 2C, the SH3 domain of DSH3PX1 interacts strongly with DAck in S2 cell extracts, suggesting that at least one of DAck’s proline-rich (PXXP) motifs is available for protein-protein interactions.

Since DAck was isolated by virtue of its ability to interact with the Dock SH2 domain, we sought to dissect the potential protein/protein interactions between Dock complex proteins using a directed two-hybrid screen (5). Dock interacts strongly with full length (FL) DAck but
not with a COOH-terminally truncated DAck construct consisting of amino acids 1-507 (Table I). It was not possible to use full length DAck as a bait due to autoactivation. Since the DAck constructs encode the active kinase, removal of the COOH-terminal sequence most likely removes an auto-phosphorylation site important for Dock SH2 domain binding (Fig. 2C). Full length DSH3PX1 as well as the DSH3PX1 SH3 domain also interact strongly with full length DAck but not truncated DAck, presumably due to the removal of one or more of DAck’s COOH-terminal PXXP motifs. As previously reported, Dock and the DSH3PX1 SH3 domain are capable of interacting with Dscam (4,5). However, DAck (1-507) that contains the kinase domain plus the SH3 domain is not capable of interacting with any of the Dock complex proteins tested. Therefore, the binding partners for the DAck SH3 domain are most likely not present in this group. Interestingly, our two-hybrid results suggest that Dock is able to interact with itself. This possibility would effectively increase Dock’s ability to bring together a variety of molecules simultaneously.

Since DAck co-immunoprecipitates (Fig. 2A) and directly interacts (two-hybrid data) with DSH3PX1, we sought to determine if DSH3PX1 is an in vivo substrate for this kinase. dsRNAs were used to ablate the expression of DAck and other intracellular Src-related tyrosine kinases and the resulting extracts separated by SDS-PAGE followed by Western analysis using antibodies directed against phosphotyrosine (Fig. 3A). Equal loading of DSH3PX1 was confirmed by stripping and reprobing the Western with antibody recognizing DSH3PX1 (Fig. 3A). In S2 cell extracts, ablation of DAck, Src42A, or Src64 each reduce the level of tyrosine phosphorylated DSH3PX1. Simultaneous ablation of all three kinases completely obliterates Tyr phosphorylation of DSH3PX1. Alternatively, removal of Shark and Abl did not effect the level of SH3PX1’s tyrosine phosphorylation. These results suggest that Src 42A, Src64 and DAck are upstream of DSH3PX1 phosphorylation. Our ability to co-immunoprecipitate DSH3PX1 and DAck leads us to speculate that DAck is an important cellular kinase for DSH3PX1 tyrosine
phosphorylation. Furthermore, it has been reported that the Src-like kinase, Fyn, phosphorylates and activates mammalian ACK1 (8) and that ACK2 activity is similarly controlled by Src phosphorylation (12). Therefore, the reduction in DSH3PX1 phosphorylation observed in the Src RNAi examples may be the result of reduced DAck activity. In order to demonstrate that DAck can directly phosphorylate DSH3PX1, DAck engineered to contain an NH$_2$-terminal FLAG tag was stably expressed in S2 cells. This kinase was immunoprecipitated from S2 cells using FLAG beads followed by extensive washing in high salt buffer to remove any co-precipitating proteins. Precipitated DAck was then added to an in vitro kinase assay containing bacterially expressed DSH3PX1 as the substrate (Fig. 3B). Under these experimental conditions, DAck is able to efficiently phosphorylate DSH3PX1. A kinase dead version of DAck made by mutating Lys 156 to Ala, similarly expressed and immunoprecipitated from S2 cells, was not able to phosphorylate itself or DSH3PX1 (Fig. 3B). This indicated that DAck and not a co-precipitating kinase was responsible for DAck phosphorylation. In addition, immunoprecipitated DAck only phosphorylates itself in the absence of added substrate, demonstrating that no potential substrates have co-immunoprecipitated with the active kinase under the experimental conditions used. Taken together, DSH3PX1 is an in vitro substrate for DAck and our RNAi data suggest that it is also an in vivo substrate.

In order to elucidate the role of DAck phosphorylation on DSH3PX1 function, the site of phosphorylation by DAck was determined. Comparison of the amino acid sequences between mammalian, C. elegans, and Drosophila SH3PX1 highlighted the conservation of several tyrosine residues including Y9, Y56, and Y256 (Drosophila numbering) (5). These Tyr residues were mutated to Phe in the context of the bacterial expression vector, GST-DSH3PX1. Mutant and wild type proteins were expressed in bacteria and subjected to the in vitro kinase assay described above. FLAG-tagged wild type DAck was immunoprecipitated from S2 extracts as previously described and used in the in vitro kinase assays along with the bacterially expressed

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wild type and mutant DSH3PX1 proteins (Fig. 3C). Mutation of Tyr 56 to Phe (Y56F) significantly reduced the ability of DAck to phosphorylate DSH3PX1 indicating that Y56 is a major site of Tyr phosphorylation (Fig. 3C). Residual phosphorylation persists, however, suggesting that more than one Tyr in DSH3PX1 is phosphorylated by DAck. However, mutation of Y9 and Y256 do not dramatically reduce the level of DSH3PX1 tyrosine phosphorylation indicating that these residues are not candidates for DAck phosphorylation (Fig. 3C). It is interesting that Y56 falls in the poly-proline recognition site of the DSH3PX1 SH3 domain. Based on mutational studies on Src-family SH3 domains (21), we predict that phosphorylation of Y56 would interfere with the ability of the DSH3PX1 SH3 domain to bind proline-rich ligands. Since we previously demonstrated that DSH3PX1 interacts with WASP via its SH3 domain, we predict that phosphorylation of Y56 will interfere with WASP binding. Similar results have been reported for the SH3 domain interactions of PSTPIP and Bruton’s tyrosine kinase (Btk) with WASP (22,23). In the case of PSTPIP, phosphorylation of Tyr 367 which resides in its SH3 domain poly-proline binding pocket interferes with WASP binding (22). For Btk, autophosphorylation of a specific tyrosine residue in its SH3 domain abolishes its interaction with WASP while preserving its ability to interact with c-Cbl (23). These reports provide evidence that a common theme may exist for regulating SH3 domain-polyproline interactions involving WASP.

In order to test this theory, DSH3PX1 phosphorylated in vitro by DAck was allowed to interact with either Dock or WASP immobilized on affinity resins. After extensive washing, the amount of DSH3PX1 bound to each protein was visualized by Western analysis. In the case of Dock, His-tagged protein was produced in bacteria and attached to Ni-agarose resin. Since we could not produce recombinant WASP in bacteria, V5-tagged WASP was over-expressed and immunoprecipitated from S2 cells as described (5). As can be seen in Figure 4A, phosphorylated (P) DSH3PX1 binds efficiently to epitope-tagged Dock while non-phosphorylated (NP)
DSH3PX1 does not. This demonstrates that phosphorylation of DSH3PX1 by DAck generates a binding site for the Dock SH2 domain. On the other hand, NP-DSH3PX1 binds efficiently to WASP while P-DSH3PX1 displays reduced binding (Fig. 4A). The interaction between P-DSH3PX1 and WASP can be explained by the incomplete phosphorylation of DSH3PX1 in our kinase assay and from the ability of NP-DSH3PX1 to dimerize with P-DSH3PX1. Therefore the above data suggest that binding of DSH3PX1 to WASP may be regulated by phosphorylation of Tyr 56. In order to further test this possibility, Tyr 56 was mutated to either aspartate (D) or glutamate (E) to mimic the incorporation of the negatively charged phosphate group at this position. Modeling of the DSH3PX1 SH3 domain indicates that Tyr 56 is solvent exposed suggesting that these mutations should not globally effect the structure of the SH3 domain.

DSH3PX1 containing either the Y56D or the Y56E mutation is no longer able to bind to WASP (Fig. 4B). As expected, the conservative Y56F mutation does not affect the ability of DSH3PX1 to bind to WASP. Taken together, our results suggest that WASP most likely interacts with non-phosphorylated DSH3PX1 while phosphorylated DSH3PX1 is targeted for Dock interactions. It is intriguing that dimers of DSH3PX1 could create bridges between PXXP motif containing proteins such as Wasp and SH2 domain containing proteins such as Dock depending upon the phosphorylation state of their SH3 domains.

In summary, we have identified DAck as a member of a complex of proteins involved in axonal guidance via its association with Dock. It is important to note that experiments in fly embryos using dsRNAs directed against Dock and DAck result in similar axonal pathfinding defects as assayed by Bolwig’s organ development (JCC, manuscript in preparation). Given DAck’s ability to interact with DSH3PX1, a potential sorting nexin that associates with the clathrin coat adaptor protein, AP-50 (5, 24), and mammalian ACK1’s ability to interact with clathrin (9, 11), it is tempting to speculate that DAck is involved in regulating the extracellular presentation of Dscam and/or other Dock-associated receptors by endocytosis via clathrin coated
This speculation is further supported by the role of *C. elegans* ARK-1 in down-regulating Let 23, the *C. elegans* EGFR orthologue (13). Furthermore, we identify DSH3PX1 as a substrate for DAck and demonstrate that phosphorylation of DSH3PX1 likely increases its interaction with Dock while decreasing its interaction with WASP. The protein complex consisting of Dscam, Dock, DAck and DSH3PX1 and their known protein-protein interactions are summarized in Figure 5. In this scenario, DAck acts as a molecular switch to control DSH3PX1 protein-protein interactions. Non-phosphorylated DSH3PX1 interacts strongly with WASP, a known modulator of the actin cytoskeleton. When phosphorylated, DSH3PX1 interacts preferentially with Dock. In addition, the PX domain of DSH3PX1 interacts with phospholipids thereby targeting DSH3PX1 to specific cellular membranes (data not shown (reviewed in 25)). We are interested in understanding how the Dock SH2 domain chooses among its binding partners i.e, Dscam versus DAck versus DSH3PX1, and how the resulting protein complexes ultimately influence neurite outgrowth. For now, the complexity of the protein-protein interactions involving Dock preclude us from directly linking a specific Dock protein complex to specific changes in the actin cytoskeleton. Nevertheless, Dock and the proteins recruited by Dock, are clearly instrumental in signaling changes in the actin cytoskeleton that are required for directed axonal growth.

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Figure Legends

Figure 1. Amino acid sequence of p145. p145 is a Drosophila orthologue of mammalian ACKs (DAck). DAck consists of a tyrosine kinase domain (shaded gray) an SH3 domain (shaded charcoal), a clathrin-binding motif (shaded gray with white lettering) and a UBA domain (shaded black). The proline-rich sequences are boxed and the endocytotic signals are in bold type. The amino acid sequences obtained for the tryptic peptides are underlined. Domain predictions were generated by SMART (26).

Figure 2. In vivo and in vitro analyses of DAck’s association with Dock and DSH3PX1. (A) DAck and Dock associate with DSH3PX1 in S2 cells. Dock and DAck immunoprecipitates from S2 cell extracts were analyzed by Western blotting using DSH3PX1 antibodies. (B) Dock immunoprecipitates with DAck from S2 cells. Dock and DAck immunoprecipitates from S2 cell extracts were analyzed by Western blotting with DAck antibodies. (C) In vitro analysis of the ability of Dock or DSH3PX1 GST fusions to interact with DAck. GST fusion proteins of the indicated Dock domains prepared in bacteria were mixed with extracts prepared from S2 cells. The ability of the GST fusion proteins to bind DAck was assessed by Western analysis using antibodies directed against DAck. The amount of Dock or DSH3PX1 GST fusion proteins present in each reaction is displayed in the Coomassie stained panel.

Figure 3. DAck, Src42A and Src64 are cellular tyrosine kinases for DSH3PX1. (A) Profile of tyrosine phosphorylated DSH3PX1 in S2 cell extracts treated with dsRNAs for various tyrosine kinases. Protein extracts from untreated S2 cells or S2 cells treated with the indicated dsRNAs directed against cytoplasmic tyrosine kinases were analyzed by SDS-Page and Western blot using pTyr antibodies and DSH3PX1 antibodies. The top panel represents the p-Tyr signal obtained for DSH3PX1 and the bottom panel represents
the signal obtained for anti-DSH3PX1. (B) In vitro kinase assay using immunoprecipitated DAck. NH$_2$-terminal FLAG-tagged kinase dead or wild type DAck was stably expressed in S2 cells. Wild type and mutant DAck were immunoprecipitated from S2 cells using FLAG beads and were combined with approximately 5 µg of recombinant GST-DSH3PX1 in an in vitro kinase reaction (Materials and Methods). Phosphorylated proteins were separated by SDS-Page and Coomassie stained to verify equal loading. Phosphorylation was visualized by autoradiography overnight at −80°C with a screen. (C) DAck in vitro kinase assay with DSH3PX1 mutants. Conserved Tyr residues in GST-DSH3PX1 were mutated to Phe and the recombinant proteins expressed and purified from bacteria. FLAG-tagged DAck was immunoprecipitated from S2 cells and mixed with recombinant DSH3PX1 in an in vitro kinase assay as described in Materials and Methods. Phosphorylated proteins were separated by SDS-Page and Coomassie stained to verify equal loading. Phosphorylation was visualized by autoradiography overnight at −80°C with a screen.

Figure 4. Phosphorylation of DSH3PX1 determines its binding partners. (A) Equal amounts of non-phosphorylated (NP) GST-DSH3PX1 or phosphorylated (P) GST-DSH3PX1 were allowed to interact with bacterially expressed His-Dock or immunoprecipitated V5-tagged WASP in a pull-down analysis. The stoichiometry of phosphorylation of DSH3PX1 for this experiment was 48%. The load of His-Dock and immunoprecipitated WASP is shown in the silver stained panels. (B) Wild type (WT) GST-DSH3PX1 or the indicated Y56 mutants were allowed to interact with WASP in a pull-down analysis. In each case, DSH3PX1 binding was analyzed by Western analysis.

Figure 5. DAck complex schematic. Green lines indicate protein-protein interactions specified by SH3 domains interacting with proline-rich sequences (boxes containing horizontal hatches). Red lines indicate protein-protein interactions involving
P-Tyr residues interacting with SH2 domains. The loops in the Dscam schematic represent IgG2 repeats and the blackened ovals, fibronectin repeats. In DSH3PX1, the PHOX-homology domain is labeled PX while the abbreviation for a coiled-coil is CC.
References


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Asterisks denote partial overlap of data published in Worby et al. (5)
Figure 1, Worby et al.

MTSTSAVDGGGLGSETAWLEDLLREVQLEQFLDRIRDDLQVTRLAHFDYVLPDDLERCGLG

KPAIRRLMEAVRKKAHQWRKNILSKLIGGQKPSSKQSSAARESSQNGTQLTCLIHE

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PKNNAYSSTASLYDAVAAASTAGSTYYGQVPNGSAGVLYDEVQQDDYLRTRPAPLAPPF

SAQQIQRMEKMRLLQQQQQLDGAHQLYPVPDSGVREGSEQKLTQQMLQEGSSAVEQDVRNA

LRAASGDVLATRFYKIDQLARLGVAGRQCEALQQTNWSLEVAELLLNAG
Figure 2, Worby et al.

A.

\[ \text{Western: } \alpha-\text{DSH3PX1} \]

B.

\[ \text{Western: } \alpha-\text{DAck} \]

C.

\[ \text{Western: } \alpha-\text{DAck} \]

Coomassie stain
Figure 3, Worby et al.

A.

dsRNA Treatments

<table>
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B.

kDa

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P32-DACK

P32-DSH3PX1

GST-DSH3PX1

C.

kDa

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P32-DACK

P32-DSH3PX1

GST-DSH3PX1

Coomassie stain
Figure 4, Worby et al.

A.

Dock pull-downs

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WASP pull-downs

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</table>

GST-DSH3PX1

Dock load

WASP load

Silver stain

B.

WASP pull-downs

GST-DSH3PX1

GST-DSH3PX1

mutant load
Figure 5, Worby et al.
DAck targets its substrate, the sorting nexin, DSH3PX1, to a protein complex involved in axonal guidance
Carolyn A. Worby, Nancy Simonson-Leff, James C. Clemens, Donald Huddler, Jr, Marco Muda and Jack E. Dixon

*J. Biol. Chem.* published online December 28, 2001

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