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 Schneider 2 cells. A protein designated p145 was identified and shown to be a tyrosine kinase with sequence similarity to mammalian Cdc-42-associated tyrosine kinases. We demonstrate that Drosophila Ack (DAck) can be co-immunoprecipitated with Dock and DSH3PX1 from fly cell extracts. The domains responsible for the in vitro interaction between Drosophila Ack 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 while enabling DSH3PX1 to associate with Dock. Furthermore, when Tyr-56 is mutated to aspartate or glutamate, the binding to Wiskott-Aldrich Syndrome protein is abrogated. These results suggest that the phosphorylation of DSH3PX1 by DAck targets this sorting nexin to a protein complex that includes Dock, an adaptor protein important for axonal guidance.

To form precise patterns of connections among 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 intracellular signal transduction pathways of the neuron (2). Thus, understanding how individual neurons transduce late 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 masses of 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 domains containing protein, DSH3PX1 (5). DSH3PX1 is a member of the sorting nexin superfamily and shares extensive sequence similarity to 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 masses of 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).

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

¶ Howard Hughes Medical Institute, Dept. of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109-0606

Published, JBC Papers in Press, December 28, 2001, DOI 10.1074/jbc.M110172200

This paper is available on line at http://www.jbc.org

9422 This paper is available on line at http://www.jbc.org

The abbreviations used are: Dock, Drosophila orthologue of the mammalian SH3/SH2 adaptor protein Nck; Dscam, Down’s Syndrome cell adhesion molecule; PX, PHOX homology domain; WASP, Wiskott-Aldrich Syndrome protein; DAck, Drosophila Ack; ACK, Cdc-42-associated tyrosine kinase; GST, glutathione S-transferase; RNAi, RNA interference; S2, Schneider 2 cells; RIPA, radioimmune precipitation buffer; RACE, rapid amplification of cDNA ends; UBA, ubiquitin-associated; dsRNA, double-stranded RNA.
DSH3PX1 Is a Substrate for DAck

RESULTS AND DISCUSSION

Dock was first described by Garrity et al. (3) as an adaptor protein playing a critical role in axonal pathfinding. Because 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 overexpressed as a His-tagged protein in Drosophila S2 cells (4, 5).2 Protein lysates were prepared from S2 cells and subjected to two-hybrid screen—The selected two-hybrid screen was run as described previously (5). Approximately 0.5–1 μg of His-tagged Dock and immunoprecipitated WASP were used for each 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 1 h at 4 °C followed by three 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).

2 J. C. Clemens, manuscript in preparation.

Downloaded from http://www.jbc.org/ by guest on July 22, 2018
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, the COOH-terminal sequence of DAck contains four proline-rich (PXXP) motifs (Fig. 1, clear boxes), which could be involved in interactions with SH3 domains. There are also six YXXH motifs (boldface) that 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 and the presence of a UBA domain in the Drosophila enzyme. The apparent absence of a Cdc42-binding domain in DAck raises the intriguing possibility that this enzyme may be activated by a different mechanism from mammalian ACKs.

Because 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 DAck from S2 cell extracts (Fig. 2A) (5). Furthermore, DAck can also be co-immunoprecipitated with Dock (Fig. 2B). Because DAck contains several proline-rich motifs, we wanted to know whether the SH3 domains of Dock and/or its SH2 domain 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 Dock was assessed by Western analysis using antibodies directed against Dock. The amount of Dock or DSH3PX1 GST fusion proteins present in each reaction is displayed in the Coomassie Blue-stained panel.

**Fig. 1.** Amino acid sequence of p145. p145 is a Drosophila orthologue of mammalian ACKs. DAck consists of a tyrosine kinase domain (gray shade), an SH3 domain (charcoal shade), a clathrin-binding motif (gray shade with white lettering), and a UBA domain (black shade). The proline-rich sequences are boxed, and the endocytotic signals are in bold. The amino acid sequences obtained for the tryptic peptides are underlined. Domain predictions were generated by SMART (26).
and Teo et al. (9) most probably results from the type of assay employed. It is possible that DACK isolated from S2 cell extract is conformationally unavailable for binding to the SH3 domains of Dock. However, we also tested the ability of the DSH3PX1 SH3 domain to interact with DACK. As can be seen in Fig. 2C, the SH3 domain of DSH3PX1 interacts strongly with DACK in S2 cell extracts, suggesting that at least one of the proline-rich (PXXP) motifs of DACK is available for protein-protein interactions.

Because 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 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 because of autoactivation. Because the DACK constructs encode the active kinase, the removal of the COOH-terminal sequence most probably removes an auto-phosphorylation site that is 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 with truncated DACK, presumably because of the removal of one or more of the COOH-terminal PXXP motifs of DACK. As reported previously (4, 5), Dock and the DSH3PX1 SH3 domain are capable of interacting with Dscam. However, DACK (amino acids 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 probably not present in this group. Interestingly, our two-hybrid results suggest that DACK is able to interact with itself. This possibility would effectively increase the ability of Dock to bring together a variety of molecules simultaneously.

Because DACK co-immunoprecipitates (Fig. 2A) and directly interacts (Table I) with DSH3PX1, we sought to determine whether DSH3PX1 is an in vivo substrate for this kinase. dsRNAs were used to ablate the expression of Dock 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, the 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, the removal of Shark and Abl did not effect the level of SH3PX1 tyrosine phosphorylation. These results suggest that Src42A, 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. To demonstrate that DACK can directly phosphorylate DSH3PX1, DACK engineered to contain an NH2-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 finding 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.

To elucidate the role of DACK phosphorylation on DSH3PX1 function, the site of phosphorylation by DACK was determined. A comparison of the amino acid sequences among mammalian, D. melanogaster, and D. pseudoobscura indicated the conservation of several tyrosine residues including Tyr-9, Tyr-56, and Tyr-256 (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 as described above. FLAG-tagged wild type DACK was immunoprecipitated from S2 extracts as described under “Materials and Methods” and used in the in vitro kinase assays along with the bacterially expressed wild type and mutant DSH3PX1 proteins (Fig. 3C). The mutation of Tyr-56 to Phe (Y56F) significantly reduced the ability of DACK to phosphorylate DSH3PX1, indicating that Tyr-56 is a major site of Tyr phosphorylation (Fig. 3C). Residual phosphorylation persists, suggesting that more than one Tyr in DSH3PX1 is phosphorylated by DACK. However, the mutation of Tyr-9 and Tyr-256 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 Tyr-56 falls in the polyproline recognition site of the DSH3PX1 SH3 domain. Based on mutational studies on Src-family SH3 domains (21), we predict that the phosphorylation of Tyr-56 would interfere with the ability of the DSH3PX1 SH3 domain to bind proline-rich ligands. Because we previously demonstrated that DSH3PX1 interacts with WASP via its SH3 domain, we predict that the phosphorylation of Tyr-56 will interfere with WASP binding. Similar results have been reported for the SH3 do-

**Table I**

| Bait      | Dock | DACK (FL) | DSH3PX1 | Dscam | VP16 |
|-----------|------|-----------|---------|-------|------|
| DACK      | +++  | +++       | −       | +++   | −    |
| DSH3PX1 (1–507) | −    | −         | −       | −     | −    |
| DSH3PX1 (SH3 domain) | +    | +         | −       | −     | +    |
| Lamin     | −    | −         | −       | −     | −    |

* Partial overlap of data published in Worby et al. (5) is shown.
main interactions of PSTPIP and Bruton’s tyrosine kinase with WASP (22, 23). In the case of PSTPIP, the phosphorylation of Tyr-367, which resides in its SH3 domain polyproline-binding pocket interferes with WASP binding (22). For Bruton’s tyrosine kinase, 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.

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. Because we could not produce recombinant WASP in bacteria, V5-tagged WASP was overexpressed and immunoprecipitated from S2 cells as described previously (5). As can be seen in Fig. 4A, phosphorylated DSH3PX1 binds efficiently to epitope-tagged Dock, whereas nonphosphorylated-DSH3PX1 does not. This demonstrates that the phosphorylation of DSH3PX1 by DAck generates a binding site for the Dock SH2 domain. On the other hand, nonphosphorylated DSH3PX1 binds efficiently to WASP, whereas P-DSH3PX1 displays reduced binding (Fig. 4A). The interaction between P-DSH3PX1 and WASP can be explained by the incomplete phosphorylation of DSH3PX1 by DAck as described under “Materials and Methods.” Phosphorylated proteins were separated by SDS-PAGE and stained with Coomassie Blue to verify equal loading. Phosphorylation was visualized by autoradiography overnight at −80 °C with a screen.
A Dock complex schematic. Green lines indicate protein-protein interactions specified by SH3 domains interacting with proline-rich sequences (horizontal hatched boxes). 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 represent fibronectin repeats. In DSH3PX1, CC represents coiled-coil.

Fig. 5. Dock complex schematic. Neck 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 represent fibronectin repeats. In DSH3PX1, CC represents coiled-coil.

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 probably interacts with non-phosphorylated DSH3PX1, whereas phosphorylated DSH3PX1 is targeted for Dock interactions. It is intriguing that the dimers of DSH3PX1 could create bridges between PX domain 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 Dock 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 Dock result in similar axonal pathfinding defects as assayed by Bolwig's organ development. Given the ability of Dock to interact with DSH3PX1, a potential sorting nexin that associates with the clathrin-coated adaptor protein 50 (5, 24), and the ability of mammalian ACK1 to interact with clathrin (9, 11), it is tempting to speculate that Dock is involved in regulating the extracellular presentation of Dscam and/or other Dock-associated receptors by endocytosis via clathrin-coated pits. This speculation is further supported by the role of C. elegans ACK-related tyrosine kinase-1 in down-regulating Let-23, the C. elegans epidermal growth factor receptor orthologue (13). Furthermore, we identify DSH3PX1 as a substrate for Dock and demonstrate that phosphorylation of DSH3PX1 probably increases its interaction with Dock while decreasing its interaction with WASP. The protein complex consisting of Dscam, Dock, Dock, and Dock has been summarized in Fig. 5. In this scenario, Dock acts as a molecular switch to control DSH3PX1 protein-protein interactions. Nonphosphorylated 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 Ref. 25). We are interested in understanding how the Dock SH2 domain chooses among its binding partners, i.e. Dscam versus Dock 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.

Acknowledgment—We thank Matthew Wishart for critical reading of the manuscript.

REFERENCES
1. Tessier-Lavigne, M., and Goodman, C. S. (1996) Science 274, 1123–1133
2. Ming, G. L., Song, H. J., Berning, B., Holt, C. E., Tessier-Lavigne, M., and Poo, M. M. (1997) Neuron 19, 1225–1235
3. Garrity, P. A., Rauf, Y., Salecker, I., McGlade, J., Pawson, T., and Zipursky, S. L. (1996) Cell 85, 639–650
4. 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
5. Worby, C., Simonson-Leff, N., Clemens, J., Kruger, R., Muda, M., and Dixon, J. (2001) J. Biol. Chem. 276, 41782–41789
6. Manser, E., Leung, T., Salti, H., Tan, L., and Lim, L. (1998) Nature 363, 364–367
7. Yang, W., and Cerione, R. A. (1995) J. Biol. Chem. 270, 24819–24824
8. Linseman, D. A., Heidenreich, K. A., and Fisher, S. K. (2001) J. Biol. Chem. 276, 5622–5628
9. M. M., and Goodman, C. S. (1996) Science 274, 1123–1133
10. Yang, W., Lin, Q., Guan, J. L., and Cerione, R. A. (1999) J. Biol. Chem. 274, 8524–8530
11. Yang, W., Lo, C. G., Dispensa, T., and Cerione, R. A. (2001) J. Biol. Chem. 276, 17468–17473
12. Yang, W., Lin, Q., Zhan, J., Guan, J. L., and Cerione, R. (2001) J. Biol. Chem. 276, 43987–43993
13. Hopper, N. A., Lee, J., and Sternberg, P. W. (2000) Mol. Cell 6, 65–75
14. Worby, C., Simonson-Leff, N., and Dixon, J. (2001) Science’s STKE, stke .sciencemag.org/cgi/content/full/OC_sigsigtran; 2001/95/11
15. Worby, C., Clemens, J., and Dixon, J. (1997) in Cells: A Laboratory Manual (Spector, D. L., and Leitward, L. A., eds) Vol. 1, pp. 1–10, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. Desai, C. J., Gindhart, J. G., Goldstein, L. S., and Zinn, K. (1996) Cell 84, 589–609
17. Krueger, N. X., Van Vactor, D., Wan, H. I., Gelbart, W. M., Goodman, C. S.,
22. Morrogh, L., Hinshelwood, S., Costello, P., Cory, G., and Kinnon, C. (1999) *Eur. J. Immunol.* **29**, 2269–2279
23. Wu, Y., Spencer, S., and Lasky, L. (1998) *J. Biol. Chem.* **273**, 5765–5770
24. Zhang, Y. Q., and Broadie, K. (1999) *Gene (Amst.)* **233**, 171–179
25. Wishart, M., Taylor, G., and Dixon, J. (2001) *Cell* **105**, 817–820
26. Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 5857–5864
Drosophila Ack 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. 2002, 277:9422-9428.
doi: 10.1074/jbc.M110172200 originally published online December 28, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M110172200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 25 references, 12 of which can be accessed free at http://www.jbc.org/content/277/11/9422.full.html#ref-list-1