Cell, Vol. 97, 853–863, June 25, 1999, Copyright ©1999 by Cell Press

Pak Functions Downstream of Dock to Regulate Photoreceptor Axon Guidance in *Drosophila*

Huey Hing,*‖ Jian Xiao,*‖
Nicholas Harden,† Louis Lim,†‡
and S. Lawrence Zipursky*§
*Howard Hughes Medical Institute
Department of Biological Chemistry
School of Medicine
University of California, Los Angeles
Los Angeles, California 90095
†Institute of Molecular and Cell Biology
National University of Singapore
10 Kent Ridge Crescent
Singapore 0511
‡Institute of Neurology
1 Wakefield Street
London WC1N 1PJ
United Kingdom

Summary

The SH2/SH3 adaptor protein Dock has been proposed to transduce signals from guidance receptors to the actin cytoskeleton in *Drosophila* photoreceptor (R cell) growth cones. Here, we demonstrate that *Drosophila p*21-*a*ctivated *k*inase (Pak) is required in a Dock pathway regulating R cell axon guidance and targeting. Dock and Pak colocalize to R cell axons and growth cones, physically interact, and their loss-of-function phenotypes are indistinguishable. Normal patterns of R cell connectivity require Pak's kinase activity and binding sites for both Dock and Cdc42/Rac. A membrane-tethered form of Pak (Pak^{myr}) acts as a dominant gain-of-function protein. Retinal expression of Pak^{myr} rescues the R cell connectivity phenotype in *dock* mutants. These data establish Pak as a critical regulator of axon guidance and a downstream effector of Dock in vivo.

Introduction

Neurons form precise patterns of connections that emerge through the interaction between the growth cone and extracellular signals in the developing nervous system. Guidance receptors detect short- and long-range signals and activate specific intracellular signal transduction pathways that control growth cone motility (reviewed in Tessier-Lavigne and Goodman, 1996). These signals can act in a graded fashion as either attractants or repellents (e.g., de la Torre et al., 1997) and, in combination, can elicit unique responses in the growth cone (Winberg et al., 1998). Recent studies indicate that whether a given guidance signal acts as a repellent or an attractant depends on the activities of intracellular signal transduction pathways (e.g., Ming et al., 1997).

The control of the actin cytoskeleton lies at the core of axon guidance. Pharmacological and cell biological studies have underscored the importance of regulating the assembly and disassembly of actin microfilaments, not only as the "engine" for motility, but also for growth cone steering (see Mitchison and Cramer, 1996; Suter and Forscher, 1998). Rho family GTPases have been proposed to play a key role in transmitting extracellular signals to changes in actin microfilament structure and motility in growth cones. These proteins are evolutionarily conserved molecular switches that regulate the actin cytoskeleton (Hall, 1998). They are active in the GTP-bound form and inactive when bound to GDP. Despite strong evidence for the importance of Rho family GTPases in axon guidance (Luo et al., 1994; Zipkin et al., 1997), little is known about the mechanisms by which they couple to guidance receptors and signal to downstream effectors to regulate growth cone motility.

We previously proposed that Dreadlocks (Dock), the fly homolog of mammalian Nck (an SH3/SH2 adaptor protein), links guidance signals to changes in the actin-based cytoskeleton in photoreceptor (R cell) growth cones (Garrity et al., 1996). The compound eye of the fly contains some 800 simple eyes called ommatidia. Each ommatidium contains eight R cells (R1–R8) that project in a retinotopic fashion to two different layers in the brain. R1–R6 terminate in the lamina, whereas R7 and R8 terminate in the medulla. While *dock* mutant R cells extend axons into the optic lobe normally, they form abnormal patterns of connections in both the lamina and medulla with defects in topographic map formation and ganglion target specificity (i.e., lamina versus medulla).

By analogy to Grb2, an adaptor protein that couples receptor tyrosine kinases (RTK) to Ras, we hypothesized that Dock links guidance receptors to the related Rho family GTPases, thereby modulating the actin cytoskeleton. While *Drosophila* guidance receptors linked to Dock have not been identified, the SH2 domain of Nck can bind to the cytoplasmic regions of several mammalian guidance receptors upon ligand stimulation, including c-Met (Kochhar and Iyer, 1996) and EphB1 (Stein et al., 1998). In addition, mammalian Nck binds through its SH3 domains to proteins such as Pak and Prk2, which in turn bind to and are activated by Rho family GTPases (McCarty, 1998). As a step toward understanding how Dock regulates downstream effectors, we set out to critically assess the function of a *Drosophila* homolog of one of these, Pak (*p*21-*a*ctivated *k*inase), in R cell axon guidance.

Paks are evolutionarily conserved regulators of the actin cytoskeleton. The yeast Pak Ste20 regulates polarized cell growth in response to mating pheromone (Leeuw et al., 1998), and mammalian Paks reorganize the actin cytoskeleton when overexpressed in tissue culture cells (Sells et al., 1996; Manser et al., 1997). Pak consists of an N-terminal regulatory region that inhibits the activity of the C-terminal kinase domain (Frost et al., 1998; Zhao et al., 1998). The regulatory region contains binding sites for at least three signaling proteins. An N-terminal proline-rich sequence (PXXP) binds to Nck (Bokoch et al., 1996; Galisteo et al., 1996); a CRIB

§To whom correspondence should be addressed (e-mail: zipursky@hhmi.ucla.edu).
‖These authors contributed equally to this work.

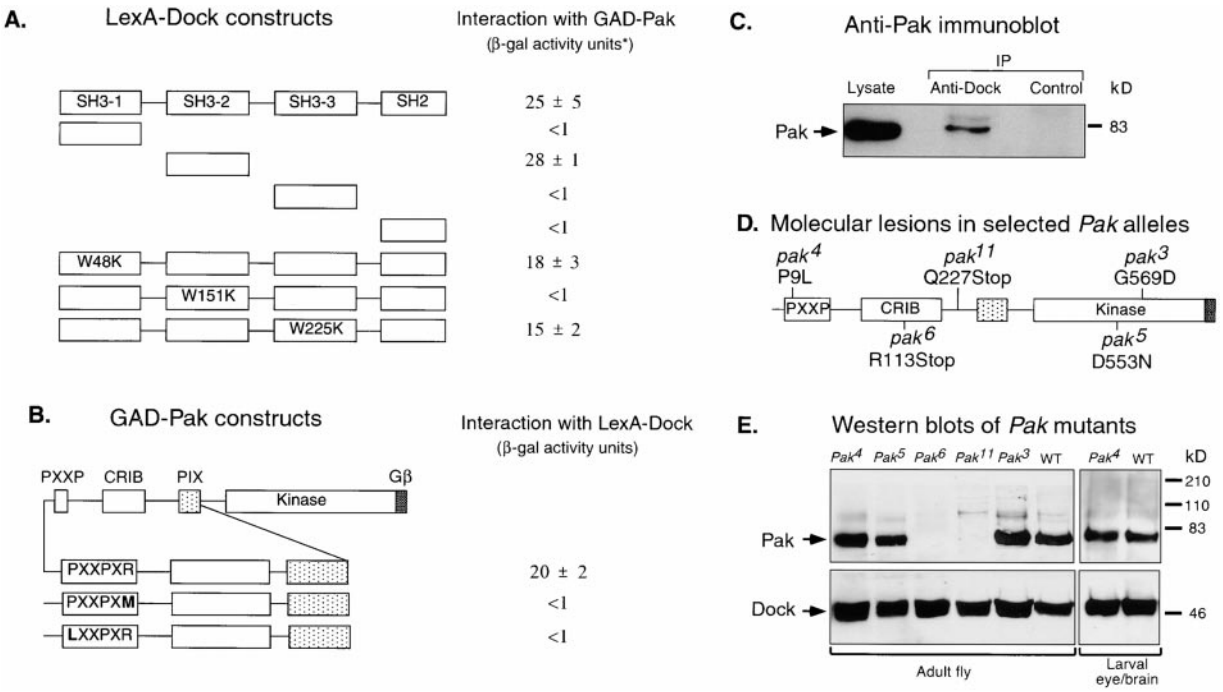

Figure 1. Dock/Pak Interactions and Molecular Characterization of *Pak* Mutants

(A) Dock interacts with Pak through its second SH3 domain (SH3-2). Different forms of Dock fused to the LexA DNA-binding domain were tested for interactions with Pak fused to the Gal4 transcriptional activation domain in a yeast two-hybrid assay.

(B) Pak interacts through its N-terminal PXXP site with Dock. N-terminal fragments of Pak fused to the Gal4 activation domain were tested for interactions with full-length Dock fused to LexA. PXXP, N-terminal proline-rich sequence (PAPPVR); CRIB, Cdc42/Rac interacting domain; Pix, Pix guanine nucleotide exchange factor–binding site; Gβ, binding site for Gβ subunit of trimeric G proteins.

(C) Pak and Dock coimmunoprecipitate from S2 cells. S2 cell lysates were immunoprecipitated with anti-Dock antibody or a control serum and analyzed by Western blotting with anti-Pak antibody.

(D) Schematic diagram of molecular lesions in *Pak* mutants (see text for isolation of mutants). Complete coding sequences of the indicated mutant alleles were determined.

(E) Pak protein levels in *Pak* mutants. Western blots of *Pak* mutant and wild-type extracts were probed with anti-Pak (upper panel) and anti-Dock (control; lower panel) antibodies.

*β-galactosidase activity is indicated in Miller units (Bartel and Fields, 1995; see Experimental Procedures).

(*C*dc42/*R*ac *i*nteractive *b*inding) motif binds to GTP-bound forms of Cdc42 and Rac (Manser et al., 1994; Burbelo et al., 1995); and a proline-rich motif is constitutively bound to Pix, a guanine nucleotide exchange factor specific to Rac and Cdc42 (Manser et al., 1998). In the inactive state, an autoinhibitory sequence adjacent to the CRIB motif inhibits Pak kinase activity. These biochemical studies provided a basis for a model of Pak function in growth cones. Recruitment of the Pak/Pix complex to the membrane by Dock (Nck) in response to guidance receptor activation would promote GTP binding and activation of Cdc42/Rac by Pix. Cdc42/Rac can, in turn, bind the CRIB motif and induce a conformational change in Pak. This displaces the autoinhibitory peptide, thereby activating Pak kinase. Hence, recruitment of the Pak/Pix complex to the membrane may lead to the activation of both Rho family GTPases and Pak.

In this paper, we provide evidence in support of this model. We demonstrate that Pak functions downstream of Dock to regulate R cell axon guidance. Pak function requires its kinase activity and the Dock and Cdc42/Rac interaction sites. We propose that Dock, Pak, and Cdc42/Rac comprise an evolutionarily conserved signaling pathway controlling actin microfilament dynamics in growth cones.

Results

Dock and Pak Physically Interact

Mammalian Paks bind Nck (Bokoch et al., 1996; Galisteo et al., 1996). The interaction sites have been mapped to the SH3-2 domain of Nck and the N-terminal-most PXXP site in Pak. Three proteins highly related to mammalian Paks have been identified in *Drosophila*. Two of these, Mbt (Melzig et al., 1998) and DPak2 (G. Suh et al., unpublished observations), neither bind to Dock nor are required for R cell axon guidance. The third, called Pak, contains an N-terminal PXXP site highly related to mammalian Paks, which bind to Nck (Harden et al., 1996). We tested whether *Drosophila* Pak interacts with Dock through these sites in a yeast two-hybrid assay (Figures 1A and 1B). First, we demonstrated that Pak and Dock do, indeed, interact. The DNA-binding domain of Lex A fused to full-length Dock (LexA-Dock) interacted strongly with a fusion protein containing the Gal4 activation domain and full-length Pak (GAD-Pak). Second, we demonstrated that the SH3-2 domain is necessary for interaction with Pak. LexA-Dock fusion proteins carrying mutations in the SH3-1 and SH3-3 domains designed to disrupt interactions with proline-rich sequences exhibited a similar level of interaction to that seen for LexA-Dock. Conversely, the same mutations introduced into

the SH3-2 domain abolished this interaction. And, third, we demonstrated that the SH3-2 domain is not only necessary, but it is sufficient for binding to Pak. While Lex-SH3-2 interacts strongly, neither LexA-SH3-1 nor LexA-SH3-3 interacts with GAD-Pak.

The requirement for the N-terminal PXXP site of Pak for these interactions was demonstrated in a separate series of experiments in which LexA-Dock was tested for interactions with three different forms of the N-terminal region of Pak fused to GAD (Figure 1B). The N-terminal region of Pak interacts strongly with LexA-Dock. Conversely, LexA-Dock did not interact with Pak N-terminal fragments containing point mutations in which arginine 14 was changed to methionine (R14M) or proline 9 was changed to leucine (P9L).

To assess whether these proteins can associate in vivo, immunoprecipitation experiments were carried out in *Drosophila* S2 cells (Figure 1C). Both Dock and Pak are endogenously expressed in these cells. S2 cell lysates were incubated with either anti-Dock antibody (Clemens et al., 1996) or a control antiserum and precipitated with protein A beads. The immunoprecipitates were analyzed on Western blots with anti-Pak antibodies. Pak was found in anti-Dock, but not in control, immunoprecipates.

Pak Localizes to Axons and Growth Cones in the Developing Visual System

If Dock and Pak function in the same signaling pathway in developing R cells to regulate growth cone motility, we expect them to colocalize in these cells. R cells extend their axons into the optic lobe during the third instar of larval development (Figure 2A). In previous studies, we demonstrated that Dock staining is markedly enriched in the lamina and medulla neuropils, consistent with its localization to R cell axons and growth cones (Garrity et al., 1996; see Figures 2E and 2F). In contrast, Dock is only expressed at low levels in the cell bodies of developing R cells as well as in neuronal cell bodies in the cortical regions in the lamina and medulla. A similar staining pattern was observed with two different anti-Pak antibodies, raised to different epitopes. No staining was seen in Pak protein null mutants (data not shown; see isolation and characterization of Pak mutants below). While strong staining in the neuropils was observed (Figures 2A and 2D), only weak staining was seen in the R cell bodies in the eye disc (data not shown) and in cell bodies of lamina and medulla neurons surrounding the optic lobe neuropils (Figures 2A and 2D). The lamina and medulla neuropils contain no neuronal cell bodies and comprise axonal processes and growth cones (Figure 2A). Both anti-Dock and anti-Pak antibodies stain the medulla neuropil uniformly, indicating that these proteins are expressed on many visual system fibers. Since the R7 and R8 axons only contribute a small fraction of the total number of fibers in the medulla, it is not possible to assess whether Pak and Dock are coexpressed in these axons (Figure 2B). In contrast, at this stage in development, the vast majority of the processes in the lamina neuropil belong to R cells, including the expanded R1–R6 growth cones and axons of R7 and R8 (Figures 2B and 2C). Hence, Pak, like Dock, preferentially localizes to axons and growth cones.

Pak Loss-of-Function Mutations Are Lethal

To assess whether *Pak* is required for growth cone guidance, mutations disrupting its function were identified. We assumed that, like *dock*, null mutations in *Pak* would cause recessive lethality. Accordingly, we identified lethal mutations in a small region of the chromosome (83E1,2–84A4,5) within which *Pak* mapped, defined by a deficiency, $Df(3R)Win^{11}$ (see Experimental Procedures). From 9440 mutant lines containing randomly mutagenized third chromosomes, 238 lethal mutations mapping to the deficiency were isolated. These were then tested against the same deficiency chromosome bearing a Pak-containing cosmid. The cosmid rescued 21 mutations. These fell into two groups based on complementation tests. A *Pak* cDNA expressed under the control of the heat shock promoter rescued the lethality associated with one complementation group, indicating that these mutations disrupted *Pak* function. These alleles are designated Pak^{1} to Pak^{13}. Flies homozygous or transheterozygous for *Pak* mutations die as pharate adults, although occasional adult escapers were seen. They are uncoordinated and have crumpled wings but are otherwise wild type in appearance.

Five *Pak* alleles were sequenced. Missense mutations in highly conserved residues in Pak^{3}, Pak^{4}, and Pak^{5} and nonsense mutations in Pak^{6} and Pak^{11} were identified (Figure 1D; for analysis and discussion of these mutations see below). Full-length Pak protein is missing in extracts of both Pak^{6} and Pak^{11} tissues analyzed by Western blots (Figure 1E); a truncated polypeptide of about 46 kDa is detected in Pak^{11} extracts (data not shown). Full-length proteins expressed at normal levels are detected in Pak^{3}, Pak^{4}, and Pak^{5}.

R Cell Axon Projection Defects in *Pak* and *dock* Mutants Are Indistinguishable

To assess R cell projections in *Pak* mutants, eye–brain complexes from transheterozygous larvae were stained with the R cell–specific antibody mAb24B10. In wild type (Figure 3A) R cell axons grow from the eye disc, through the optic stalk, and into the optic ganglia during the third instar of larval development. The eight R cell axons from each ommatidium form a single bundle. These bundles spread out upon entering the optic lobe and form a smooth topographic map that reflects the arrangement of ommatidia in the eye. In the view presented in Figure 3A, growth cones of R1–R6 are seen as a band of immunoreactivity. In contrast, individual R8 growth cones are readily observed in the medulla neuropil. They are evenly spaced and exhibit a characteristic expanded morphology. At this stage of development, few of the R7 axons stain with mAb24B10.

In *Pak* strong loss-of-function mutants (Figure 3B), R cell axons extend into the brain normally. However, these fibers do not spread evenly within the lamina and medulla. As a result, some regions are hyperinnervated while others lack innervation. In the medulla neuropil, R cell axons fail to find their proper targets but instead, terminate as thick, blunt-ended fascicles. Hence, in contrast to wild type, Pak mutant R cells do not elaborate a smooth topographic map in the lamina and medulla neuropils. A small fraction of the R2–R5 neurons project through the lamina and into the medulla as assessed using the $Ro\text{-}lacZ^{tau}$ marker (data not shown; Garrity et al., 1999), indicating a modest disruption in ganglion

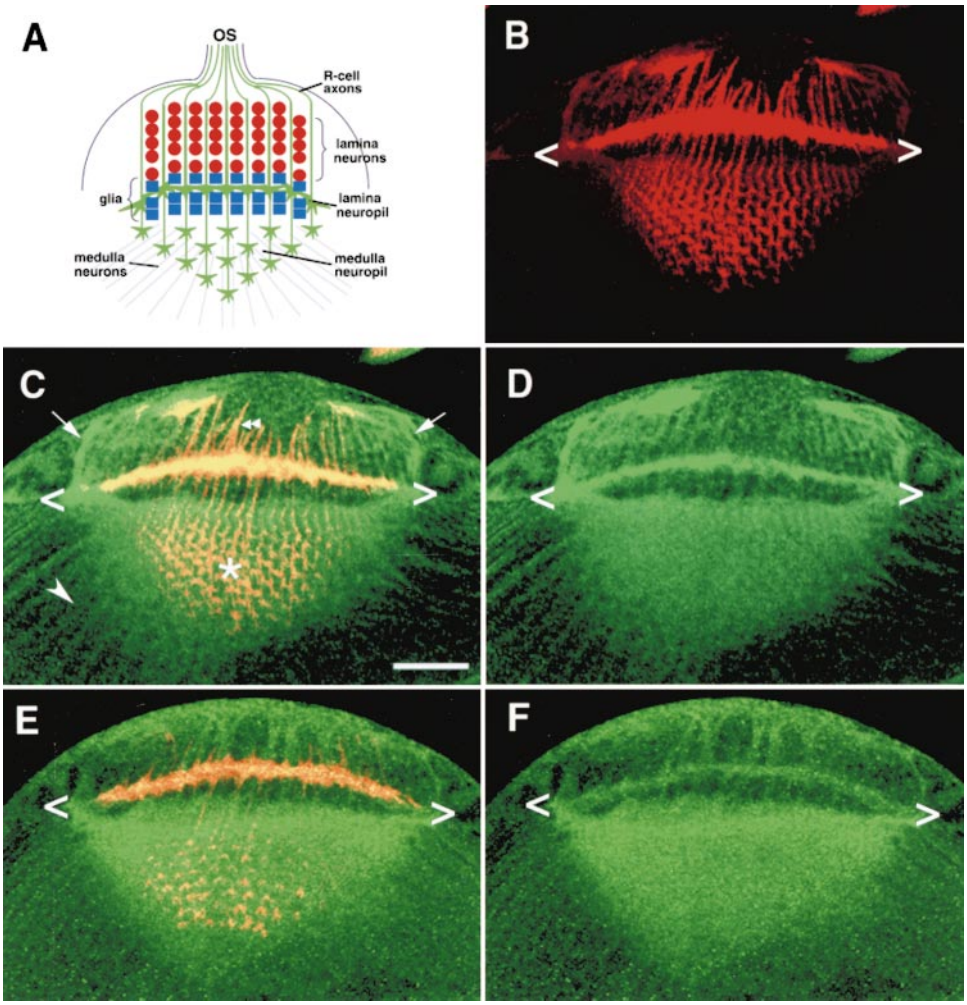

Figure 2. Pak Is Localized to the Neuropil in Developing Optic Lobes

(A) Schematic representation of a third instar larval optic lobe. R cell axon bundles project from the eye disc (data not shown) through the optic stalk (OS) into the optic lobe. The bundles fan out over the surface of the optic lobe and then project to their topographically appropriate positions in the lamina and medulla. R1–R6 axons terminate in the lamina neuropil, where they have expanded growth cones that are nestled between layers of lamina glial cells. At this time the lamina neuropil is comprised largely of R cell axons and growth cones. R7 and R8 axons terminate in the medulla neuropil, where their growth cones expand. Medulla neurons also extend axons and growth cones into the medulla neuropil.

(B–D) An optic lobe stained with both mAb24B10 (red), which recognizes an R cell–specific antigen, and an anti-Pak antibody (green). Strong overlap between the staining patterns (yellow) is seen in the lamina neuropil (brackets). Many R8 growth cones in the medulla neuropil (asterisk) also appear yellow. Double staining is also observed in older R cell axons coursing through the lamina neuron layer (double arrowheads). Interestingly, the edges of the lamina neuropil stained only with the anti-Pak antibody. These edges contain the youngest R cell axons and growth cones, and they do not yet express the mAb24B10 antigen, which is a late R cell axon and growth cone marker. Pak is also expressed in R cell (arrows) and medulla (arrowhead) axons as they enter the developing neuropil. No Pak staining is seen in Pak protein null mutants (data not shown).

(E and F) For comparison, anti-Dock staining (green) is shown with mAb24B10 (red) and alone in (E) and (F), respectively.

Bar, 40 μm.

target specificity. Variation in phenotypic severity between different alleles was not observed, and all alleles behaved as strong loss-of-function mutations. For instance, the projection defects of the two truncation alleles in *trans* to each other (Pak^{6}/Pak^{11}) were indistinguishable from those of either allele in *trans* to $Df(3R)Win^{11}$. Eye-specific expression of a wild-type *Pak* cDNA under the control of the *GMR* promoter ($GMR\text{-}Pak^{wt}$) rescued the mutant phenotype (Figure 3C). These data indicate that Pak is required for axon targeting but that it is not required for axon outgrowth as R cell axons extend in the correct direction and into the target region. The *Pak* phenotypes are essentially indistinguishable from those previously described in *dock* mutants (see Figure 3D; Garrity et al., 1996).

While *Pak* has a profound effect on R cell projections, it does not disrupt R cell fate determination or differentiation. This was shown by determining the expression of various markers in the eye disc (data not shown) and in plastic sections of the compound eye of adult escapers. Pak mutant ommatidia are largely indistinguishable from wild type (Figures 4A and 4D); of 898

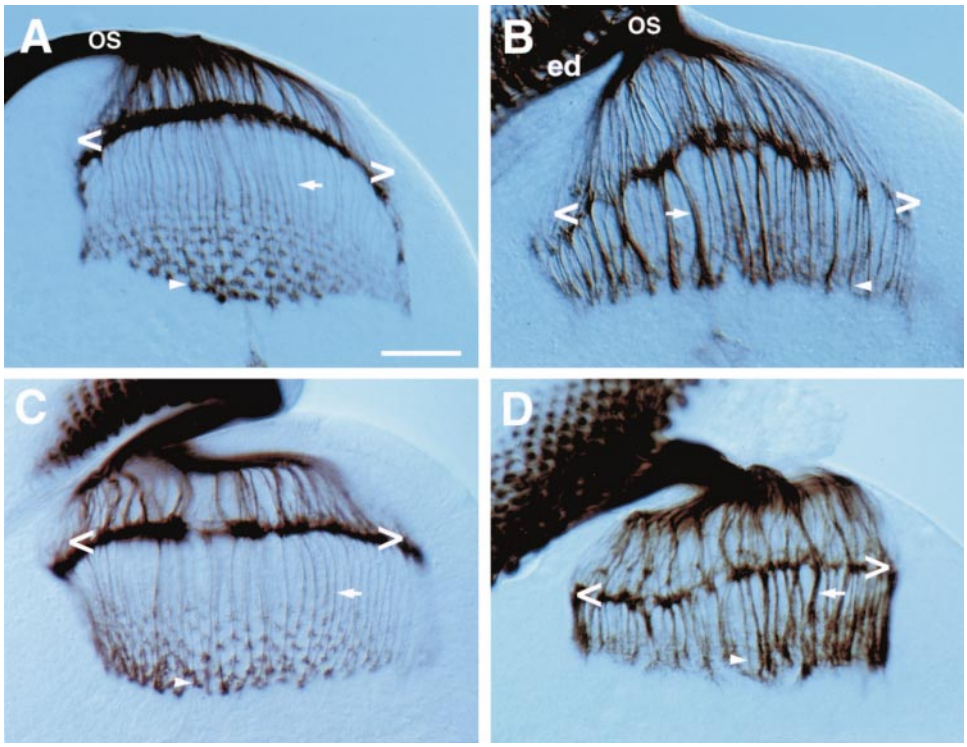

Figure 3. R Cell Projections in *dock* and *Pak* Are Similar

R cell projection patterns were visualized using mAb24B10, in eye–brain complexes of wild type (A), Pak^{6}/Pak^{11} (B), *GMR-Pak/+;* Pak^{6}/Pak^{11} (C), and *dock* (D). In all panels, R cells project from the eye disc (ed) (upper left) through the optic stalk (OS) and into the brain. In wild type (A), R1–R6 axons terminate in the lamina, where their growth cones form a continuous line of staining, the lamina neuropil (brackets). R8 axons terminate in the medulla neuropil, forming a topographic array. Few, if any, R7 axons stain at this stage of development. Axons projecting between the lamina and medulla are thin (arrow) with elaborate expanded growth cones at their ends (arrowhead). In the *Pak* mutant (B), some R1 to R6 axons fail to stop in the lamina (see text), resulting in gaps in the lamina plexus. In addition, R cell axons form abnormally thick bundles between the lamina and medulla (arrow). In contrast to wild type, axons terminate with a blunt-ended morphology (arrowhead). Expression of a *Pak* cDNA using the eye-specific *GMR* promoter ($GMR\text{-}Pak^{wt}$) rescues the *Pak* mutant phenotype (C), indicating *Pak* function is required in the eye. The *Pak* mutant phenotype is remarkably similar to *dock* (D). Bar, 40 μm.

ommatidia counted in four *Pak* mutant eyes, only 9 ommatidia lacked a single R cell. A similar level of missing R cells is seen in most connectivity mutants affecting R cell projections (e.g., *dock* and *PTP69D*; Garrity et al., 1996, 1999). R cells in *Pak* mutant ommatidia assume their normal trapezoidal arrangement with normal dorsoventral polarity. In addition, R cell morphology is normal. As in wild type, R cell bodies extend out toward the periphery of the ommatidia, and the rhabdomere, a hexagonal stack of actin-based microvilli containing rhodopsin, extends toward the center. While the rhabdomere fine structure was not analyzed using electron microscopy, defects in rhabdomere structure in other eye mutants are typically visible even at the light microscope level. Hence, *Pak*, like *dock*, is not required for R cell differentiation.

R cell axons not only target to specific regions of the developing optic ganglia, they play an essential role in inducing optic ganglion development (see Salecker et al., 1998). They induce the proliferation of neuronal precursor cells in the lamina, and subsequently they induce neuronal differentiation. R cell axons also induce lamina glial cell differentiation. These inductive processes were assessed in *Pak* mutants using BrdU labeling to detect proliferating lamina precursor cells (data not shown), and anti-Elav (Figures 4B and 4E) and anti-Repo staining (Figures 4C and 4F) to assess lamina neuron and glial cell differentiation, respectively. These steps occur normally in *Pak* mutants. Hence, like *dock*, *Pak* is not required for lamina induction.

In summary, *Pak* and *dock* are required specifically for R cell axon guidance and targeting but not for R cell differentiation, axon outgrowth, or target induction.

Pak Guidance Function Requires Dock- and Cdc42/Rac-Binding Sites and Kinase Activity

The requirements for different domains of Pak in R cell axon guidance were assessed through analysis of EMS-induced alleles and in rescue experiments using *GMR-Pak* transgenes carrying specific mutations (Figure 5). Transgenic Paks driven by the *GMR* promoter were expressed at higher levels than endogenous Pak, as assessed by immunohistochemistry and Western blot analyses of eye–brain complexes from transgenic animals. Hence, the failure of Pak mutant transgenes to rescue the mutant phenotypes is not a consequence of reduced levels of protein expression. None of the mutant *GMR-Pak* transgenes used in these experiments induced dominant phenotypes. The analyses in this section assess the requirement of three regions of Pak in

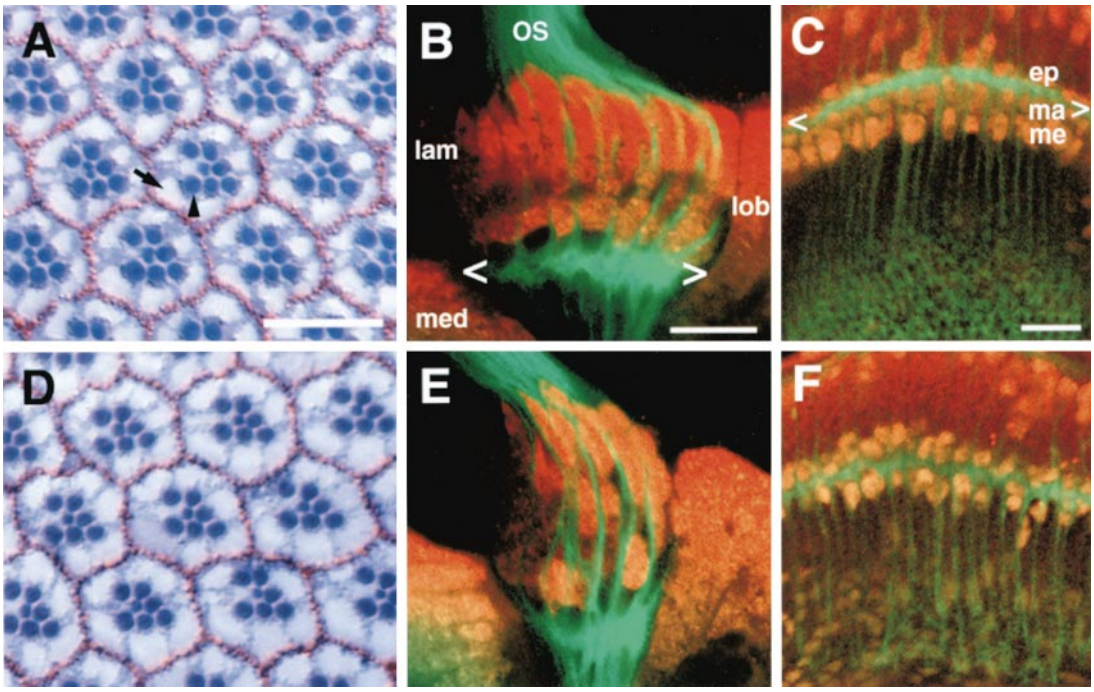

Figure 4. R Cell Differentiation and Target Development Occur Normally in *Pak* Mutants

Preparations of wild type (A–C) and Pak^{6}/Pak^{11} (D–F) are shown. R cell differentiation was assessed in toluidine blue–stained sections of adult eye (A and D). An R cell body and rhabdomere are indicated by the arrow and arrowhead, respectively. R cell innervation of the target region induces lamina neuron and glial cell differentiation (see Salecker et al., 1998). These occur largely normally in *Pak* mutants. (B and E) R cell axons and growth cones, stained with mAb24B10 (green), signal precursor cells to express the pan-neuronal nuclear marker ELAV (red), visualized using 7E8A10. (C and F) R cell axons and growth cones (green) also induce glial cells to express a glial nuclear antigen, Repo (red). Brackets in (B) and (C) indicate lamina neuropil. ep, epithelial glia; lam, lamina neurons; lob, lobula neurons; ma, marginal glia; me, medulla glia; med, medulla neurons; OS, optic stalk. Bars, 15 μm in (A); 20 μm in (B) and (C).

R cell axon guidance: the kinase domain, the CRIB domain, and the Dock-binding site.

The kinase activity of Pak is essential for R cell axon guidance. Two EMS-induced strong loss-of-function mutations, Pak^{3} and Pak^{5}, lead to amino acid substitutions in conserved residues in the catalytic site. The Pak^{3} mutation leads to the replacement of glycine at position 569 with aspartic acid. This change, G569D, is found within the DFG triplet conserved in all kinases (Hanks et al., 1988). The change in Pak^{5} (D553N) is in a residue conserved in all Paks. These mutations are in subdomains (VI and VII) implicated in ATP binding (Hanks et al., 1988). Both Pak^{3} and Pak^{5} proteins are expressed at wild-type levels (Figure 1E), suggesting that it is the lack of kinase activity, rather than protein instability, that leads to the mutant phenotype. This requirement for kinase activity was confirmed by the observation that a *Pak* transgene in which the invariant lysine required for the phosphotransfer reaction was changed to alanine ($GMR\text{-}Pak^{K459A}$) also failed to rescue the *Pak* mutant phenotype (Figure 5B).

Pak kinase activity is stimulated by Rac or Cdc42. This occurs through direct binding of Rac/Cdc42 in the GTP-bound form to the CRIB domain (Manser et al., 1994; Burbelo et al., 1995). Previous studies demonstrated that substituting leucines for two conserved histidines in the CRIB site of mammalian Pak prevents this interaction (Sells et al., 1996). Similarly, we have demonstrated in the yeast two-hybrid assay that this mutation prevents binding of *Drosophila* Pak to Cdc42 (J. X. and S. L. Z., unpublished observations). A *Pak* transgene carrying these mutations ($GMR\text{-}Pak^{H91,94L}$) failed to rescue the *Pak* mutant phenotype (Figure 5C), indicating that interaction between Pak and Cdc42/Rac is necessary for R cell growth cone guidance.

DNA sequence analysis revealed a missense mutation that changed a proline within the conserved Dock-binding site to a leucine (P9L) in Pak^{4}. This substitution abolishes the interaction between Dock and Pak in the yeast two-hybrid assay (Figure 1B). Pak^{4} protein is expressed at wild-type levels in eye–brain complexes (see Figure 1E) and is localized to the optic lobe neuropil as in wild type (data not shown). R cell axon guidance defects in Pak^{4}/Pak^{11} (Figure 5D) are indistinguishable from the strong loss-of-function phenotypes associated with Pak^{6}/Pak^{11} and *dock* null alleles. Hence, the Dock-binding site in Pak is essential for its function. This finding and the similarities between the *dock* and *Pak* mutant phenotypes provide strong evidence that direct interaction between Dock and Pak is essential for R cell axon guidance.

Membrane-Tethered Pak Is Dominant

To assess whether Pak acts downstream from Dock, we sought to generate a dominant gain-of-function form of Pak. Previous studies demonstrated that membrane localization of human Pak1 to the membrane leads to kinase activation (Lu et al., 1997; Manser et al., 1997).

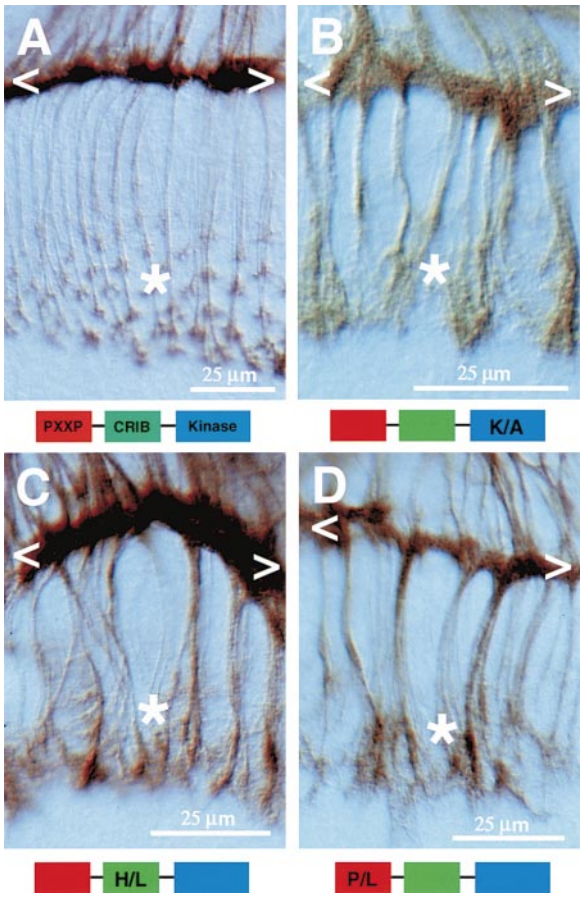

Figure 5. Pak Requires the Dock- and Cdc42/Rac-Binding Sites and Serine/Threonine Kinase Activity for R Cell Axon Guidance

(A) A *Pak* cDNA driven by the GMR promoter (*GMR-Pak*) rescues Pak^{6}/Pak^{11}.

(B) $GMR\text{-}Pak^{K459A}$ carrying a mutation that abolishes Pak kinase activity does not rescue Pak^{6}/Pak^{11}.

(C) $GMR\text{-}Pak^{H91,94L}$ carrying mutations in the CRIB domain that disrupt Cdc42/Rac binding does not rescue Pak^{6}/Pak^{11}.

(D) The P9L mutation in Pak^{4} disrupts Dock binding (see Figure 1) and gives a strong loss-of-function phenotype, shown here as a transheterozygote with Pak^{11}. Pak^{4} is expressed at levels similar to wild type (see Figure 1) and is localized to axons and growth cones (data not shown). Lamina and medulla neuropils are indicated by brackets and asterisks, respectively. The PXXP and CRIB regions are binding sites for Dock and Cdc42/Rac, respectively. Bars, 25 μm.

Accordingly, we generated a membrane-anchored version of Pak by fusing a myristylation signal from *Drosophila* Src1 to the N terminus of Pak and expressed it in developing R cells. This construct is designated $GMR\text{-}Pak^{myr}$. A single copy of $GMR\text{-}Pak^{myr}$ rescues the *Pak* mutant phenotype (data not shown), indicating that Pak^{myr} retains wild-type activity.

$GMR\text{-}Pak^{myr}$ shows a dose-dependent dominant phenotype in R cells. Wild-type larvae carrying a single copy of $GMR\text{-}Pak^{myr}$ showed mild clumping of axons in the lamina cortex (Figure 6A). The R8 projections in the medulla and the pattern of R cells in the eye disc, however, were normal. This defect is slightly enhanced in animals with two copies of $GMR\text{-}Pak^{myr}$ (data not shown). In larvae carrying four copies of $GMR\text{-}Pak^{myr}$ (Figure 6B), the R cell projection pattern was severely disrupted. In addition, the pattern of R cell clusters in the eye disc was highly abnormal. Remarkably, in these animals R cells delaminated from the eye disc epithelium and migrated through the optic stalk into the brain. Both membrane localization and kinase activity are required to induce motility, as larvae carrying four copies of either $GMR\text{-}Pak^{myr\ K459A}$ (kinase inactive) or $GMR\text{-}Pak^{wt}$ (not membrane tethered) were indistinguishable from wild type (data not shown). While kinase activity is necessary to induce guidance defects and cell motility, it is not sufficient. The R cell projection pattern in larvae carrying four copies of $GMR\text{-}Pak^{L115F}$, which encodes a constitutively active but soluble form of Pak (Brown et al., 1996; Frost et al., 1998; Zhao et al., 1998), was also indistinguishable from wild type. In summary, the ability of $GMR\text{-}Pak^{myr}$ to rescue a *Pak* mutant phenotype and to confer a dose-dependent dominant phenotype is consistent with it acting as a dominant gain-of-function allele.

Membrane-Tethered Pak Rescues *dock*

If a key step in Dock function is to recruit Pak to the membrane, then membrane-tethered Pak, $GMR\text{-}Pak^{myr}$, may rescue some aspects of the *dock* mutant phenotype. To test this, a single copy of the transgene was introduced into a *dock* null background. In *dock* mutants, R cell axons form large abnormal fascicles in the optic ganglia (Figure 6D). This leads to hyperinnervated regions separated by areas lacking innervation in both the lamina and medulla. In addition to disrupting targeting, R cell axon terminals in the medulla are thick and blunt ended. The *dock* phenotype was substantially rescued by $GMR\text{-}Pak^{myr}$ (Figure 6E). Axon bundles between the lamina and medulla were thinner. Growth cones in the medulla were expanded and spread out more evenly, giving rise to an array of terminals. Quantification of the expanded growth cones in the medulla showed an increase from less than 2 ($n = 12$ brains) in *dock* mutants to 64 ± 8 ($n = 12$ brains) in *dock* mutants carrying a copy of $GMR\text{-}Pak^{myr}$ (Figure 6F). This represents a restoration of about half the number of growth cones, compared with wild-type preparations of similar age (136 ± 12; $n = 12$ brains). Rescue requires both myristylation and kinase activity; rescue was not observed with $GMR\text{-}Pak^{wt}$, kinase inactive $GMR\text{-}Pak^{myr\ K459A}$, or constitutively active soluble $GMR\text{-}Pak^{L115F}$. Both $GMR\text{-}Pak^{wt}$ (see above) and $GMR\text{-}Pak^{L115F}$ (data not shown) are biologically active as they rescued the *Pak* mutant phenotype. Though biologically inactive, $GMR\text{-}Pak^{myr\ K459A}$ is expressed at high levels in R cell axons and growth cones. These data are consistent with models in which recruitment of Pak to the membrane by Dock is an essential regulatory step in R cell axon guidance.

Discussion

In this paper we establish that Pak plays an essential role in axon guidance and is a primary target of Dock in vivo. Several lines of evidence indicate that Dock and Pak act together to regulate axon guidance. First, *Pak* and *dock* connectivity phenotypes are identical. While *dock* and *Pak* mutant axons extend normally from the

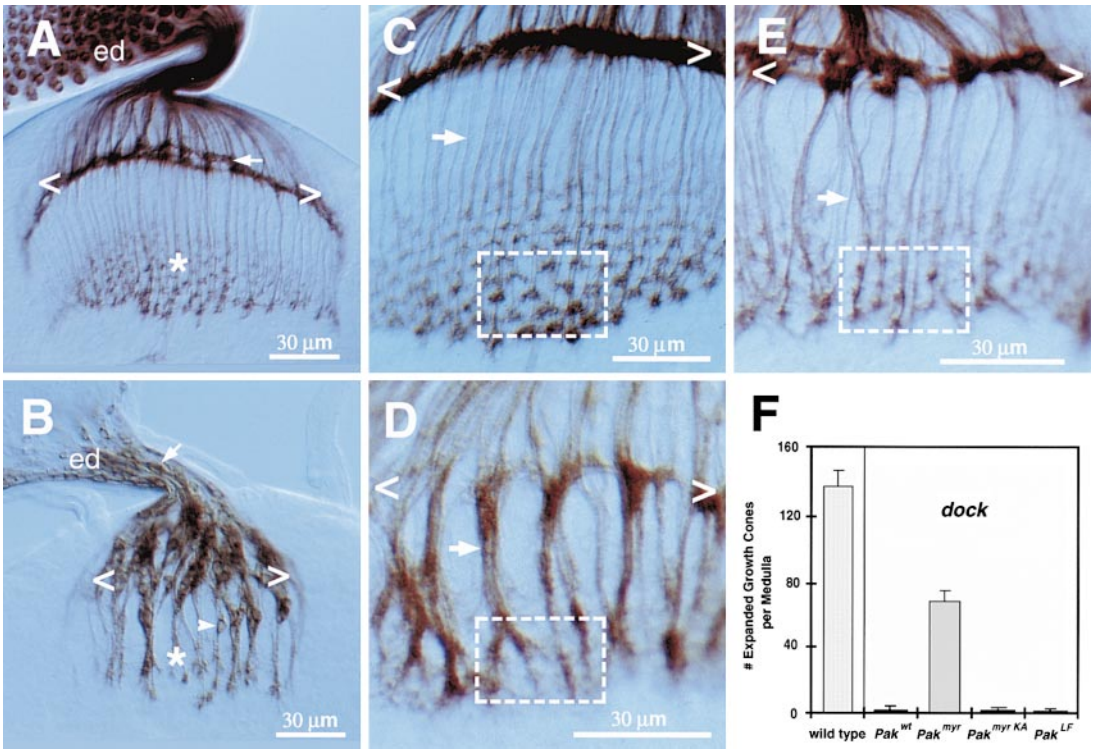

Figure 6. Gain-of-Function *Pak*^{myr} Rescues *dock* Phenotype

Third instar eye–brain complexes were stained with mAb24B10. As shown in (A) and (B), *Pak*^{myr} acts as a dominant gain-of-function mutation (see text). (A) In larvae carrying one copy of *GMR-Pak*^{myr}, R cell morphology and axon projections are largely normal. Some R cell axons in the center of the lamina (brackets) terminate prematurely (arrow). (B) In larvae with four copies of *GMR-Pak*^{myr}, the projection patterns in the lamina and medulla (asterisk) are severely disrupted. R cells delaminate from the eye disc epithelium and migrate through the optic stalk (arrow) into the brain. Some R cell bodies have migrated as far as the medulla (arrowhead). As shown in (C)–(F), *Pak*^{myr} rescues the *dock* loss-of-function phenotype. (C) Wild-type projection pattern. (D) In *dock* there are gaps in the lamina (brackets) and abnormal axon bundling (arrow) and unexpanded growth cones in the medulla (e.g., dashed rectangle). (E) This phenotype is suppressed in *dock* mutants carrying one copy of *GMR-Pak*^{myr}. Gaps in the lamina are less prominent, axons defasciculate, and a regular array of expanded growth cones forms in the medulla. (F) To quantitate rescue of *dock* by *GMR-Pak*^{myr}, the number of separated and expanded growth cones in the entire medulla field in each genetic background was determined. Wild-type Pak (*GMR-Pak*^{wt}), kinase-dead Pak (*GMR-Pak*^{myrKA}), and constitutively active soluble Pak (*GMR-Pak*^{LF}) did not rescue the *dock* mutant phenotype. The LF and KA mutations correspond to L115F and K459A, respectively. Bars, 30 μm.

eye into the brain, they form abnormal fascicles and exhibit targeting defects. Hence, Dock and Pak are not required for axonogenesis but rather for growth cone steering within the target region. Second, Dock and Pak physically interact and colocalize in R cell axons and growth cones. Third, the domains in Dock and Pak that mediate their interactions are critical for their functions in vivo. A mutation in *Pak* that disrupts the Dock interaction site gives rise to a null phenotype. Similarly, a mutation in *dock* that disrupts the Pak interaction site, the second SH3 domain, also gives rise to a null phenotype (Rao and Zipursky, 1998). Finally, a gain-of-function *Pak* allele substantially rescues the *dock* null mutant phenotype (see below for further discussion of this issue). Together, these data indicate that Dock and Pak function in the same signaling pathway, and that Pak is a key downstream effector of Dock in R cell growth cones.

Our studies provide strong evidence that Pak plays a crucial role in regulating axon guidance by modulating the actin cytoskeleton. That Pak kinase activity is stimulated by binding to Rac and/or Cdc42 led to the original proposal that Pak is a downstream effector of Rho family GTPases (Manser et al., 1994). The importance of Pak binding for cytoskeletal rearrangement was challenged, however, by the finding that mutations in the Pak-binding sites on activated Rac and Cdc42 did not prevent these proteins from promoting actin polymerization upon injection into cultured fibroblasts (e.g., Lamarche et al., 1996). Subsequently, several groups demonstrated that altered forms of Pak lead to changes in actin organization (Sells et al., 1996; Manser et al., 1997). That the CRIB domain is essential for Pak's function in regulating R cell axon guidance supports a model in which binding of activated Cdc42/Rac to Pak is necessary for the actin-cytoskeletal changes underlying growth cone guidance. Binding of Rho family GTPases to this domain is critical for stimulating Pak's serine/threonine kinase activity. Indeed, kinase activity of Pak is essential for R cell axon guidance.

The discovery that Pak is constitutively associated with Pix, a guanine nucleotide exchange factor specific for Rac/Cdc42, underscores the potential complexity of interactions between Pak and Rho family GTPases (Manser et al., 1998). Recruitment of the Pak/Pix complex to specific membrane domains in growth cones by activated receptors (e.g., through Dock) may lead to

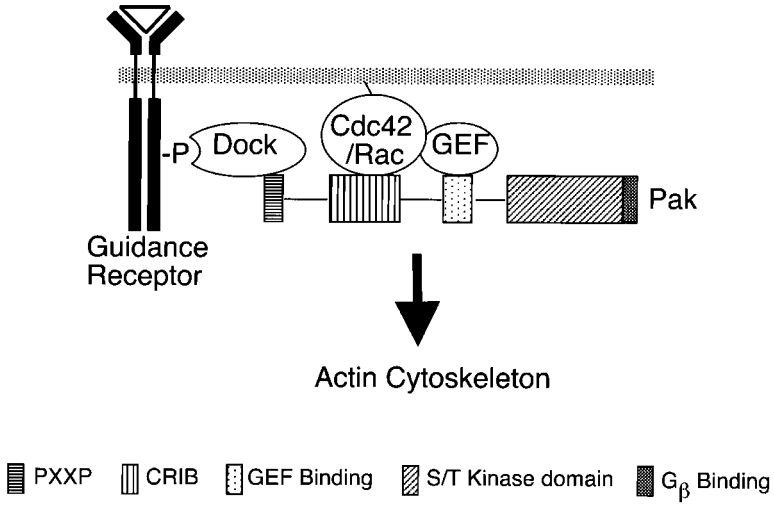

Figure 7. Model for Pak Function

Dock is proposed to bind to activated guidance receptors through its C-terminal SH2 domain. The second SH3 domain of Dock recruits Pak to the membrane. Pix, a GEF bound to Pak, can then activate Cdc42/Rac. In turn, activated Cdc42/Rac promotes Pak kinase activity. We propose that the combined activities of these proteins regulate the actin cytoskeleton in the growth cone.

local activation of Rho family GTPases. In this sense, Pak functions upstream of Cdc42/Rac. However, since the CRIB domain is essential for Pak function in R cell growth cones, it is likely that Pak must be activated by Cdc42/Rac in order to signal. From this perspective, Pak is downstream of Cdc42/Rac. We favor the view, however, that it is the coordinated activity of Pak, Pix, and Rho family GTPases in membrane-associated complexes that regulates actin rearrangements (Figure 7). Complex formation leads to Pak kinase activation, which alone may be sufficent to regulate growth cone motility. Alternatively, activated Cdc42/Rac within the complex may activate other effectors, in addition to Pak, all of which are required for normal axon guidance.

Recent overexpression studies in mammalian tissue culture cells suggest that the N- and C-terminal domains of Pak have separate, but complementary activities in regulating the actin-based cytoskeleton (Frost et al., 1998; Obermeier et al., 1998). Expression of kinase-inactive Pak mutants induces lamellipodia and membrane ruffling (Sells et al., 1996). Conversely, expression of the C-terminal kinase domain leads to dissolution of actin stress fibers (Manser et al., 1997; Frost et al., 1998). Hence, Pak can both promote the formation of specific actin structures and break down others. These properties are well suited for dynamic remodeling of the actin cytoskeleton in response to specific guidance signals. These observations also raise the intriguing possibility that Pak could function in the context of different signaling pathways to mediate both attractive and repulsive responses in the growth cone.

While our findings have established that Pak is a primary target of Dock in R cell growth cones, little is known about the upstream signals that regulate Dock and Pak function. We propose that guidance signals recruit Dock and Pak to the membrane. Two observations support this view. First, overexpression of a constitutively membrane-tethered form of Pak (Pak^{myr}) leads to marked changes in growth cone and cell motility. Second, Pak^{myr} expressed at a lower level substantially rescues the Dock null mutant phenotype. The latter result is somewhat surprising, since we envision that precise spatial regulation of Pak within the membrane by recruitment to activated guidance receptors is crucial to its guidance function, an activity that is unlikely to be replicated entirely by the myristylation tag. Perhaps some elements of spatial regulation are retained if activated guidance receptors nucleate the formation of signaling complexes containing multiple components that can recruit Pak^{myr} in the absence of Dock, albeit at lower efficiency. For instance, the Pix GEF contains an N-terminal PH domain. Activated guidance receptors may, in addition to recruiting Dock, lead to increased levels of phosphoinositides, which in turn provide an alternate route to recruit Pak/Pix into complexes induced by the activated guidance receptors. Indeed, Pak recruitment to focal complexes in cultured cells is mediated by Pix (Manser et al., 1998). Alternatively, guidance signals may independently activate Cdc42 (or Rac) in specific spatial domains within the growth cone. These proteins may subsequently recruit Pak^{myr} to these regions through the CRIB domain. Although we envision that recruitment of Pak to specific spatial domains in growth cone membranes is important in axon guidance, we cannot rule out the possibility that general localization of Pak to the membrane is sufficient to promote its function in guidance.

While our genetic studies do not provide insights into the dynamic nature of Pak recruitment to the membrane, biochemical studies in mammalian cells suggest that not only is Dock recruitment to receptors activity dependent, but so too is association of Nck and Pak. Both T cell receptor and PDGF receptor activation have been shown to lead to a rapid and transient association of Pak and Nck and Pak kinase activation (Bokoch et al., 1996; Yablonski et al., 1998). The importance of regulating Pak activity in cells is underscored by our observations that misexpression of high levels of Pak^{myr}, beyond that necessary to rescue the *dock* mutant phenotype, induces these cells to delaminate from the eye disc epithelium and migrate into the brain.

The recruitment of Pak and Dock to the membrane may nucleate formation of large signaling complexes. In addition to the potential link of Pak to tyrosine kinase signaling pathways through Dock, Pak also contains an extreme C-terminal domain that binds to the Gβ subunit

of trimeric G proteins. Indeed, the yeast Pak Ste20 is activated through this site in response to G protein signaling pathways stimulated by mating pheromone (Leeuw et al., 1998). Pix not only contains a Pak interaction site and a guanine nucleotide exchange factor activity, it also contains a PH domain that potentially links Pak to yet additional pathways, including those regulated by phosphoinositides. And finally, Dock also interacts with multiple proteins, including phosphatases (Clemens et al., 1996) and kinases (see Introduction) as well as other cytoskeletal regulators (e.g., N-WASP; McCarty, 1998). While a Dock/Pak complex is well suited to integrate multiple signals and transmit them to the changes in the actin-based cytoskeleton, Dock and Pak also may act separately in different combinations to regulate growth cone guidance.

Experimental Procedures

Yeast Two-Hybrid Assay

Assays were performed as described previously (Bartel and Fields, 1995). Dock was fused to the DNA-binding domain of *E. coli* LexA (pBTM116), and Pak was fused to the activation domain of yeast Gal4 protein (pACT for full-length Pak and pGAD424 for truncated forms of Pak). Constructs were transformed into L40 cells, and interactions between Dock and Pak were quantified in Miller units by liquid β-galactosidase activity assay as described previously (Bartel and Fields, 1995). Values shown are averages from three independent transformants.

Biochemistry

The rabbit anti-Pak antibody (Ab3119), directed against a peptide from the N-terminal region of Pak, VRLTSNRGGNERSGG, was made by Chiron technologies. The affinity-purified antiserum was used for all Western blots at a dilution of 1:500. The rabbit anti-Dock antibody was a generous gift from J. Clemens and J. Dixon (University of Michigan). Coimmunoprecipitation experiments from S2 cells were as described (Garrity et al., 1999) with the following modifications. Sepharose A beads were first preincubated with the anti-Dock antibody or a control antiserum for 40 min, washed, and then incubated with S2 cell lysates for 30 min at 4°C. After incubation, the beads were washed once with lysis and twice with wash buffer and Western blotted. Pak expression in mutants was analyzed on Western blots of whole fly or larval eye–brain homogenates probed with anti-Pak antibody.

Genetics

Df(3R)Win^{11} was obtained from the Bloomington Stock Center. Single embryo PCR experiments revealed that it uncovers *Pak*. A cosmid clone (T2) carrying an ~30 kb genomic DNA fragment encompassing the *Pak* locus was recovered from a cosmid library (a generous gift of J. Tamkun) and transformed into flies. Lines containing randomly mutagenized third chromosome (*/*TM3*) were crossed to *Df(3R)Win*^{11}/*TM3* to identify lethal mutations mapping to *Df(3R)Win*^{11}. These mutants were crossed to a *Df(3R)Win*^{11} chromosome carrying the T2 cosmid to identify mutations mapping to T2. Lines rescued by the T2 cosmid were further tested for rescue by a *Pak* cDNA driven by the heat shock promoter.

Molecular Biology

DNA sequences of *Pak* mutants were obtained by directly sequencing PCR products of genomic DNA extracted from *Pak/Df(3R)Win*^{11} hemizygotes. Rescue experiments were done using *Pak* cDNAs expressed from either the GMR or pCaSpeR-hs transformation vectors. Mutations in the various *Pak* constructs were generated using the Transformer kit from Clontech. The *Pak*^{myr} construct was made by fusing DNA encoding the first 90 amino acids from *Drosophila Src1* (Simon et al., 1985) to the first codon of *Pak*.

Immunohistochemistry

All staining procedures were essentially as described in Garrity et al. (1996). Two different affinity-purified rabbit anti-Pak antibodies (1:3000) were used for Pak immunolocalization: Ab3119 (see above) and an antiserum directed against the N-terminal 166 amino acids of Pak (Harden et al., 1996). R cell projections were visualized with mAb24B10 at a dilution of 1:200. Lamina neurons were stained with a rat anti-Elav antibody, 7E8A10 (Developmental Studies Hybridoma Bank), at a dilution of 1:25. Glia were stained using a rat anti-Repo antibody at a dilution of 1:100. All secondary antibodies were used at 1:200 dilution. HRP-conjugated goat anti-mouse was from BioRad. FITC-conjugated goat anti-rabbit, Texas red–conjugated goat anti-mouse, FITC-conjugated goat anti-mouse, and CY3-conjugated goat anti-rat antibodies were from Jackson Laboratories.

Acknowledgments

We thank Owen Witte and the members of the Zipursky lab for comments on the manuscript, Karen Ronan for help on manuscript preparation, and Linda Ballard and Susan Tran for technical assistance. This work was supported by National Institutes of Health training grants in Biotechnology (J. X.) and Tumor Biology (H. H.). S. L. Z. is an Investigator of the Howard Hughes Medical Institute.

Received March 29, 1999; revised May 13, 1999.

References

Bartel, P.L., and Fields, S. (1995). Analyzing protein-protein interactions using two-hybrid systems. Methods Enzymol. *254*, 241–263.

Bokoch, G.M., Wang, Y., Bohl, B.P., Sells, M.A., Quilliam, L.A., and Knaus, U.G. (1996). Interaction of the Nck adaptor protein with p21-activated kinase (PAK1). J. Biol. Chem. *271*, 25746–25749.

Brown, J.L., Stowers, L., Baer, M., Trejo, J., Coughlin, S., and Chant, J. (1996). Human Ste20 homologue hPAK1 links GTPases to the JNK MAP kinase pathway. Curr. Biol. *6*, 598–605.

Burbelo, P.D., Drechsel, D., and Hall, A. (1995). A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. J. Biol. Chem. *270*, 29071–29074.

Clemens, J.C., Ursuliak, Z., Clemens, K.K., Price, J.V., and Dixon, J. (1996). A Drosophila protein-tyrosine phosphatase associates with an adaptor protein required for axonal guidance. J. Biol. Chem. *271*, 17002–17005.

de la Torre, J.R., Hopker, V.H., Ming, G.-L., Poo, M.-M., Tessier-Lavigne, M., Hemmati-Brivanou, A., and Holt, C.E. (1997). Turning of retinal growth cones in a netrin-1 gradient mediated by the netrin receptor DCC. Cell *19*, 1211–1224.

Frost, J.A., Khokhlatchev, A., Stippec, S., White, M.A., and Cobb, M.H. (1998). Differential effects of PAK1-activating mutations reveal activity-dependent and -independent effects on cytoskeletal regulation. J. Biol. Chem. *273*, 28191–28198.

Galisteo, M.L., Chernoff, J., Su, Y.C., Skolnik, E.Y., and Schlessinger, J. (1996). The adaptor protein Nck links receptor tyrosine kinases with the serine-threonine kinase Pak1. J. Biol. Chem. *271*, 20997–21000.

Garrity, P.A., Rao, Y., Salecker, I., McGlade, J., Pawson, T., and Zipursky, S.L. (1996). Drosophila photoreceptor axon guidance and targeting requires the Dreadlocks SH2/SH3 adaptor protein. Cell *85*, 639–650.

Garrity, P.A., Lee, C.-H., Salecker, I., Robert, C.D., Zinn, K., and Zipursky, S.L. (1999). Retinal axon target selection in Drosophila is controlled by a receptor protein tyrosine phosphatase. Neuron *22*, 707–717.

Hall, A. (1998). Rho GTPases actin cytoskeleton. Science *279*, 509–514.

Hanks, S.K., Quinn, A.M., and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science *241*, 42–52.

Harden, N., Lee, J., Loh, H.Y., Ong, Y.M., Tan, I., Leung, T., Manser, E., and L.L. (1996). A Drosophila homolog of the Rac- and Cdc42-activated serine/threonine kinase PAK is a potential focal adhesion

and focal complex protein that colocalizes with dynamic actin structures. Mol. Cell. Biol. *16*, 1896–1908.

Kochhar, K.S., and Iyer, A.P. (1996). Hepatocyte growth factor induces activation of Nck and phospholipase C-gamma in lung carcinoma cells. Cancer Lett. *104*, 163–169.

Lamarche, N., Tapon, N., Stowers, L., Burbelo, P.D., Aspenstrom, P., Bridges, T., Chant, J., and Hall, A. (1996). Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. Cell *87*, 519–529.

Leeuw, T., Wu, C., Schrag, J.D., Whiteway, M., Thomas, D.Y., and Leberer, E. (1998). Interaction of a G-protein beta-subunit with a conserved sequence in Ste20/PAK family protein kinases. Nature *391*, 191–195.

Lu, W., Katz, S., Gupta, R., and Mayer, B.J. (1997). Activation of Pak by membrane localization mediated by an SH3 domain from the adaptor protein Nck. Curr. Biol. *7*, 85–94.

Luo, L., Liao, Y.J., Jan, L.Y., and Jan, Y.N. (1994). Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. Genes Dev. *8*, 1787–1802.

Manser, E., Leung, T., Salihuddin, H., Zhao, Z.-S., and Lim, L. (1994). A brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature *367*, 40–46.

Manser, E., Huang, H.Y., Loo, T.H., Chen, X.Q., Dong, J.M., Leung, T., and Lim, L. (1997). Expression of constitutively active alpha-PAK reveals effects of the kinase on actin and focal complexes. Mol. Cell. Biol. *17*, 1129–1143.

Manser, E., Loo, T.H., Koh, C.G., Zhao, Z.S., Chen, X.Q., Tan, L., Tan, I., Leung, T., and Lim, L. (1998). PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. Mol. Cell *1*, 183–192.

McCarty, J.H. (1998). The Nck SH2/SH3 adaptor protein: a regulator of multiple intracellular signal transduction events. BioEssays *20*, 913–921.

Melzig, J., Rein, K.H., Schafer, U., Pfister, H., Jackle, H., Heisenberg, M., and Raabe, T. (1998). A protein related to p21-activated kinase (PAK) that is involved in neurogenesis in the Drosophila adult central nervous system. Curr. Biol. *8*, 1223–1226.

Ming, G.L., Song, H.-J., Berninger, B., Holt, C.E., Tessier-Lavigne, M., and Poo, M.-M. (1997). cAMP-dependent growth cone guidance by netrin-1. Neuron *19*, 1225–1235.

Mitchison, T.J., and Cramer, L.P. (1996). Actin-based cell motility and cell locomotion. Cell *84*, 371–379.

Obermeier, A., Ahmed, S., Manser, E., Yen, S., Hall, C., and Lim, L. (1998). PAK promotes morphological changes by acting upstream of Rac. EMBO J. *17*, 4328–4339.

Rao, Y., and Zipursky, S.L. (1998). Domain requirements for the Dock adaptor protein in growth-cone signaling. Proc. Natl. Acad. Sci. USA *95*, 2077–2082.

Salecker, I., Clandinin, T.R., and Zipursky, S.L. (1998). Hedgehog and Spitz: making a match between photoreceptor axons and their targets. Cell *95*, 587–590.

Sells, M.A., Knaus, U.G., Bagrodia, S., Ambrose, D.M., Bokoch, G.M., and Chernoff, J. (1996). Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. Curr. Biol. *7*, 202–210.

Simon, M.A., Drees, B., Kornberg, T., and Bishop, J.M. (1985). The nucleotide sequence and the tissue-specific expression of Drosophila c-src. Cell *42*, 831–840.

Stein, E., Huynh-Do, U., Lane, A.A., Cerretti, D.P., and Daniel, T.O. (1998). Nck recruitment to Eph receptor, EphB1/ELK, couples ligand activation to c-Jun kinase. J. Biol. Chem. *273*, 1303–1308.

Suter, D.M., and Forscher, P. (1998). An emerging link between cytoskeletal dynamics and cell adhesion molecules in growth cone guidance. Curr. Opin. Neurobiol. *8*, 106–116.

Tessier-Lavigne, M., and Goodman, C.S. (1996). The molecular biology of axon guidance. Science *274*, 1123–1133.

Winberg, M.L., Mitchell, K.J., and Goodman, C.S. (1998). Genetic analysis of the mechanisms controlling target selection: complementary and combinatorial function of netrins, semaphorins and IgCAMs. Cell *93*, 581–591.

Yablonski, D., Kane, L.P., Qian, D., and Weiss, A. (1998). A Nck-Pak1 signaling module is required for T-cell receptor-mediated activation of NFAT, but not of JNK. EMBO J. *17*, 5647–5657.

Zhao, Z.S., Manser, E., Chen, X.Q., Chong, C., Leung, T., and Lim, L. (1998). A conserved negative regulatory region in alphaPAK: inhibition of PAK kinases reveals their morphological roles downstream of Cdc42 and Rac1. Mol. Cell. Biol. *18*, 2153–2163.

Zipkin, I.D., Kindt, R.M., and Kenyon, C.J. (1997). Role of a new Rho family member in cell migration and axon guidance in C. elegans. Cell *90*, 883–894.