Dishevelled-1 Regulates Microtubule Stability: A New Function Mediated by Glycogen Synthase Kinase-3β

Olga Krylova,*† Marcus J. Messenger,* and Patricia C. Salinas*‡

*The Randall Institute, King’s College London, London, United Kingdom, WC2B 5RL; and ‡Department of Biochemistry, Imperial College of Science, Technology and Medicine, London, United Kingdom, SW7 2AY

Abstract. Dishevelled has been implicated in the regulation of cell fate decisions, cell polarity, and neuronal function. However, the mechanism of Dishevelled action remains poorly understood. Here we examine the cellular localization and function of the mouse Dishevelled protein, DVL-1. Endogenous DVL-1 colocalizes with axonal microtubules and sediments with brain microtubules. Expression of DVL-1 protects stable microtubules from depolymerization by nocodazole in both dividing cells and differentiated neuroblastoma cells. Deletion analyses reveal that the PDZ domain, but not the DEP domain, of DVL-1 is required for microtubule stabilization. The microtubule stabilizing function of DVL-1 is mimicked by lithium-mediated inhibition of glycogen synthase kinase-3β (GSK-3β) and blocked by expression of GSK-3β. These findings suggest that DVL-1, through GSK-3β, can regulate microtubule dynamics. This new function of DVL-1 in controlling microtubule stability may have important implications for Dishevelled proteins in regulating cell polarity.

Key words: WNT • granule cells • cerebellum • cytoskeleton • nocodazole

Introduction

Dishevelled was originally characterized as a Drosophila mutation that affects planar polarity and epithelial patterning in the early embryo (Perrimon and Mahowald, 1987; Adler, 1992; Klingensmith et al., 1994). Dishevelled is a cytoplasmic protein implicated in the Notch and WNT signaling pathways. In the Notch pathway, Dishevelled acts as a repressor, whereas it is a positive regulator of the WNT signaling pathway (Klingensmith et al., 1994; Noordermeer et al., 1994; Siegfried et al., 1994; Axelrod et al., 1996). In Drosophila and Xenopus embryos, overexpression of dishevelled confers a similar phenotype to Wnt overexpression, suggesting that Dishevelled activates WNT signaling (Rothbacher et al., 1995; Sokol et al., 1995; Yanagawa et al., 1995). The current model for the canonical pathway proposes that Wg, the Drosophila homologue of WNT, activates Dishevelled through the Frizzled receptor to control epithelial patterning in Drosophila. Activation of Dishevelled results in the inhibition of Shaggy (Sgg), a serine/threonine kinase, and the homologue of glycogen synthase kinase-3β (GSK-3β). Inhibition of GSK-3β/Sgg leads to increased levels of β-catenin/Armadillo and the activation of transcription via T-cell factor/Pangolin factors (Cadigan and Nusse, 1997; Arias et al., 1999). In planar cell polarity, however, Dishevelled activates the c-Jun NH2-terminal protein kinase (JNK) pathway in an Armadillo-Pangolin–independent manner (Boutros et al., 1998). Different domains of Dishevelled are required for signaling through these different pathways. It has been proposed that Dishevelled function is to interpret signals coming from the plasma membrane and to direct them to different signaling pathways (Boutros and Mlodzik, 1999).

Dishevelled proteins possess three conserved domains: a DIX domain, present in the WNT antagonizing protein Axin (Zeng et al., 1997), a PDZ domain involved in protein–protein interactions (Ponting et al., 1997), and a DEP domain found in proteins that regulate Rho GTPases (Ponting and Bork, 1996). In the canonical WNT pathway, the PDZ domain is required. Whereas the DEP domain is...
essential for activation of the planar polarity pathway (for review, see Boutros and Mlodzik, 1999). Mutation in the DEP domain of **dishevelled** results in the random orientation of hair cells, suggesting that Dishevelled may control the reorganization of the cytoskeleton in planar cell polarity (Axelrod et al., 1998). Epistatic analyses support the role of RhoA in this process (Strutt et al., 1997). Thus, Dishevelled may regulate the actin cytoskeleton through the JNK pathway. However, the role of Dishevelled in regulating the cytoskeleton through other pathways has not been established. In the mouse, three **dishevelled** genes, **Dvl-1**, **Dvl-2**, and **Dvl-3** have been identified (Sussman et al., 1994; Klingensmith et al., 1996; Yang et al., 1996). **Dvl-3** is expressed throughout development, but functional studies are missing (Tsang et al., 1996). **Dvl-2** is ubiquitously expressed during embryonic development and in many adult tissues (Klingensmith et al., 1996). More recently, **Dvl-2** has been shown to be highly expressed in the outer root sheath and hair precursor cells (Millar et al., 1999). Studies using transgenic mice show that mouse Dishevelled protein, **DVL-2**, mimics WNT-3 function and therefore suggest that **DVL-2** is part of the WNT signaling pathway involved in hair development (Millar et al., 1999). Like **Dvl-2**, **Dvl-1** is ubiquitously expressed at early stages of development (Sussman et al., 1994). In the central nervous system (CNS), **Dvl-1** is highly expressed in areas of high neuronal density at embryonic and postnatal stages of development (Sussman et al., 1994). Analysis of the **Dvl-1** null mouse shows that **Dvl-1** is not required for early development. However, **Dvl-1** null mice exhibit behavioral abnormalities and neurological deficits, suggesting that **DVL-1** is required for the formation and/or function of specific neuronal pathways (Lijam et al., 1997).

Neurons are highly polarized cells with stereotypic dendritic arborizations and axons. The neuronal cytoskeleton is essential for the formation and maintenance of this polarized morphology. Recent studies on cerebellar neurons have demonstrated that WNT-7a regulates axonal morphology. WNT-7a increases growth cone size and axonal branching, while decreasing axon length (Lucas and Salinas, 1997; Hall et al., 2000). These changes are mediated through the inhibition of GSK-3β. The axonal remodelling activity of WNT-7a is associated with changes in microtubule (MT) organization (Hall et al., 2000), and the GSK-3β–mediated phosphorylation of the microtubule-associated protein, MAP-1B (Lucas et al., 1998). GSK-3β also phosphorylates Tau, an axonal microtubule-associated protein that, like MAP-1B, is involved in MT stability (Hanger et al., 1992). These findings suggest that GSK-3β regulates the organization of neuronal MTs by changing the phosphorylation of MAPs. However, the mechanisms controlling GSK-3β activity during MT reorganization remain poorly understood.

Here we examine the expression and function of **DVL-1**, a regulator of GSK-3β, in developing neurons. In the CNS, **DVL-1** is localized to neurons of the cortex, hippocampus, pons, and cerebellum. Three isoforms of **DVL-1** are differentially expressed during neuronal maturation. **DVL-1** colocalizes with axonal MTs and sediments with brain MTs. Expression of **DVL-1** protects stable MTs from depolymerization by nocodazole. The PDZ domain and, to a lesser extent, the DIX domain of **DVL-1** are required for MT stabilization. Furthermore, this process is mediated by the inhibition of GSK-3β. These findings demonstrate a novel function for **DVL-1** in regulating MT organization.

**Materials and Methods**

**Production of DVL-1 Antibodies**

Polyclonal **DVL-1** antibody was made against the carboxy-terminal 46 amino acids of **DVL-1** fused to glutathione S-transferase protein (GST-DVL-46; kindly provided by Karl Willeit and Roel Nusse, Stanford University, Stanford, CA). Specific **DVL-1** antibody was affinity purified using CNBr-Sepharose bead coupled to GST-DVL-46. The obtained anti-serum was run through a GST column, and then affinity purified on a GST-DVL-46 column (Pierce Chemical Co.). This antibody, but not the preimmune serum, recognized the **DVL-1** protein in Western blots. Anti-body specificity was confirmed by the lack of **DVL-1** immunoreactivity in cerebellar protein extracts isolated from the **DVL-1** null mutant mice (Lijam et al., 1997) and by blocking with purified GST-DVL-46 peptide.

**Neuronal Cell Cultures**

Cerebellar granule cells were isolated from newborn mice and purified using Percoll gradients (Hatten, 1985). Neurons were plated onto lamin-coated dishes at a density of 10⁵ cells/cm² and grown in serum-free medium for 2 d, as described previously (Lucas et al., 1998). Cells were fixed either in 4% formaldehyde in PBS or in 3% paraformaldehyde, 0.2% glutaraldehyde, 0.2% Triton X-100, 10 mM EGTA in PBS (detergent fixation) and stored in PBS at 4°C.

**Immunofluorescence Microscopy**

Fixed cultures were incubated with primary antibodies overnight at 4°C. Goat anti-rabbit IgG and goat anti–mouse IgG antibodies labeled with Alexa 488 and Alexa 568 and Alexa 488, respectively (Molecular Probes), and anti–rat IgG-FITC from Vector or anti–rat IgG-AMCA (Chemicon) were used as secondary antibodies. Images were obtained with a microscope (BX60; Olympus) or with a laser scanning confocal microscope (DMR; Leica). Photographic images were scanned, stored as TIFF files, and analyzed using Photoshop 4.0 (Adobe Systems Inc.). Other primary antibodies used were against acetylated tubulin (Sigma-Aldrich), growth associated protein-43 (GAP-43; monoclonal, Boehringer; polyclonal from Dr. Graham Wilkin, Imperial College, London, UK), and hemagglutinin (HA; Boehringer). For immunohistochemistry, brain tissues were fixed with 4% paraformaldehyde (PFA), embedded in polyfreeze tissue embedding medium (Polysciences, Inc.), and stored at ~70°C. Cryosections of 18 μm were fixed again in 4% PFA and incubated with **DVL-1** antibody overnight. Antibody was visualized with the ABC kit (Vector Laboratories) and DAB. No staining was obtained with **DVL-1** antibody previously blocked with purified GST-DVL-46 peptide.

**Taxol Polymerization and Cycling of Microtubules**

For taxol experiments, crude MTs were prepared from mouse cerebellum using a method adapted from Fuji et al. (1990). Dissected cerebella were homogenized on ice in MES buffer (100 mM MES, pH 6.5, 0.5 mM magnesium acetate, 1 mM dithiothreitol, 1 mM GTP. After centrifugation at 100,000 g for 1 h at 4°C, the homogenate was added to an equal volume of PEGM buffer containing 8 M glycerol, 1 mM EGTA, and 1 mM MgCl₂ with 1 mM ATP, and 0.1 mM GTP. After centrifugation at 100,000 g for 1 h at 4°C, the homogenate was spun again for 1 h at room temperature to collect the polymerized MT
fraction (P1) and supernatant (S1). MTs were depolymerized by incubating on ice for 1 h, and then spun down at 100,000 g, 4°C, to collect the cold stable MT fraction (CSP1). The supernatant was then incubated at 37°C for 30 min to polymerize MTs again, and spun down to obtain supernatant (S2) and MT pellet (P2). The P2 fraction was then subjected to a further cycle of temperature-dependent depolymerization and repolymerization. In a second set of experiments, DVL-HA, obtained from cell lysates of DVL-HA–transfected COS cells, was added to the crude MT suspension before two successive cycles of cold/warm exposure. Aliquots were removed for Western analysis. Endogenous DVL-1 protein was detected with DVL-1 antibody and DVL-HA with the HA antibody.

**Western Blot Analysis**

Mouse cerebella or whole brains were dissected and homogenized in cold sample buffer. Boiled tissue samples or cultured cell extracts were run on 10% SDS-PAGE gels and proteins were transferred to Hybond-C super (Amersham Pharmacia Biotech) membranes and probed with antibodies against DVL-1, MAP-1B (kindly provided by P. Gordon-Weeks, King’s College, London, UK), α-tubulin, and extracellular signal-regulated kinase (Erk), and developed using HRP-coupled anti–rabbit (Sigma-Aldrich) or anti-mouse (Amersham Pharmacia Biotech) antibodies and enhanced chemiluminescence reagent (Pierce Chemical Co.). Protein levels were normalized using the BSA protein assay (Pierce Chemical Co.) and Coomassie blue or by staining the membranes with Ponceau red.

**Plasmids and Transfections**

DVL-HA, DVL-1 deletion mutants, and GSK-3β-HA constructs were generated by PCR using the high fidelity thermostable DNA polymerase plaque-forming units (Stratagene or Promega) and cloned into the pc22+ expression vector. Constructs were verified by DNA sequencing. The expression of green-fluorescent protein (GFP), DVL-HA constructs, and GSK-3β-HA were driven by the cytomegalovirus promoter. DVL-1, without the tag, was driven by the SV40 promoter. Plasmids for transfection were isolated using a maxi-prep endotoxin-free kit (QIAGEN). COS cells were transfected using the calcium-phosphate technique and cultured for another 48 h. Neuroblastoma 2a (NB2a) cells were transfected using Lipofectamin (GIBCO-BRL), and then differentiated for another 48 h. Neuroblastoma 2a (NB2a) cells were transfected using Lipofectamin (GIBCO-BRL), and then differentiated for another 48 h. Neuroblastoma 2a (NB2a) cells were transfected with nucodazole (10 or 5 μM) for 1 h and fixed in 3% formaldehyde, 0.2% glutaraldehyde, 0.2% Triton X-100, and 10 mM EGTA in PBS.

**Results**

**DVL-1 Is Expressed during Postnatal Development**

To examine the function of DVL-1 in neuronal development, we generated an affinity-purified polyclonal antibody against a peptide encoding the 46 carboxy-terminal amino acids of DVL-1. Using this antibody, we found that DVL-1 is localized to several neuronal populations of the adult mouse CNS. High levels of DVL-1 protein were found in neurons of the cerebral cortex, hippocampus, pons, and cerebellum (Fig. 1, A–J). In the cortex, DVL-1 is localized to the cell body and processes of neurons (Fig. 1 A). High levels of DVL-1 were found in pyramidal neurons (Fig. 1 A, insert). In the cerebellum, we examined in more detail the distribution of DVL-1 during postnatal cerebellar development (Fig. 1, D–J). At birth, DVL-1 was found in the external granular cell layer (EGL) and in the forming internal granule cell layer (IGL) of the cerebellum (Fig. 1 D). At P7, the level of DVL-1 is higher in the EGL as granule cells migrate to the IGL. DVL-1 was also observed in the forming IGL (Fig. 1 E). By P14, DVL-1 is highly expressed in the granule cell layer and this expression is maintained in adult life (Fig. 1, F–J). DVL-1 is also expressed in the Purkinje cell layer, when Purkinje cells begin to mature and develop a complex dendritic tree (Fig. 1, F–J). From P21, low levels of DVL-1 were found in the molecular layer (Fig. 1, G–I). In Dvl-1 null mutant mice, no immunoreactivity was observed (Fig. 1 K).

Figure 1. DVL-1 expression in postnatal mouse brain. Brain sections were immunostained for DVL-1 (A–J). The highest level of DVL-1 was detected in neurons of the cerebral cortex, hippocampus, pons, and cerebellum (A–C and I). (A, inset) DVL-1 localization in cell bodies and processes of pyramidal neurons. In P0 cerebellum, DVL-1 is expressed in the EGL and forming IGL (D). At P7, DVL-1 immunoreactivity increases in the EGL (E). At P14, DVL-1 was mainly localized in the granule cell layer (GCL) and in the Purkinje cell (PC) layer (F). This pattern of DVL-1 expression was maintained throughout life (G–I). A low level of DVL-1 expression was detected in the molecular layer (ML) from P21 (G–I). At P21, DVL-1 is mainly localized in granule cells and Purkinje cells cell bodies, as shown at higher magnification (J). No immunoreactivity was detected in the cerebellum of adult Dvl-1 null mutant mice (K). Preincubation of the antibody with DVL-46 peptide completely abolished immunostaining in adult cerebellum (L). AD, adult; DG, dentate gyrus. Bar, 100 μM.
abolishes DVL-1 staining in adult mouse cerebellum (Fig. 1 L). These results show that DVL-1 is mainly localized to neurons throughout life.

We next examined the levels of DVL-1 in the developing cerebellum by Western analysis. The DVL-1 antibody recognizes three main species migrating with apparent molecular weights of 96, 88, and 83 kD. None of the three species were detected in cerebellar samples isolated from Dvl-1 null mutant mice, used as a negative control, reveal the absence of the three DVL-1 isoforms. Expression of DVL-1 in QT-6 cells reveals the presence of the high molecular weight isoforms (DVL-1). The same blot was reprobed with an antibody against extracellular signal-regulated kinase to control for loading.

**Figure 2.** DVL-1 isoforms are developmentally regulated. Western blot analysis of cerebellar extracts from P0, P6, P14, P21, and adult mice reveals changes in the level of three DVL-1 isoforms. DVL-1 antibody recognizes three DVL-1 species of 96 kD (arrow), 88 kD (arrowhead), and 83 kD (asterisk). At birth, the three forms of DVL-1 are present, but the 83-kD isoform is expressed at low levels. During cerebellar maturation, expression of the 83-kD isoform increases, while the two higher molecular weight isoforms decrease. Cerebella samples from Dvl-1 null mutant mice, used as a negative control, reveal the absence of the three DVL-1 isoforms. Expression of DVL-1 in QT-6 cells reveals the presence of the high molecular weight isoforms (DVL-1). The same blot was reprobed with an antibody against extracellular signal-regulated kinase to control for loading.

**Figure 3.** The subcellular distribution of DVL-1 in maturing cerebellar granule cell neurons. Granule cell cultures were grown for 2 d and stained with antibodies to DVL-1 (A, D, G, and J), GAP-43 (B and E), and acetylated tubulin (H and K). DVL-1 has a punctate distribution in the neuronal cell bodies and along the axon shaft (A–C). High levels of DVL-1 were detected in the central domain of the growth cone (D and F). DVL-1 immunostaining colocalizes with acetylated tubulin along the axon shaft (G–I). In neurons fixed in the presence of detergent, a pool of DVL-1 remains colocalized with acetylated tubulin (J–L). CB, cell body; AS, axon shaft; GC, growth cone. Bar: A–C and G–I, 20 μM; D–F and J–L, 10 μM.

**DVL-1 Colocalizes with Axonal Microtubules**

To determine the localization of DVL-1 in neurons, we examined DVL-1 in cerebellar granule cell cultures. Double labeling for GAP-43, a protein that reveals neuronal morphology, shows that DVL-1 protein has a punctate distribution in the cell body, along the axon shaft and at the growth cone (Fig. 3, A–F). At the growth cone, the highest levels of DVL-1 were found in the central domain that contains numerous MTs (Fig. 3, D–F). In the axon shaft, DVL-1 immunoreactivity seems to localize to regions of the axon where MTs are present (Fig. 3 A). To visualize MTs, we used acetylated tubulin antibody that labels stable MTs (Bulinski et al., 1988). Double labeling for acetylated MTs and DVL-1 reveals that high levels of DVL-1 appear to colocalize with axonal MTs (Fig. 3, G–L). To test whether DVL-1 interacts with MTs, cerebellar granule cells were detergent-extracted during fixation, a procedure that leaves the cytoskeleton and its associated proteins intact yet removes cytoplasmic and membrane-associated proteins. DVL-1 immunoreactivity was retained after detergent extraction, although at a lower level, suggesting that a fraction of DVL-1 is associated with the cytoskeleton (Fig. 3, J–L). In contrast, the membrane-bound protein, GAP-43, was removed by this treatment (data not shown). Most of the DVL-1 immunoreactivity colocalizes with acetylated MTs (Fig. 3, J–L). However, at the growth cone, low levels of DVL-1 were found in areas lacking acetylated MTs, suggesting that DVL-1 may also be associated with other cytoskeleton components (Fig. 3, J–L). These results suggest that a pool of DVL-1 colocalizes with axonal MTs.

To investigate the possible association of DVL-1 with axonal MTs, we examined whether endogenous DVL-1 cosediments with taxol-polymerized MTs from mouse brain. Indeed, DVL-1 was found in taxol-stabilized MT fractions (Fig. 4, A and B). In adult brain lysate, similar levels of the 83-kD isoform were found in the soluble and pellet fractions when taxol was absent (Fig. 4 A). Addition of taxol leads to MT polymerization and sedimentation of most of the 83-kD protein into the pellet MT fraction (Fig. 4 A). We also examined the distribution of endogenous DVL-1 at early postnatal stages when all three isoforms of DVL-1 are present. The soluble fraction from P5 brain contains low levels of the 96-kD DVL-1 protein. However, MT fractions contain the 88-kD pro-
tein and low levels of the 96- and 83-kD DVL-1 proteins (Fig. 4 B). These results show that a higher proportion of DVL-1 becomes associated with MTs in adult brain compared with younger brain (compare Fig. 4, A and B).

To test further the association of DVL-1 with MTs, we performed MT depolymerization and repolymerization experiments in which MTs are depolymerized by cold temperature and repolymerized by GTP at 37°C. After polymerization of MTs from adult brain lysate, the 96-kD isoform and, more abundantly, the 83-kD DVL-1 isoform cosediment with polymerized MTs (Fig. 4 C). However, the 83-kD DVL-1 protein was also found in the soluble fraction (S1). Interestingly, when the MT pellet (P1) was subjected to another round of depolymerization, high levels of DVL-1 immunoreactivity were found in the cold stable MT pellet. Cold stability is a property of neuronal MTs that have a high proportion of stable MTs (Webb and Wilson, 1980). These findings suggest that DVL-1 is tightly associated with the more stable pool of MTs (Fig. 4 C). In further cycles, no DVL-1 was detected (Fig. 4 C). In contrast, the MT-associated protein, MAP-1B, cosediments with MTs after a few cycles of depolymerization/repolymerization (Fig. 4 C), although at much lower levels in the second and third cycles (Fig. 4 C). The lack of detection of DVL-1 in further cycles could be due to the low levels of endogenous DVL-1 in brain samples as compared with MAP-1B. To test this possibility, we added exogenous DVL-1 protein to the crude MT fraction. We found that addition of a lysate from DVL-HA–expressing COS cells increases the level of DVL-1 in the cold stable pellet; however, no DVL-1 was detected in the second cycle of polymerization (data not shown). Taken together, these experiments show that endogenous DVL-1 protein, especially the 83-kD isoform, is associated with taxol-stabilized MT fractions and with cold-stable MTs from the adult brain.

**DVL-1 Stabilizes Microtubules**

The interaction of endogenous DVL-1 with neuronal MTs suggests its possible role in controlling MT organization or, alternatively, that MTs are just used to transport DVL-1 along the axon. We obtained evidence in favor of the former possibility when we examined the effect of expression of Dvl-HA on MTs in COS cells. Consistent with pre-
vious studies, DVL-HA forms vesicle-like structures in transfected cells (Fig. 5, A and G; Fagotto et al., 1999; Smalley et al., 1999). Cells expressing DVL-1 have similar levels of acetylated MTs than neighboring nonexpressing cells (Fig. 5, A–C). Similar results were obtained with DVL-1 without the epitope tag HA (data not shown). We then examined the effect of DVL-1 on MTs in cells that have been treated with 10 μM nocodazole, an MT depolymerizing drug (Hoebeke et al., 1976; Lee, 1990). Expression of GFP (control) does not prevent nocodazole-induced MT depolymerization as GFP-expressing cells, like untransfected cells, have no stable MTs (Fig. 5, D–F). However, cells expressing DVL-HA have a significant number of stable MTs after nocodazole treatment (Fig. 5, G–I). We often observed that DVL-1 induces the formation of processes (Fig. 5, G–I). Interestingly, most of the DVL-HA that colocalizes with MTs is in small vesicle-like structures (Fig. 5 I, insert). The stabilizing effect of DVL-1 was quantified by counting the number of transfected cells displaying stable MTs after nocodazole treatment. While expression of GFP does not stabilize microtubules, DVL-HA driven by the cytomegalovirus promoter stabilizes MTs in 74.7% of transfected cells after nocodazole treatment (Fig. 6 C). These results show that DVL-1 can protect MTs from nocodazole and can therefore act as an MT stabilizer.

The PDZ Domain of DVL-1 Is Required for Microtubule Stabilization

Dishevelled proteins have three conserved domains that are required for signaling through distinct pathways.
The Microtubule Stabilizing Activity of DVL-1 Is Mediated through GSK-3β

In the WNT signaling pathway, activation of Dishevelled results in the inhibition of GSK-3β (Cadigan and Nusse, 1997; Arias et al., 1999). GSK-3β itself phosphorylates a number of MT-associated proteins such as Tau, MAP-1B, and MAP-2 that control MT stability (Hanger et al., 1992; Mandelkow et al., 1992; Lucas et al., 1998). Therefore, DVL-1 might regulate MT stability through inhibition of GSK-3β. Consistent with this idea, expression of GSK-3β blocks the ability of DVL-1 to protect MTs against nocodazole (Fig. 7, A–C). This effect of GSK-3β depends on its kinase activity as a kinase-dead, dominant-negative form of GSK-3β (dnGSK-3β) fails to block the MT stabilizing effect of DVL-1 (Fig. 7, D–F and J). Neither GSK-3β-HA nor dnGSK-3βHA when expressed alone have a detectable effect on MT stability in nocodazole-treated cells (data not shown). Expression of DVL-1 under the SV40 promoter prevents MT depolymerization in 65.6% of transfected cells (Fig. 7 J), whereas 15.2% of cells expressing DVL-1 and GSK-3β-HA display stable MTs after nocodazole treatment (Fig. 7 J). dnGSK-3β, when coexpressed with DVL-1, protects MTs against depolymerization by nocodazole in 74.4% of cells (Fig. 7 J). Interestingly, coexpression of dnGSK-3β and DVL-1 increases the number of cells that retain their MTs (Fig. 7 J), suggesting that dnGSK-3β enhances DVL-1 function. These results suggest that DVL-1 stabilizes MTs through the inhibition of GSK-3β. To test further this idea, we examined the effect of lithium, a direct inhibitor of GSK-3β and an activator of the WNT pathway (Klein and Melton, 1996). Control NaCl has no effect on MT stability (Fig. 7 G). In contrast, lithium chloride stabilizes MTs in COS cells treated with nocodazole (Fig. 7, H and I). Lithium has a concentration-dependent MT stabilizing effect. At 20 mM lithium, few MTs are stabilized (Fig. 7 H), whereas more MTs are stabilized in the presence of 40 mM lithium (Fig. 7 I). The addition of myo-inositol to lithium-treated cells did not affect the MT stabilizing effect of lithium, suggesting that depletion of inositol, another known effect of lithium (Atack et al., 1995), is not involved in this process (data not shown). These results suggest that the MT stabilizing activity of DVL-1 is mediated by the inhibition of GSK-3β.

DVL-1 Stabilizes Axonal Microtubules

DVL-1 colocalizes with axonal MTs in maturing neurons (Fig. 3). To test whether DVL-1 also regulates the stability of axonal MTs, we examined the effect of expression of DVL-1-HA in differentiated NB2a neuroblastoma cells. Untransfected and GFP-expressing NB2a cells have bundles of acetylated MTs along the axon (Fig. 8, B and C, and data
Previous studies in Xenopus embryos have shown that Dsh-GFP moves along MTs, suggesting that Dishevelled interacts with MTs, although biochemical evidence was not provided (Miller et al., 1999). Here we demonstrate a physical association of endogenous DVL-1 with MTs isolated from brain tissues. Consistent with these findings, DVL-1 is mainly localized to neurons that contain high levels of stable MTs. Thus, Dishevelled proteins associate with MTs in different cell types, suggesting a general role for Dishevelled in regulating MT organization.

Three isoforms of DVL-1 are expressed during postnatal cerebellar development with the 83-kD DVL-1 isoform becoming more predominant in adult cerebellum. This isoform is tightly associated with cold stable MT fractions, representing axonal MTs (Webb and Wilson, 1980; Brady et al., 1984). In agreement with this finding, immunofluorescence studies show that DVL-1 colocalizes with MTs during developing axons. The association with cold stable MTs has been observed with STOP proteins and Doublecortin, both microtubule-associated proteins that regulate MT stability (Guillaud et al., 1998; Francis et al., 1999; Gleeson et al., 1999). DVL-1 colocalizes with axonal MTs and sediments with MT fractions after nocodazole treatment (arrow), whereas untransfected cells have no acetylated MTs (asterisk). Bar, 25 μM.

**Discussion**

In this paper, we demonstrate a novel function of DVL-1 in regulating MT stability. In neurons, endogenous DVL-1 colocalizes with axonal MTs and sediments with MT fractions. Our studies show that DVL-1 stabilizes MTs, a process mediated by the inhibition of GSK-3β. These findings suggest a possible mechanism by which Dishevelled proteins could regulate cell morphology, cell polarity, and asymmetric cell divisions.

**Dishevelled Is Associated with Microtubules**

Dishevelled is a major component of the Wnt signaling pathway, a PDZ domain present in a number of signaling factor. Takemura et al., 1992). Thus, DVL-1 acts as a MT stabilizer.

**DVL-1 Stabilizes Microtubules through GSK-3β Inhibition**

DVL-1 regulates MT stability as expression of DVL-1 increases the level of acetylated MTs and protects MTs from depolymerization by nocodazole in COS and neuroblastoma 2a cells. Similar results have been observed with microtubule-associated proteins such as Tau, MAP-1B, and MAP-2, which increase MT stability (Baas et al., 1994; Takemura et al., 1992). Thus, DVL-1 acts as a MT stabilizing factor.

How does DVL-1 regulate MT stability? Dishevelled proteins contain three conserved domains, a DIX domain also present in Axin, a negative regulator of the WNT signaling pathway, a PDZ domain present in a number of junctional proteins of the PSD-95 and Disc-large family,...
and a DEP domain present in the Caenorhabditis elegans protein Egl-10 and in Pleckstrin (for review, see Boutros and Mlodzik, 1999). The PDZ domain is required for Armadillo stabilization through the canonical WNT pathway (Sokol et al., 1995; Yanagawa et al., 1995). In contrast, the DEP domain, but not the PDZ domain, is required for JNK signaling (Boutros et al., 1998; Li et al., 1999). Here, we show that the PDZ domain is required for MT stabilization, while deletion of the DIX domain has a weaker effect. In contrast, deletion of the DEP domain does not affect the MT stabilizing function of DVL-1. The requirement of the PDZ domain suggests that DVL-1 may be acting through the canonical pathway. Consistent with these findings, DVL-1 stabilizes MTs through the inhibition of GSK-3β, a downstream component of the canonical WNT pathway. This mechanism is supported by three findings. Firstly, expression of GSK-3β blocks the MT stabilizing activity of DVL-1. Secondly, dnGSK-3β enhances DVL-1 function. Thirdly, lithium, an inhibitor of GSK-3β (Klein and Melton, 1996), mimics the MT stabilizing activity of DVL-1. Inhibition of GSK-3β by DVL-1 could lead to changes in the phosphorylation of microtubule-associated proteins such as Tau, MAP-1B, and MAP-2 that are direct targets of GSK-3β, a downstream component of the canonical WNT pathway. This mechanism is supported by three findings. Firstly, expression of GSK-3β blocks the MT stabilizing activity of DVL-1. Secondly, dnGSK-3β enhances DVL-1 function. Thirdly, lithium, an inhibitor of GSK-3β (Klein and Melton, 1996), mimics the MT stabilizing activity of DVL-1. Inhibition of GSK-3β by DVL-1 could lead to changes in the phosphorylation of microtubule-associated proteins such as Tau, MAP-1B, and MAP-2 that are direct targets of GSK-3β (Hanger et al., 1992; Lucas et al., 1998; Sanchez Martin et al., 1998). Studies of Tau and MAP-1B show that GSK-3β-mediated phosphorylation decreases the ability of Tau to bind to MTs and decreases MT stabilization by MAP-1B (Wagner et al., 1996; Hong et al., 1997; Goold et al., 1999). Thus, inhibition of GSK-3β by DVL-1 may change the ability of MAPs to stabilize MTs. Although inhibition of GSK-3β by lithium stabilizes MTs, DVL-1 has a stronger stabilizing effect than high levels of lithium. Thus, it is likely that DVL-1 promotes MT stability via inhibition of GSK-3β, but an alternative pathway may also contribute to this function of DVL-1. Dishevelled has recently been found to colocalize with actin fibers (Torres and Nelson, 2000). More importantly, Dishevelled has been proposed to influence the actin cytoskeleton through the JNK pathway (Strutt et al., 1997; Axelrod et al., 1998). As MT dynamics is influenced by actin, Dishevelled could regulate MTs indirectly through the JNK pathway by changing the actin cytoskeleton. However, this mechanism seems unlikely, as the DEP domain of DVL-1, essential for the JNK pathway, is not required for MT stability. Consequently, our data suggest that MT stabilization by DVL-1 is mediated through GSK-3β signaling and a parallel, as yet unidentified, pathway.

In maturing neurons, endogenous DVL-1 colocalizes with axonal MTs that contain high levels of stable MTs. More importantly, DVL-1 increases the level of stable MTs in differentiated NB2a neuroblastoma cells and protects axonal MTs against nocodazole treatment. These findings suggest that neuronal DVL-1 may stabilize axonal MTs in vivo. Consistent with this view, a higher proportion of endogenous DVL-1 becomes associated with MTs from adult brain samples, which are enriched in stable MTs. Moreover, DVL-1 sediments with cold stable MT fractions representing the most stable pool of MTs. Thus, the localization of DVL-1 to MTs correlates with increased MT stability in maturing axons. Further studies are needed to address the in vivo role of DVL-1 in microtubule dynamics in developing neurons.

Dishevelled in Cell Polarity and Movement

Dishevelled has been implicated in three processes in which the reorganization of the cytoskeleton is essential: planar polarity, cell movement, and mitotic spindle orientation. In Drosophila, the epithelial planar polarity mutant phenotype is characterized by the random orientation of cells within the epithelia in the wing, leg, and eye. In the wing, epithelial cells are oriented along the proximal-distal axis and each develops a hair pointing towards the distal end. Mutations in dishevelled or frizzled result in the random orientation of this hair (Adler, 1992). In the Drosophila eye, Fz/Dsh signaling affects mirror-symmetric arrangement of ommatidia (Cooper and Bray, 1999; Fant and Mlodzik, 1999). Dishevelled lies upstream of RhoA/Rac and the JNK kinase pathway in the control of planar polarity in the eye and wing (Strutt et al., 1997; Boutros et al., 1998). In addition, mutational analyses have demonstrated that Armadillo/β-catenin or pangolin/T-cell factor are not required for planar polarity in the eye (Boutros et al., 1998). However, the role of shaggy, the Drosophila homologue of GSK-3, is less clear. Although shaggy mutants do not exhibit a planar polarity phenotype in the wing (Axelrod et al., 1998), overexpression studies showed that shaggy produces a similar but weaker planar polarity phenotype in the eye to those observed in fz or dsh (Tomlinson et al., 1997). Therefore, it is possible that Dishevelled functions through shaggy/GSK-3 in planar polarity depending on the cellular context.

Recent studies in Xenopus and zebrafish embryos have shown that wnt-11 regulates convergent extension movements through Dishevelled (Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000). Dishevelled does not signal through the canonical pathway, as β-catenin or Tcf-3 are not required (Tada and Smith, 2000). Although the role of GSK-3 has not been tested in this process, the requirement of the DEP domain, but not the PDZ domain, suggests that Dishevelled is signaling through a similar pathway to that involved in Drosophila planar polarity.

Dishevelled also regulates the orientation of the mitotic spindle. Mutations in the Drosophila dishevelled and frizzled result in random orientation of the mitotic spindle in the asymmetric cell divisions during the development of mechanosensory organs (Gho and Schweighuth, 1998). In C. elegans, components of the Wnt pathway upstream and downstream of dishevelled regulate the orientation of the mitotic spindle during asymmetric cell divisions. gsk-3, mom-5/fz, and mom-1/porc regulate the orientation of the mitotic spindle (Schlesinger et al., 1999). Although the role of dishevelled has not been demonstrated in this system, these findings strongly suggest that WNT/FZ signaling through GSK-3 regulates the organization and/or orientation of MT structures. The mechanism by which the cytoskeleton is reorganized by WNT/FZ-mediated signaling remains elusive.

In the present work, we demonstrate that endogenous DVL-1 associates with axonal MTs that contain a large pool of stable MTs. DVL-1 also stabilizes MTs in dividing cells and differentiated neurons. Our data is consistent with the idea that activation of Dishevelled, depending on the cellular context, results in MT stabilization through the
inhibition of GSK-3β. The localization of Dishevelled to MTs may target its function within the cell. Local inhibition of GSK-3β by DVL-1 may result in local changes in the phosphorylation of GSK-3β targets that regulate MT dynamics. This novel MT stabilizing function of DVL-1 provides a possible mechanism for regulating cell polarity.

We thank Karl Willert and Roel Nusse for providing the DVL-1-GST fusion construct for antibody production, Daniel Sussman for the Dvl-1 cDNA, Peter Klein for GSK-3β cDNA, and Tony Wydmaw-Boris and Daniel Sussman for the DVL-1 mutant mouse. We also thank Denis Bra
y and Peter Baas for useful discussions and Simon Hughes and members of our lab for comments on the manuscript.

This work was supported by a grant from the Wellcome Trust. Submitted: 21 December 1999

References

Adler, P.N. 1992. The genetic control of tissue polarity in Drosophila. Bioes- says. 14:735–741.
Arias, A.M., A.M. Brown, and K. Brennan. 1999. Wnt signalling: pathway or network? Curr. Opin. Genet. Dev. 9:487–474.
Atack, J.R., H.B. Broughton, and S.J. Pollack. 1995. Inositol monophosphatase: a putative target for Li+ in the treatment of bipolar disorder. Trends Neuro- sci. 18:333–334.
Axelrod, J.D., K. Matsuno, S. Artavanis-Tsakonas, and N. Perrimon. 1996. Interaction between Wingless and Notch signaling pathways mediated by dishevelled. Science. 271:1826–1832.
Axelrod, J.D., R.R. Miller, J.M. Shulman, R.T. Moon, and N. Perrimon. 1998. Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. Genes Dev. 12:2610–2622.
Baas, P.W., T.P. Pienkowski, K.A. Cimbalik, K. Toyama, S. Bakalis, F.J. Ah- mad, and K.S. Kosik. 1999. Tau confers drug stability but not cold stability to microtubules in living cells. J. Cell Sci. 107:155–163.
Boutros, M., and M. Mlodzik. 1999. Dishevelled: at the crossroads of divergent intracellular signaling pathways. Mech. Dev. 83:27–37.
Boutros, M., N. Parcio, D.I. Strutt, and M. Mlodzik. 1998. Dishevelled activ- ates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. Cell. 94:109–118.
Brady, S.T., M. Tytell, and R.J. Lasek. 1984. Axonal tubulin and axonal micro- tubules: biochemical evidence for cold stability. J. Cell Biol. 99:1716–1724.
Bulinski, J.C., J.E. Richards, and G. Piperno. 1988. Posttranslational modifications of alpha-tubulin: deoxyribosylation and acetylation differentiate populations of interphase microtubules in cultured-cells. J. Cell Biol. 100:1213–1220.
Cdagan, K.M., and R. Nusse. 1997. Wnt signalling: a common theme in animal development. Genesis. 11:3286–3305.
Cooper, M.T., and S.J. Bray. 1999. Frizzled regulation of Notch signaling po- larizes cell fate in the Drosophila eye. Nature. 397:526–530.
Dreger, G., A. Ebnet, C. R. Moser, and M.M. Moline. 1998. MAPS, MARKs and mi- crotubule dynamics. Trends Biochem. Sci. 23:307–311.
Fagotto, F., E. Jho, L. Zeng, T. Kurth, T. Joos, C. Kaufmann, and F. Costantini. 1999. Domains of axin involved in protein–protein interactions, Wnt path- way inhibition, and intracellular localization. J. Cell Biol. 145:741–756.
Fanto, M., and M. Mlodzik. 1999. Asymmetric Notch activation specifies photo- receptors R3 and R4 and planar polarity in the Drosophila eye. Nature. 397:526–531.
Francis, F., A. Koulakoff, D. Boucher, P. Chafey, B. Schaar, M.C. Vinet, G. Cooper, M.T., and S.J. Bray. 1999. Frizzled regulation of Notch signalling po-

The Journal of Cell Biology, Volume 151, 2000 92
Siegfried, E., E.L. Wilder, and N. Perrimon. 1994. Components of wingless signalling in Drosophila. Nature. 367:76–79.
Smallley, M.J., E. Sara, H. Paterson, S. Naylor, D. Cook, H. Jayatilake, L.G. Fryer, L. Hutchinson, M.J. Fry, and T.C. Dale. 1999. Interaction of Axin and Dvl-2 proteins regulates Dvl-2-stimulated TCF-dependent transcription. EMBO (Eur. Mol. Biol. Organ.) J. 18:2823–2835.
Sokol, S.Y., J. Klingensmith, N. Perrimon, and K. Itoh. 1995. Dorsalizing and neuralizing properties of Xdsh, a maternally expressed Xenopus homolog of dishevelled. Development. 121:1637–1647.
Strutt, D.I., U. Weber, and M. Mlodzik. 1997. The role of RhoA in tissue polarity and Frizzled signalling. Nature. 387:292–295.
Sussman, D.J., J. Klingensmith, P. Salinas, P.S. Adams, R. Nusse, and N. Perrimon. 1994. Isolation and characterization of a mouse homolog of the Drosophila segment polarity gene dishevelled. Dev. Biol. 166:73–86.
Tada, M., and J.C. Smith. 2000. Xwnt11 is a target of Xenopus Brachyury: regulation of gastrulation movements via dishevelled, but not through the canonical Wnt pathway. Development. 127:2227–2238.
Takemura, R., S. Okabe, T. Umezawa, Y. Kanai, N.J. Cowan, and N. Hirokawa. 1992. Increased microtubule stability and alpha-tubulin acetylation in cells transfected with microtubule-associated proteins Map1b, Map2 or Tau. J. Cell Sci. 103:953–964.
Tomlinson, A., W.R. Strapps, and J. Heemskerk. 1997. Linking Frizzled and Wnt signaling in Drosophila development. Development. 124:4515–4521.
Torres, M.A., and W.J. Nelson. 2000. Colocalization and redistribution of Dishevelled and actin during Wnt-induced mesenchymal morphogenesis. J. Cell Biol. 149:1433–1442.
Tsang, M., N. Lijam, Y. Yang, D.R. Beier, A. Wynshaw-Boris, and D.J. Sussman. 1996. Isolation and characterization of mouse Dishevelled-3. Dev. Dyn. 207:253–262.
Wagner, U., M. Utton, J.M. Gallo, and C.C.J. Miller. 1996. Cellular phosphorylation of tau by GSK-3beta influences tau binding to microtubules and microtubule organization. J. Cell Sci. 109:1537–1543.
Wallingford, J.B., B.A. Rowing, K.M. Vogelt, U. Rotthacher, S.E. Fraser, and R.M. Harland. 2000. Dishevelled controls cell polarity during Xenopus gastrulation. Nature. 405:81–85.
Webb, B.C., and L. Wilson. 1980. Cold-stable microtubules from brain. Biochemistry. 19:1993–2001.
Yanagawa, S.I., F. Van Leeuwen, A. Wodarz, J. Klingensmith, and R. Nusse. 1995. The dishevelled protein is modified by wingless signaling in Drosophila. Genes Dev. 9:1087–1095.
Yang, Y., N. Lijam, D.J. Sussman, and M. Tsang. 1996. Genomic organization of mouse Dishevelled genes. Gene. 180:121–123.
Zeng, L., F. Fagotto, T. Zhang, W. Hsu, T.J. Vasicek, W.L. Perry, J.J. Lee, S.M. Tilghman, B.M. Gumbiner, and F. Costantini. 1997. The mouse fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. Cell. 90:181–192.