Functional Consequences of Wnt-induced Dishevelled 2 Phosphorylation in Canonical and Noncanonical Wnt Signaling*

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Significance: Wnt-induced phosphorylation of Dvl differentially regulates canonical and noncanonical Wnt signaling.

Dishevelled (Dvl) proteins are intracellular effectors of Wnt signaling that have essential roles in both canonical and noncanonical Wnt pathways. It has long been known that Wnts stimulate Dvl phosphorylation, but relatively little is known about its functional significance. We have previously reported that both Wnt3a and Wnt5a induce Dvl2 phosphorylation that is associated with an electrophoretic mobility shift and loss of recognition by monoclonal antibody 10B5. In the present study, we mapped the 10B5 epitope to a 16-amino acid segment of human Dvl2. Alanine substitution of these residues (P4m) eliminated the mobility shift induced by either Wnt3a or Wnt5a. The Dvl2 P4m mutant showed a modest increase in canonical Wnt/b-catenin signaling activity relative to wild type. Consistent with this finding, Dvl2 4Pm preferentially localized to cytoplasmic puncta. In contrast to wild-type Dvl2, however, the P4m mutant was unable to rescue Wnt3a-dependent neurite outgrowth in TC-32 cells following suppression of endogenous Dvl2.3. Earlier work has implicated casein kinase 16/e as responsible for the Dvl mobility shift, and a CK1δ in vitro kinase assay confirmed that Ser594, Thr595, and Ser597 of Dvl2 are CK1 targets. Alanine substitution of these three residues was sufficient to abrogate the Wnt-dependent mobility shift. Thus, we have identified a cluster of Ser/Thr residues in the C-terminal domain of Dvl2 that are Wnt-induced phosphorylation (WIP) sites. Our results indicate that phosphorylation at the WIP sites reduces Dvl accumulation in puncta and attenuates b-catenin signaling, whereas it enables noncanonical signaling that is required for neurite outgrowth.

Dishevelleds (Dvl) are multifunctional intracellular proteins that were first identified through their essential role in the transduction of signals elicited by members of the Wnt family of secreted proteins (1–3). Wnt proteins themselves are widely known critical regulators of embryonic and postnatal development. They control diverse aspects of cell behavior including cell polarity and stem cell self-renewal, and aberrant Wnt signaling is associated with numerous human diseases, including cancer (4, 5).

Intracellular signaling triggered by Wnt proteins is generally classified in two functional categories: canonical and noncanonical, based on the involvement of b-catenin as a signaling intermediate (5, 6). Canonical Wnt signaling is initiated by binding of Wnt proteins to a Frizzled/FRP5/LRP6 receptor complex (7). This leads to stabilization of cytoplasmic b-catenin by inhibiting phosphorylation events that otherwise target the protein for proteasomal destruction. The stabilized b-catenin also accumulates in the nucleus where, together with Tcf/Lef proteins, it regulates the transcription of specific target genes (5, 7). Wnt1 and Wnt3a are examples of Wnt ligands that typically activate canonical Wnt/b-catenin signaling (8), although they also stimulate other Wnt signaling pathways (9–12).

In contrast to the above mechanism, noncanonical Wnt signaling can be broadly defined as an intracellular response to Wnt proteins that does not involve stabilization of b-catenin. There are numerous instances of such signaling in development, particularly associated with planar cell polarity (PCP) and the movement of cells during morphogenesis (6, 13). More recent data also implicate noncanonical Wnt signaling in the regulation of tumor cell invasiveness and motility (14–17).
Wnt5a is currently the best studied Wnt protein that usually signals via noncanonical mechanisms (17, 18). Through genetic and biochemical studies in diverse organisms, several pathways of noncanonical Wnt signaling have been proposed, such as the Wnt/PCP, Wnt/Ca^{2+}, and Wnt/Ror2 pathways (19–22). However, many details of these pathways remain obscure, and there may be additional noncanonical signaling mechanisms.

Dishevelled (Dvl) proteins are among the few signaling intermediaries common to both modes of Wnt signaling, canonical and noncanonical. In mammals there are three isoforms: Dvl-1, 2, and 3, which are products of paralogous genes. Each has a conserved domain structure comprising DIX, PDZ, and DEP domains, followed by a C-terminal domain (CTD)\(^4\) (3). Several other proteins can bind Dvls, and the latter have thus been referred to as scaffold proteins (3, 23). Dvl proteins also have a capacity to form multimeric complexes, and this can be promoted by Wnt signaling (24–26). In canonical signaling, such multimers may be recruited to Wnt receptors to form signalosomes, which, in turn, recruit Axin and other proteins that inactivate the β-catenin destruction complex (27, 28).

All three mammalian Dvl proteins become phosphorylated in response to Wnt signals, a feature that is detectable as a mobility shift on SDS-polyacrylamide gels (12, 29). Although the phosphorylated and shifted form (psDvl) has sometimes been assumed to be a sign of “activation” in terms of downstream signaling, the kinetics of Wnt-induced Dvl phosphorylation appear too slow to be able to precede the stabilization of β-catenin in canonical signaling (12, 30–33). Moreover, we and others have shown that Dvl becomes phosphorylated, not only in response to Wnts that stimulate the canonical pathway, but also in response to those such as Wnt5a that do not stabilize β-catenin (12, 30, 33, 34). Because Wnt-induced phosphorylation of Dvl can occur in the absence of β-catenin stabilization and is independent of the LRP5/6 Wnt receptor components required for canonical Wnt signaling, we have previously argued that psDvl is a manifestation of noncanonical Wnt signaling (12). However, the functional consequences of Dvl phosphorylation for either mode of Wnt signal transduction have remained unclear.

Several kinases have been reported to bind and/or phosphorylate Dvl, including casein kinase 1δ and ε (CK1δ/ε), casein kinase 2, PAR-1, Plk1, and protein kinase C (reviewed in Ref. 3). Dozens of phosphorylation sites have been documented in Dvl proteins, and some have been identified as targets of specific kinases (35–38). Although phosphorylation sites are widely distributed in the Dvl sequence, they are particularly abundant in the CTD (35). Deletion analysis and site-directed mutagenesis have suggested that phosphorylation in this region correlates with formation of psDvl (12, 35, 39). Furthermore, it has been proposed that CK1δ/ε are primarily responsible for CTD phosphorylation and the corresponding mobility shift (39). However, CK1 is able to phosphorylate other Wnt signaling components besides Dvl and regulates their function (40, 41). Thus, it has proved difficult to investigate functional consequences of Dvl phosphorylation in isolation from pleiotropic effects of altered kinase levels.

In the present study, we identify a cluster of three Ser/Thr residues in Dvl2 that constitutes a key target of Wnt-induced phosphorylation \textit{in vivo} and is a target for CK1 phosphorylation \textit{in vitro}. Mutation of the three amino acids to Ala prevents the shift in electrophoretic mobility of Dvl2 in response to either Wnt3a or Wnt5a. Our data further indicate that the Wnt-induced phosphorylation normally observed at these residues in wild-type Dvl results in attenuation of canonical Wnt signaling and concomitant stimulation of noncanonical activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Neurite Outgrowth**—Rat2 fibroblasts and HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% FBS (Invitrogen). C57MG cells were cultured in the same medium supplemented with 10 μg/ml insulin (Invitrogen). The Ewing sarcoma family tumor cell line TC-32 was grown on cell culture dishes, cluster plates, or glass coverslips precoated with type I collagen solution (Sigma-Aldrich) as described (10). Wnt-stimulated neurite outgrowth was induced and quantified as previously described (10).

**Recombinant Protein and Conditioned Medium**—Recombinant Wnt3a was purchased from R&D Systems (Minneapolis, MN). Secreted Frizzled-related protein (sFRP-1) was prepared as described (42). Wnt5a CM was harvested from Rat2/Wnt5a cells in DMEM + 1% FBS, centrifuged at 2000 × g for 10 min, and concentrated 10-fold using Centriplus YM-10 columns (Millipore). Wnt3a CM was obtained from L/Wnt3a cells as previously described (12).

**Antibodies Used for Western Blotting**—Mouse anti-Dvl-2 (10B5), rabbit anti-Dvl-2 (H-75), mouse anti-Myc (9E10), and mouse anti-HSP70 were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Dvl2 (catalog no. 3216) and rabbit anti-Dvl3 (catalog no. 3218) were from Cell Signaling Technology, Inc. (Danvers, MA). Mouse anti-β-catenin (C19220) and mouse anti-GSK3β (clone 7) were from BD Transduction Labs (San José, CA). Mouse FLAG (M2) antibody was obtained from Sigma-Aldrich.

**Immunoblotting**—For Western blot analysis of Rat2 and HEK293T cells, lysates were prepared in radioimmunoprecipitation assay buffer and processed as previously described (12). Separation of phosphorylated forms of Dvl was achieved using 7% polyacrylamide gels in Tris-glycine buffer. For verification of siRNA knockdown of endogenous proteins, TC-32 cells transfected with siRNA were harvested 48 h after transfection and processed for SDS-PAGE and Western blot analysis as previously described (10).

**Recombinant DNA**—Human Dvl2 cDNA was cloned into pcdNA3.1-mycHisA (Invitrogen) using NotI and XhoI sites. hDvl2 deletion constructs were then generated by PCR using 3’-specific primers. Site-directed mutagenesis for production of hDvl2 mutants was performed using a QuikChange II mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA), and all mutations were verified by DNA sequencing. Myc-tagged hDvl2 mutants and WT were subcloned into the retroviral vector pLNCX, using SnaB1 and StuI sites for stable expression in Rat2 cells (see below), pCS2+ FLAG-mDvl2 WT

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\(^4\) The abbreviations used are: CTD, C-terminal domain; WIP, Wnt-induced phosphorylation; MS\(^2\), MS/MS; MS\(^3\), MS/MS/MS; CM, conditioned medium.
Wnt-induced Phosphorylation Sites in Dvl2 Regulate Signaling

was kindly provided by X. He (Harvard University), and mDvl2 P4m was generated from this by site-directed mutagenesis as above. pcDNA3.3 Myc-tagged mCK1δ and mCK1ε were prepared as described (43). For pCMV32 lentiviral constructs, Gateway entry clones were first generated from pCS2 + FLAG-mDvl2 WT and P4m, and lentiviral expression clones were then constructed using multisite Gateway recombinational cloning (Invitrogen).

Retroviral and Lentiviral Expression—LNCX retroviral vectors expressing Myc-tagged WT or mutant hDvl2 were packaged in BOSC23 cells, and the viruses were used to transduce Rat2 cells with selection in G418 (Geneticin) (44). Lentiviral particles were produced by transient transfection of HEK293T cells and concentrated 10-fold with Amicon Ultra-15 (Millipore) and left for 24 h. Selection was performed in pGEX-4T-2 vector (Amersham Biosciences). Similarly, a Smal-NotI fragment of human CK1δ was generated by PCR from pCS2 + -Myc6-CK1δ (a gift of D. Virshup, Duke-NUS Graduate Medical School, Singapore) and inserted into pGEX-4T-2. GST-Dvl2 and GST-CK1δ were purified from Escherichia coli BL21 (DE3) with GSH-agarose (Sigma). In vitro kinase reactions were carried out in 50 mM Tris-Cl (pH 7.5), 10 mM MgCl2, 5 mM dithiothreitol, 2 mM EGTA, 0.5 mM Na3VO4, and 20 mM p-nitrophenyl phosphate in the presence of 100 μM ATP at 30 °C for 30 min. The samples were separated by 10% SDS-PAGE. The GST-Dvl2 band was excised and in-gel digested with trypsin (Promega). Tryptic peptides were separated using nano-flow reversed phase liquid chromatography, coupled online to an LTQ linear ion trap or LTQ-Orbitrap XL mass spectrometer (Thermo Electron, San Jose, CA) for MS/MS and MS/MS/MS analysis. The mass spectrometer was operated in a data-dependent mode to sequentially acquire MS, MS2, and neutral phosphate loss-dependent MS3 spectra with dynamic exclusion. Normalized collision energy was 35% for both MS2 and MS3. The raw MS2 and MS3 data were searched using SEQUEST (Thermo Electron) to identify phosphopeptides. These were subjected to validation of peptide sequence and phosphorylation sites by examining the corresponding MS2 and/or MS3 spectra.

Statistical Analysis—The significance of differences in data was determined with Student’s t test. The differences were considered significant when the p value was less than 0.05.

RESULTS

Mapping Wnt-induced Phosphorylation Sites in Dvl2 Associated with Mobility Shift—The phosphorylation of Dvl induced by Wnt signaling results in altered mobility of the protein with the phosphorylated and shifted form, psDvl2, being noticeably retarded on polyacrylamide gels. We have previously reported that the Dvl2 mAb 10B5 is selectively deficient in recognizing psDvl2, although it readily detects the unmodified, or faster retarded on polyacrylamide gels. We have previously reported that the Dvl2 mAb 10B5 is selectively deficient in recognizing psDvl2, although it readily detects the unmodified, or faster migrating, band (12). This phenomenon is illustrated in Fig. 1A, both for endogenous Dvl2 in Rat2 cells and for exogenous Myc-tagged human Dvl2. The inability of 10B5 to detect psDvl2 suggests that the epitope is masked by Wnt-induced phosphorylation. To identify the site of this phosphorylation, we mapped the 10B5 epitope. Because the antibody was raised against the C-terminal domain of Dvl2 (amino acids 594–736), a series of C-terminal deletion mutants of Dvl2-Myc was generated and analyzed by Western blotting with both Myc antibody and mAb 10B5 (Fig. 1, B and C). The shortest construct that still reacted with both antibodies was Dvl2Δ127, containing amino acids 1–609. Further truncation of Dvl2 eliminated recognition by 10B5 (Fig. 1C). Thus, our results placed the epitope within a 16-amino acid sequence from residues 594 to 609 (Fig. 1D). This sequence contains two serine and two threonine residues formed as previously described (10). Mouse FLAG (M2) antibody was from Sigma-Aldrich, and Alexa Fluor 488 goat anti-mouse IgG was from Invitrogen. Fluorescent images were collected as described (43).

In Vitro Kinase Assay and Mass Spectrometry Analysis—Mouse Dvl2 cDNA was generated by PCR from pCS2 + –FLAG-mouse Dvl2 (a gift of S. B. Lee, National Institutes of Health, Bethesda, MD) and cloned into a pGEX-4T-2 vector (Amersham Biosciences). Similarly, a Smal-NotI fragment of human CK1δ was generated by PCR from pCS2 + -Myc6-CK1δ (a gift of D. Virshup, Duke-NUS Graduate Medical School, Singapore) and inserted into pGEX-4T-2. GST-Dvl2 and GST-CK1δ were purified from Escherichia coli BL21 (DE3) with GSH-agarose (Sigma). In vitro kinase reactions were carried out in 50 mM Tris-Cl (pH 7.5), 10 mM MgCl2, 5 mM dithiothreitol, 2 mM EGTA, 0.5 mM Na3VO4, and 20 mM p-nitrophenyl phosphate in the presence of 100 μM ATP at 30 °C for 30 min. The samples were separated by 10% SDS-PAGE. The GST-Dvl2 band was excised and in-gel digested with trypsin (Promega). Tryptic peptides were separated using nano-flow reversed phase liquid chromatography, coupled online to an LTQ linear ion trap or LTQ-Orbitrap XL mass spectrometer (Thermo Electron, San Jose, CA) for MS/MS and MS/MS/MS analysis. The mass spectrometer was operated in a data-dependent mode to sequentially acquire MS, MS2, and neutral phosphate loss-dependent MS3 spectra with dynamic exclusion. Normalized collision energy was 35% for both MS2 and MS3. The raw MS2 and MS3 data were searched using SEQUEST (Thermo Electron) to identify phosphopeptides. These were subjected to validation of peptide sequence and phosphorylation sites by examining the corresponding MS2 and/or MS3 spectra.

Statistical Analysis—The significance of differences in data was determined with Student’s t test. The differences were considered significant when the p value was less than 0.05.
as potential phosphorylation sites, three of which are clustered as STRS at position 594–597. Because all three Dvl proteins undergo Wnt-induced phosphorylation and mobility shift (12, 34), we examined the equivalent regions of Dvl1 and Dvl3. The STRS motif is perfectly conserved in Dvl1, whereas the equivalent sequence in Dvl3 is SNRS (Fig. 1D).

To investigate whether in vivo phosphorylation of specific amino acid residues within the 16-amino acid 10B5 epitope is necessary for the Wnt-induced mobility shift of Dvl2, we individually mutated each of the four Ser and Thr residues to Ala in a Myc-tagged human Dvl2 cDNA and stably expressed the constructs in Rat2 cells by retroviral transduction. The mobility of the mutant proteins was then examined on Western blots of cells treated with or without Wnt3a conditioned medium (CM). No individual Ser/Thr mutation prevented the phosphorylation-dependent mobility shift. However, for each of the mutants S594A and S597A only ~50% of the protein showed slower mobility in response to Wnt3a, indicating that the absence of either of these serines partially impairs formation of psDvl2 (Fig. 2). In contrast, we observed little or no inhibition of psDvl2 formation in T595A or T604A mutants. Apart from the Ser and Thr residues, the only other amino acid within the epitope region that is conserved in all three Dvl proteins is Arg603. When this residue was mutated, Wnt-induced formation of psDvl2 was unaffected, as detected by Myc antibody. However, even in the absence of Wnt treatment, the mutant protein was undetectable by the 10B5 antibody (Fig. 2, lanes 13 and 14). This indicates that Arg603 is a critical residue in the 10B5 epitope but is unrelated to Wnt-induced phosphorylation.

We next tested combinations of Ser → Ala and Thr → Ala mutations, again using a retrovirus vector to express Myc-tagged human Dvl2 proteins. Remarkably, mutation of all four Ser/Thr residues (P4m) of the epitope produced a protein that showed no shift in mobility in response to either Wnt3a or Wnt5a signaling (Fig. 3, A and B). Reagents based on this set of mutations were used in most of the subsequent experiments. For comparison, the Wnt-dependent mobility shift of endogenous Dvl3 was maintained in the cell lines expressing the Dvl2 P4m mutant (Fig. 3, A and B). We subsequently determined that a mutant, Dvl2 P3m, having alanine substitutions only at Ser594, Thr595, and Ser597, also failed to undergo a Wnt-dependent mobility shift (Fig. 3C). Because Dvl2 P3m and P4m are deficient in the Wnt-induced phosphorylation that results in a mobility shift, we refer to them as WIP mutants.

The amino acid sequence of the 10B5 epitope is perfectly conserved between human and mouse Dvl2. To confirm the properties of the Dvl2 P4m WIP mutation in mouse Dvl2, we introduced the same four Ser → Ala and Thr → Ala changes into a FLAG-tagged mDvl2 cDNA and expressed it in the human Ewing sarcoma family tumor cell line TC-32 by lentiviral transduction. These cells display significant endogenous Wnt activity, which is associated with a basal level of neurite outgrowth (10). Accordingly, exogenous mDvl2 WT protein was spontaneously phosphorylated and shifted on Western blots (Fig. 3D). However, this shift was abolished by treating the
cells for 3 h with the Wnt ligand antagonist sFRP1, consistent with the mobility shift being due to endogenous Wnt production (Fig. 3D, lane 3). In contrast, mDvl2 P4m protein showed no phosphorylation-related mobility shift and was unaffected by sFRP1 (Fig. 3D, lanes 4–6). This confirmed that the P4m mutations eliminate the phosphorylation-dependent mobility shift in Dvl2 caused by Wnt signaling.

WIP Mutant Dvl2 Shows Elevated Activity in Canonical Wnt Signaling—The WIP mutant is not only resistant to Wnt-induced phosphorylation at the 10B5 epitope, but it fails to undergo the conformational change that presumably underlies the altered electrophoretic mobility. This mutant is therefore well suited for studying the functional impact of Dvl2 phosphorylation in Wnt signaling.

Although the ability of Wnts to induce Dvl phosphorylation is associated with noncanonical signaling, it also occurs in response to Wnts that concurrently activate the β-catenin pathway (12). To investigate the functional consequences of Dvl phosphorylation in Wnt signaling, we first tested the hypothesis that the Dvl2 WIP mutant might show altered activity in canonical signaling assays. As previously noted (25), transient overexpression of hDvl2 WT in 293T cells gives robust activation of the TCF/β-catenin-dependent luciferase reporter TOPflash in a dose-dependent manner (Fig. 4A). Parallel transfections with the WIP mutant Dvl2 P4m also activated TOPflash. However, at lower doses of input DNA, the signal from hDvl2 P4m was significantly stronger than from hDvl2 WT (Fig. 4A). This suggested that the WIP mutant is moderately...
hyperactive in β-catenin signaling. We also analyzed cytosolic β-catenin by immunoblotting to compare the responsiveness of these cells to varying Wnt3a concentrations. Although a 1:125 dilution of Wnt3a CM elicited only a small accumulation of β-catenin in cells expressing hDvl2 WT, we observed a larger increase in cells expressing P4m (Fig. 4B). Similarly, when mDvl2 WT and P4m constructs were transiently expressed in C57MG mouse mammary epithelial cells, the Wnt3a-dependent induction of β-catenin target genes Axin2 and RhoU (47) was stronger with Dvl2 P4m than Dvl2 WT (Fig. 4, C and D). Although the differential effects were not dramatic, these experiments suggested that expression of the Dvl2 WIP mutant enhanced the sensitivity of cells to Wnt3a. This implies that Wnt-induced Dvl2 phosphorylation at the identified sites has a modest negative effect on canonical signaling.

WIP Mutant Dvl2 Preferentially Accumulates in Puncta—Both endogenous and overexpressed Dvl proteins are known to form cytoplasmic puncta (48, 49). These puncta are thought to result from dynamic polymerization of Dvl proteins and to some extent correlate with activation of Wnt/β-catenin signaling (24–26). When we examined the intracellular distribution of FLAG-tagged mDvl2 WT and mDvl2 P4m stably expressed in TC-32 cells, we observed three different FLAG staining patterns: 1) diffuse cytoplasmic; 2) combination of cytoplasmic and puncta; and 3) puncta dominant (Fig. 5A). Quantitative analysis revealed that 64% of cells expressing mDvl2 WT had a diffuse cytoplasmic pattern, 32% had a mixed pattern, and 3% were puncta dominant. In contrast, only 15% of cells expressing mDvl2 P4m exhibited a diffuse cytoplasmic distribution, whereas 78% had a mixed pattern, and 6% were puncta dominant (Fig. 5B). Similar profiles were seen in the presence and absence of Wnt3a treatment with or without siRNA knockdown of endogenous Dvl2/3 expression (data not shown). The enhanced accumulation of P4m in puncta is consistent with its stimulation of β-catenin signaling.

WIP Mutant Dvl2 Does Not Mediate Wnt3a-dependent Neurite Outgrowth in TC-32 Cells—Wnt3a induces neurite outgrowth in TC-32 cells via noncanonical Wnt signaling, and Dvl proteins are required for this process (10). Moreover, a Wnt3a-dependent Dvl mobility shift correlates with neurite outgrowth in these cells (43). Using this experimental model as a readout for noncanonical Wnt signaling, we compared the ability of siRNA-resistant mDvl2 WT versus P4m proteins to rescue Wnt-induced neurite outgrowth after knockdown of endogenous Dvl2/3. Dvl2/3 siRNA markedly blocked Wnt3a-dependent neurite outgrowth in parental TC-32 cells (Fig. 6A). How...
ever, neurite extension was not inhibited in cells expressing mDvl2 WT. In contrast, mDvl2 P4m failed to rescue neurite outgrowth. Moreover, expression of P4m also blocked Wnt3a-induced neurite outgrowth in the presence of endogenous Dvl2/3 (Fig. 6A). mDvl2 WT and P4m proteins were expressed at similar levels, and knockdown of endogenous Dvl2/3 proteins was comparable in the various cell lines, indicating that differences in expression did not account for their distinct effects (Fig. 6B). Interestingly, levels of both FLAG-tagged Dvl2 WT and P4m were significantly increased, and to a similar

**FIGURE 5.** Intracellular distribution of FLAG-tagged mDvl2 WT and mDvl2 P4m in TC-32 cells. A, representative micrographs of FLAG-mDvl2 distribution illustrating three patterns: cytoplasmic, cytoplasmic and puncta, and puncta predominant. B, cumulative quantitative analysis of distribution patterns for mDvl2 WT and P4m. N indicates the number of cells examined for each construct.

**FIGURE 6.** Contrasting activity of FLAG-tagged mDvl2 WT versus mDvl2 P4m in neurite outgrowth assay. A, parental TC-32 cells and cells stably expressing FLAG-tagged mDvl2 WT or mDvl2 P4m were treated with siRNA reagents targeting luciferase (negative control) or Dvl2 and Dvl3, and 48 h later incubated with medium ± Wnt3a (100 ng/ml) for 3 h. Following fixation, neurite outgrowth was assessed in dozens of cells from each treatment group. The results from three separate experiments are indicated as the means ± S.D. Statistical significance of selected pairwise comparisons is indicated. B, Western blot analysis of Dvl2/3 expression in cells treated as described in A. The efficacy of knockdown of endogenous Dvl2/3 by siRNA is evident. HSP70 was a loading control. The positions of molecular mass markers are indicated. IB, immunoblotting.
the WIP sites identified here were phosphorylated by CK1\(\delta\) in an in vitro kinase assay. Others have documented CK1 phosphorylation sites elsewhere in Dvl protein (36), and we also detected phosphorylated residues of other sites in our in vitro kinase assay (data not shown). In view of the abundance of potential phosphorylation targets throughout the Dvl2 amino acid sequence, therefore, the abolition of mobility shifting by mutation of these three residues alone is perhaps unexpected. However, we suggest that phosphorylation of the WIP sites may be a necessary priming event that allows further phosphorylation at other sites and/or that WIP site phosphorylation in conjunction with other phosphorylated sites creates a critical mass of charged residues sufficient to bring about the presumed conformational change in Dvl protein that causes its electrophoretic retardation. In this regard it is notable that, in overexpression experiments, CK1\(\delta\) and CK1\(\epsilon\) can induce phosphorylation at sites in Dvl2 other than the WIP sites and that this in turn can cause mobility shifting (data not shown). A similar finding was recently reported for a Dvl3 CTD deletion mutant (39). Nevertheless, our results demonstrate that when Dvl phosphorylation is triggered by Wnt ligands, mutation of the WIP sites prevents mobility shifting in cells expressing endogenous levels of CK1\(\delta\)/\(\epsilon\). Thus, these sites are likely to have physiologic relevance.

Mutation of the WIP sites of Dvl2 provided a unique opportunity to investigate the functional consequences of preventing Wnt-induced phosphorylation of mammalian Dvl without alterations in the activity of kinases that might have pleiotropic effects. To some extent our conclusions differ from an extensive analysis of Dishevelled (Dsh) phosphorylation in Drosophila, which concluded that Ser/Thr phosphorylation did not have functional significance in \(\beta\)-catenin signaling or planar cell polarity (35). However, the authors of that study acknowledged the possibility that phosphorylation of mammalian Dvl3 at residues not conserved in Drosophila might be functionally relevant, especially in pathways that were not operative in their assays. These caveats apply to the 594STR597 sequence that we identified in Dvl2, because it is located in a region poorly conserved in Drosophila but well conserved in mammals and other vertebrates (51).

Our functional testing of Dvl2 WIP mutants suggests that phosphorylation of these three Ser/Thr residues has a mild inhibitory effect on canonical Wnt/\(\beta\)-catenin signaling, because the alanine substitution mutant Dvl2 P4m appeared more active than Dvl2 WT in assays of \(\beta\)-catenin-mediated transcription. More strikingly, and in contrast, Dvl2 P4m was unable to rescue Wnt3a-induced neurite outgrowth in TC-32 cells. We have previously reported that Wnt-mediated neurite extension in these cells does not require the canonical Wnt/\(\beta\)-catenin pathway receptor LR5/6 and instead is mediated by noncanonical Wnt pathways that rely on JNK and atypical PKC\(\zeta\) (10, 54). In the accompanying manuscript (54), we further show that Dvl2 P4m, in contrast to Dvl2 WT, fails to co-immunoprecipitate with PKC\(\zeta\), and this may account for its inability to mediate neurite outgrowth. The inactivity of Dvl2 P4m in the neurite assay indicates that it cannot transduce an essential noncanonical signal and hence that phosphorylation of the Dvl2 WIP sites normally promotes this function.

### DISCUSSION

In this study we have identified Wnt-induced phosphorylation (WIP) sites at Ser594, Thr595, and Ser597 in Dvl2 that have functional consequences for both canonical and noncanonical signaling. These sites have particular significance among other phosphorylation sites that have been mapped on Dvl proteins in the past. First, phosphorylation of the WIP sites is induced by Wnt ligand signaling rather than by overexpression of individual kinases. Second, the phosphorylation of these sites is required for the Wnt-mediated Dvl2 electrophoretic mobility shift that is routinely used as an assay for Dvl phosphorylation and also serves as an assay for noncanonical Wnt signal transduction events (12, 20). Although two amino acids in the DIX domain of Dvl2, Lys68 and Glu69, were previously shown to be required for formation of psDvl2, these residues are not phosphorylated but instead affect subcellular Dvl2 distribution (50).

Several previous studies have implied that CK1\(\delta\) and CK1\(\epsilon\) are primarily responsible for the Wnt-dependent mobility shift of Dvl2/3 (30, 39, 43). Consistent with this notion, we found...
In apparent contrast to our results concerning the impact of Wnt-induced, CK1ε-dependent, Dvl2 phosphorylation on β-catenin signaling, a number of previous studies have implicated CK1δ/ε as a positive mediator of the canonical pathway (52, 53). One explanation of this may stem from the ability of overexpressed CK1δ/ε to phosphorylate several other Wnt signaling components besides Dvl and thus to exert a combination of positive and negative effects on the pathway as a whole (40). In addition, there appear to be differential effects of CK1-mediated phosphorylation at different sites within Dvl proteins. For example, a recent report indicated that CK1ε phosphorylation of Dvl3 at upstream sites correlated with stimulation of canonical signaling, whereas phosphorylation in the CTD was associated with attenuation of the signal (39). The consequences we describe here for phosphorylation of the WIP sites in the CTD of Dvl2 are consistent with that report.

We also examined the effect of WIP site mutation on subcellular distribution of Dvl2 protein, especially with regard to formation of the cytoplasmic puncta that are associated with Wnt/β-catenin signaling (24, 25, 27, 50). Our observation that Dvl2 P4m preferentially localized to puncta relative to Dvl2 WT (Fig. 5) is consistent with this view, because the mutant was more potent in assays of canonical Wnt/β-catenin signaling. Our findings are also in agreement with those of Bernatik et al. (39), who noted increased localization in puncta of a phosphorylation- and shifting-deficient Dvl3 deletion derivative that showed enhanced activity in the β-catenin pathway. The results from these studies together reinforce the idea that phosphorylation in the CTD is associated with diminished β-catenin signaling. Others have not specifically addressed the possibility that phosphorylation of the CTD would have a positive effect on other Dvl activities, and this is an important additional conclusion from our study. Phosphorylation at the CTD WIP sites might therefore serve as a switch to redirect Dvl function toward noncanonical signaling. This may have particular relevance for Wnt ligands that can stimulate both canonical and noncanonical pathways (12). In view of the 20–30-min time lag before induction of psDvl2, there may be attenuation of canonical signaling over time via this mechanism.

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