Dishevelled (Dsh/Dvl) proteins are known to mediate Wnt signaling by up-regulating β-catenin levels and stimulating T cell factor (TCF)/LEF-1-dependent transcription. We have identified a new Dvl-mediated signaling pathway in that mouse Dvl proteins, when expressed in COS-7 cells, stimulate c-Jun-dependent transcription. This suggests that Dvl may activate JNK via Cdc42 and Rac1 could not inhibit Dvl-induced JNK activation, because the dominant negative mutants do not appear to play a major role in Dvl-mediated transcription.

The Wnt family of secretory glycoproteins plays important roles in embryonic induction, generation of cell polarity, and specification of cell fate (see reviews, Refs. 1–6). Genetic studies in Drosophila revealed the involvement of Dishevelled (Dsh), armadillo (Arm), and pangolin (Pan, DTCf) in Wingless (Wg) signaling. A genetic order of these signal transducers has been established in which Wg acts through Dsh to inhibit Zw-3, thus relieving the suppression of Arm by Zw-3, with a net result of up-regulation of Arm (1–6). Recent results suggest that the Frazzled (Fz) family of cell surface proteins might function as the receptors for Wnt, because the null mutation of Drosophila fz gene has similar phenotypes as some Dsh mutants (7–10). In addition, heterologous expression of Dfz2 (Drosophila fz homolog) in cells that normally do not express Dfz2 and do not respond to Wg reconstituted the responsiveness to Wg (11).

The Wnt signaling mechanism appears to be conserved in mammals. In addition to the existence of a large number of Wg homologs, there are mammalian homologs for Fz, Dsh, Zw-3, Arm, and Pan. More than eight mammalian Fz homologs have been cloned and sequenced (12, 13). Although studies using immunostaining suggest that various Fz may bind to various Wnt proteins (11), it is not clear whether Fz by itself is sufficient to transduce signals. In addition, there is little information about the specificity in interactions between different Fz proteins and Wnt proteins, because affinities cannot be measured without purified Wnt proteins. However, there appears to be certain selectivity in the interactions between Fz and Wnt proteins, because Wnt-5A-induced axial duplication in Xenopus requires Fz-5 (14).

Molecular cloning also revealed several mammalian Dsh homologs (Dvl), including three from the mouse (15–17). Amino acid sequence comparison of all known Dsh/Dvl molecules across species revealed several highly conserved regions. Most notable is the one located in the central part of the molecule referred to as the disc-large homology region or PDZ domain, which was found in a number of proteins including PSD-95, ZO-1, and Discs-large (18). Studies have shown that the PDZ domain in PSD-95 can bind to a C-terminal motif of four amino acids (Xaa-Thr/Ser-Xaa-Val) (19). However, ligands for the PDZ domain of Dsh/Dvl remain unknown. At the C-terminal side of the PDZ domain is a DEP (Dishevelled, egl-10,leckstrin) domain. Similar DEP motifs are also found in a number of other proteins (20). The N-terminal conserved domain shares homology to a newly identified protein, Axin, which also plays an important role in Wnt signaling (21–24). This N-terminal domain is referred to as the DIX (Dishevelled and Axin) domain. Although a mouse knock-out of Dvl-1 did not display any of the dramatic developmental phenotypes exhibited by many of the Wnt knock-outs (5), behavioral and neurological abnormalities were observed (25). Despite great interest, the molecular mechanism by which Dsh/Dvl is involved in signal transduction of Wnt is largely unknown, especially in mammalian systems. One study using cultures of Drosophila cells showed that overexpression of Dsh can induce elevation of the Arm levels and that the N-terminal portion of Dsh, containing the DIX and PDZ domains, is required for such activity (26).

Dishevelled Proteins Lead to Two Signaling Pathways

REGULATION OF LEF-1 AND c-Jun N-TERMINAL KINASE IN MAMMALIAN CELLS*

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The mammalian homologs to Zw-3 and Arm are GSK-3β and β-catenin, respectively. GSK was found to form a complex with β-catenin and the product of adenomatous polyposis coli (APC) gene (27, 28). Recent evidence indicates that Axin and its
homolog, Conductin, may also be part of the complex (22–24). GSK was shown to phosphorylate APC and destabilize the complex (5). APC appears to play a role in promoting the degradation of β-catenin via ubiquitination pathways (29), and a similar role was also proposed for Conductin (24). APC was originally identified as a tumor suppressor gene (30), and mutations in APC that are associated with human colorectal cancers appear to lose the ability to destabilize β-catenin (27). β-Catenin has been shown to bind high mobility group box transcription factors of the TCF-LEF-1 family. Complexes between β-catenin and Tcf/LEF-1 have strong transcriptional activity on reporter gene constructs containing the Tcf/LEF-1 recognition sequences (31–33).

Genetic studies in Drosophila and Caenorhabditis elegans suggested that there might exist Wnt signaling pathways independent of β-catenin and TCF/LEF-1 (5). Drosophila fz and dsh genes function in the tissue polarity pathway, which regulates cell orientation. However, eg, arm (β-catenin homolog), and dtef do not appear to be involved in this pathway, in which a number of other genes, fuzzy, int, rhoa, and bsk (DJNK) are involved (5, 34). Thus, Dsh appears to mediate two pathways of different functions. Mammalian JNK belongs to the superfamily of mitogen-activated protein kinases (see reviews, Refs. 35 and 36). JNK phosphorylates the N terminus of proto-oncogene c-Jun and its related transcription factor ATF2, leading to stimulation of the transcriptional activity. The best-characterized pathways leading to JNK activation in mammalian cells are those mediated by small GTP-binding proteins Rac and Cdc42 (37, 38). Rac and Cdc42 belong to the Rho family, which also includes Rhoc, however, is a weak activator of JNK but a strong activator of serum response factor (SRF) (37, 38, 40).

To determine whether Dvl can interact with JNK- and RhoA-linked pathways in mammalian cells, we tested the effects of ectopically expressed Dvl on c-Jun- and SRF-mediated transcription activity. We found that Dvl can stimulate c-Jun-mediated but not SRF-mediated transcription of the reporter genes, suggesting that Dvl may regulate c-Jun. This is confirmed by the observation that Dvl activates JNK. Moreover, we also found that the DEP domain of Dvl-1 is required for JNK activation. By contrast, all three conserved domains, including DIX, PDZ, and DEP, are required for up-regulation of β-catenin and for stimulation of LEF-1-mediated transcription.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection, and Luciferase Assay—**COS-7 and NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37 °C under 5% CO2. For transfection, cells (5 × 104 cells/well) were seeded into 24-well plates the day before transfection. Cells were transfected with 0.25 µg of DNA/well for 3T3 cells and 0.5 µg of DNA/well for NIH3T3 cells using LipofectAMINE Plus (Life Technologies, Inc.), as suggested by the manufacturer. The transfection was stopped by switching to normal growth medium after 3 h. Cell extracts were collected 24 h later for luciferase assays, kinase assays, and Western analysis.

Luciferase assays were performed using Boehringer Mannheim Constant Light luciferase assay kit as instructed. Cell lysates were first taken for determining fluorescence intensity emitted by green fluorescence protein (GFP) proteins in a Wallac multi-counter, which is capable of counting fluorescence and luminescence. Then, luciferase substrate was added to the cell lysates, and luciferase activities were determined by measuring luminescence intensity using the same counter. Luminescence intensity was normalized against fluorescence intensity.

**Construction of Expression Plasmids and Mutagenesis—**The wild type and deletion mutants of mouse Dvl1 were generated by polymerase chain reaction with the mouse Dvl-1 cDNA as the template using the high fidelity thermostable DNA polymerase Ffu (Stratagene, CA). Myc epitope tags (EQKLISEEDL) were introduced to the C termini of the full-length and mutant Dvl molecules. The expression of Wnt1, wild type Dvl, and Dvl-1 mutant was driven by a cytomegalovirus promoter.

All constructs were verified by DNA sequencing. The LEF-1 reporter gene construct was kindly provided by Dr. Grosschedl (University of California at San Francisco). Elk- and c-Jun-reporter gene systems were purchased from Stratagene, CA. For the construction of the reporter gene plasmid, SRE derivative, SER.L, was synthesized exactly as described in Hill et al. (40). SER.L was inserted in front of a TK minimal promoter-luciferase gene. LeF-1 and the LeF reporter gene plasmid were provided by Rudolf Grosschedl.

**In Vitro JNK Assay—**Cells expressing JNK-HA were lysed with the lysis buffer containing 1% Nonidet P-40, 137 mM sodium chloride, 20 mM Hepes pH 7.4, 1 mM dithiothreitol, 10% glycerol, 10 mM sodium fluoride, 1 mM pyrophosphate, 2 mM sodium vanadate, and Complete™ protease inhibitors (Boehringer Mannheim, used as the instructions suggest). The cell lysates were precluded with 20 µl of protein A/G-Sepharose beads (Santa Cruz Biotech, CA) for 0.5 h at 4 °C and then incubated with 1 µl of monoclonal anti-HA tag antibody (Berkeley Antibody Company, CA) and 20 µl of protein A/G-Sepharose beads for 3 h on ice. The immunocomplexes were pelleted and washed 3 times with cold lysis buffer in the absence of protease and phosphatase inhibitors and twice with cold kinase buffer containing 25 mM HEPES, pH 7.4, 10 mM MgCl2, and 1 mM dithiothreitol. The kinase reactions were performed for 30 min at 30 °C in the presence of 10 µCi of [γ-32P]ATP, 10 µM ATP, and an excess of the substrate, c-Jun-GST. The reactions were terminated by the addition of 4× SDS sample buffer. The samples were boiled and loaded on 12% SDS-PAGE gels. The results were visualized and quantified using a PhosphorImager (Molecular Dynamics).

**RESULTS**

**Regulation of c-Jun-mediated Transcription by Dvl-1—**To test whether Dvl is involved in JNK- and RhoA-linked pathways, the effects of overexpressed Dvl on the activities of c-Jun (regulated by JNK) and SRF (regulated by RhoA) were examined. Activation of SRF was determined by cotransfection of the Dvl cDNA with a luciferase reporter construct containing a mutated c-Fos serum response element, SER.L., upstream of the reporter gene. Because only SRF binds to SER.L, SER.L-mediated production of luciferase mainly depends on the activity of SRF (40). Regulation of c-Jun was studied using the PathDetect signal transduction reporting systems from Stratagene. In this system, transcription of the luciferase reporter gene depends on activation of c-Jun. We also tested the effect of Dvl on Elk (regulated by Ras)-dependent transcriptional activity using the same method as for c-Jun. Positive controls and specificity of activation of these reporting systems were shown in Fig. 1; the activated form of Cdc42 activates SRF and c-Jun, whereas the activated forms of Ras and mitogen-activated protein kinase kinase are potent activators of Elk. These results are consistent with previous findings (37, 38, 40). When Dvl was tested in these reporting systems, we found that it could significantly activate c-Jun-mediated transcription but not SRF or Elk-mediated transcription (Fig. 1). These data suggest that Dvl may activate c-Jun.

**Activation of JNK by Dvl—**It has been shown that JNK phosphorlylates and activates c-Jun (35). Thus, activation of JNK by mouse Dvl proteins was examined. Monkey kidney COS-7 cells were transfected with cDNA-encoding mouse Dvl-1 and JNK-HA. JNK protein immunoprecipitated via an HA tag from cells coexpressing Dvl-1 showed significantly higher activity in phosphorylating a JNK substrate than that from cells coexpressing the control β-galactosidase (LacZ) (Fig. 2). The expression of mouse Dvl-1 is shown in Fig. 2C. Heterologous expression of mouse Dvl-2 and 3 (12, 13) could also activate JNK (Fig. 2). Activation of JNK was also observed when Dvl-1 was expressed in NIH3T3 cells (data not shown).

**C-terminal Portion of Dvl-1 Is Required for JNK Activation—**To determine which portions of the Dvl-1 molecule are responsible for activation of JNK, truncation mutants of mouse Dvl-1 were generated. Amino acid sequence homology analysis of mammalian and Drosophila Dvl/Dvl molecules suggests...
that these molecules can be roughly divided into three parts, each of which contains one highly conserved domain (Fig. 2A).

Therefore, three mutants were generated by deleting each of the conserved domains separately. The mutants are: N, an N-terminal truncation mutant; C, a C-terminal truncation mutant; and PDZ, in which the PDZ domain was deleted (Fig. 2A).

The ability of these Dvl-1 mutants to activate JNK was tested by coexpression with JNK-HA in COS-7 cells. The expression of the Dvl-1 mutants is shown in Fig. 2C. Both N and PDZ were able to activate JNK (Fig. 2), whereas C could not (Fig. 2). This result suggests that the C-terminal region of Dvl-1 may be involved in regulation of JNK.

DEP Domain Is Required for JNK Activation—To test whether the C-terminal portion of Dvl is sufficient for JNK activation, the C-terminal 320 amino acids of mouse Dvl-1, designated as C1, were expressed in COS-7 cells (Fig. 3C) and tested for JNK activation. As shown in Fig. 3, C1, which contains the DEP domain, is sufficient for activation of JNK. Two additional Dvl-1 deletion mutants, which are depicted in Fig. 3C, were generated to further delineate the sequences that are important for JNK regulation. When the Dvl mutant, C2, which encompasses the sequence downstream of the DEP domain, was expressed in COS-7 cells, it was unable to activate JNK (Fig. 3). Because the only difference between C2 and C1 is the lack of the DEP domain (Fig. 3C), the failure of C2 to activate JNK suggests that the DEP domain is involved in JNK regulation. This hypothesis is further confirmed by the observation that the Dvl mutant N, when expressed in COS-7 cells, could activate JNK (Fig. 3).

There is a nonlethal dsh1 allele in Drosophila. Homozygous dsh1 appears to cause only planar polarity but not tag-like phenotypes (8, 41, 42). The mutation in dsh1 was identified as the substitution of Met-438 for Lys (34). Because the JNK pathway was implicated in regulation of planar polarity, we tested whether this mutation affects JNK activation. A Dvl mutant (DvlKM) containing the equivalent mutation was thus generated. We found that DvlKM activated JNK as effectively as the wild type (Fig. 4). DvlKM and its wild type were even compared at two different expression levels (Fig. 4). Thus, although the dsh1 mutation (at residue 438) lies inside the DEP domain, the residue Lys-438 does not appear to play a significant role in JNK activation.

All Three Conserved Dvl-1 Domains Are Required for Up-regulation of β-Catenin—It is well established that Dsh can signal through β-catenin and TCF/LEF-1 (2, 4–7). A previous study indicated that the C-terminal region of Drosophila Dsh, corresponding to C1, was incapable of up-regulating the β-catenin levels in cultured Drosophila cells (26). This finding, together with our results that C1 is sufficient for JNK activation, suggests that Dvl can lead to two different signaling pathways. Nevertheless, we investigated the effects of Dvl and its mutants on the β-catenin-TCF/LEF-1-linked pathway in mammalian cells. We first tested whether ectopic expression of Wnt-1 and Dvl-1 can up-regulate the levels of β-catenin in Cos-7 cells as Wg and Dsh do in Drosophila cells (11, 26). Cells were cotransfected with cDNA encoding Wnt-1 or Dvl-1 along with cDNA encoding Myc-tagged β-catenin and GFP for 24 h. Cytosol fractions were prepared from cotransfected cells and analyzed by Western blotting using antibodies specific to the Myc tag and GFP. The levels of cytosolic recombinant β-catenin proteins in cells coexpressing Wnt-1 (Fig. 5A) or Dvl-1 (Fig. 5B) are clearly higher than those coexpressing the control LacZ. Moreover, coexpression of GSK-3β significantly decreased the levels of cytosolic β-catenin (Fig. 5A). All of these results are consistent with previous findings (5). The levels of coexpressed GFP were determined to ensure that the changes in the β-catenin levels are not because of variations in transfection (Fig. 5,
A and B). Dvl-1 mutants including C, N, PDZ, and C1 were tested for their abilities to up-regulate the β-catenin levels in COS-7 cells. As shown in Fig. 5B, none of the mutants were able to affect the levels of cytosolic β-catenin.

**All Three Conserved Dvl-1 Domains Are Required for LEF-1-mediated Transcriptional Regulation**—β-Catenin has been shown to interact with TCF/LEF-1 and regulate transcription (43). A reporter construct, in which LEF response element was placed in front of a minimal promoter and a luciferase gene, were used to assay LEF-1 activity. When this reporter construct was cotransfected with cDNAs encoding LEF-1 and Wnt-1 or Dvl-1, there was a marked increase in luciferase activity (Fig. 5C). The increase in luciferase activity was Wnt-1- or Dvl-1- and LEF-1-dependent (Fig. 5C). This suggests that the cells do not contain endogenous LEF-1 and that Dvl-1 activates reporter gene transcription through LEF-1. In addition, the observation that β-catenin is able to stimulate luciferase activity and that GSK-3β could inhibit Dvl-stimulated luciferase activity (Fig. 5C) indicates that the LEF-1 activity in this assay system can be regulated by β-catenin and GSK.

Dvl Mutants Were Tested in the LEF Reporter Gene Assay system. Dvl mutants, including C, N, PDZ, and C1, did not...
show significant stimulation of the luciferase activity regardless of the presence (Fig. 5D) or absence of LEF-1 (data not shown). This result is consistent with the one from the assay of the β-catenin levels. Only Dvl-N was able to stimulate LEF1-dependent transcription (Fig. 5D). The fact that Dvl-N can stimulate LEF1-dependent transcription indicates that Dvl sequences downstream of DEP are not required for activation of LEF1-dependent transcription. The ability of Dvl to regulate the β-catenin-LEF1 pathway was also investigated. We found that Dvl was as effective as the wild type Dvl in stimulation of LEF1-dependent transcription (Fig. 5E). Thus, the dsh1 mutation did not affect its ability to regulate its downstream effectors, including LEF1 and JNK, under our experimental conditions.

**Rac1 and Cdc42 Are Not Involved in Dvl-mediated JNK Activation**—Cdc42 and Rac, members of the Rho family of small G proteins, have been shown to mediate JNK activation by a number of stimuli (35, 36). To determine whether Cdc42 or Rac also mediates JNK activation by Dvl, Dvl-WT was coexpressed with the dominant negative mutants of Cdc42 and Rac1, Cdc42N17 and Rac1N17, respectively. Neither of the dominant negative mutants could significantly inhibit Dvl-induced JNK activation (Fig. 6, A and B). To ensure that the dominant-negative mutants of Cdc42 and Rac are effective in suppression of Cdc42 and Rac-mediated JNK activation, we tested these two dominant negative mutants in inhibition of EGF-induced JNK activation. EGF was previously shown to activate JNK through Rac1 and/or Cdc42 (37). As shown in Fig. 6, C and D, the Cdc42 dominant negative mutant suppressed EGF-induced JNK activation by more than 50%, and the Rac1 mutant almost completely abolished EGF-induced JNK activation as reported (37). Therefore, Cdc42 and Rac1 in COS-7 cells are unlikely to play a significant role in Dvl-induced JNK activation in COS-7 cells. RhoA, another member of the Rho family, has been shown by many laboratories to lack the ability to activate JNK in mammalian cells (37, 38, 40), which we also confirmed (data not shown). However, RhoA was suggested to genetically interact with JNK in Drosophila (10). Thus, we tested the dominant negative mutant of RhoA, RhoAN19, for its ability to inhibit Dvl-1-mediated JNK activation. As shown in Fig. 5A, RhoAN19 failed to inhibit Dvl-1-induced JNK activation.

**DISCUSSION**

In this report, we have described a new signal transduction pathway for Dvl. Dvl activates JNK and regulates c-Jun-dependent gene transcription in addition to its ability to regulate the β-catenin and LEF-1 pathways. Moreover, we found that the DEP domain is required for JNK activation. Our findings agree with two recent reports (10, 34) using genetic epistasis analysis in Drosophila showing that Dsh interacts with Drosophila JNK. JNK was shown to be required for correct establishment of planar polarity in Drosophila epidermal tissues, which does not require Arm or Pan. In addition, it was shown that Dvl activates JNK in mammalian cells (34). Our findings are also largely consistent with the study (26) showing that deletion of the conserved domains of Drosophila Dsh including DIX and PDZ resulted in the loss of the ability in up-regulation of the Arm levels. However, there is an apparent discrepancy with regard to the role of the DEP domain in up-regulation of β-catenin. In contrast to our findings, the study by Yanagawa et al. (26) suggested that deletion of the part of Drosophila Dsh corresponding to C1 including the DEP domain could still up-regulate the levels of Arm. This discrepancy may result from the use of different assay systems in cells from different species.

The Drosophila dsh1 allele shows strong planar polarity phenotypes (8, 41, 42). However, the dsh1 allele does not appear to affect the Wg signaling pathways (8, 41, 42). The mutation in dsh1 was identified, and Dsh1 was shown to have less potency in JNK activation than wild type Dsh1 (34). However, our data indicate that there are no significant differences between Dvl and Dvl in JNK activation or in activation of LEF1-dependent transcription (Figs 4 and 5). In our experiments, the effects of Dvl on the Wg signaling pathways (Dvl1) and Dvl1 were compared at the same expression levels of Dvl1 and Dvl1, whereas Boutros et al. (34) had significantly higher levels of wild type Dsh as compared with Dsh1. If our findings are correct, there may be several possible explanations to the genetic data showing that the dsh1 allele only affects the JNK pathway but not the β-catenin pathway (8, 41, 42). 1) The dsh1 mutation may play different roles in Dvl and Dsh; 2) The mutation may disrupt the coupling of Dvl to its upstream regulators (such as receptors) that are involved only in regulation of the JNK pathway. Thus, only the JNK pathway but not the β-catenin pathway is affected; 3) The mutation may partially reduce the coupling of Dvl to its upstream regulators that are involved in both the β-catenin and JNK pathways. However, there may still be sufficient activity to maintain normal functions required for the β-catenin pathway but not enough to maintain the functions required for the JNK pathway, thus leading solely to a planar polarity phenotype.

Although Drosophila RhoA may be involved in JNK activation (although there is lack of biochemical data), mammalian RhoA is clearly not involved in JNK activation (35, 36). Mammalian RhoA is involved in the regulation of SRF via yet-to-be identified pathways (39, 40). In mammals, JNK was found to be regulated by two other small G proteins belonging to the Rho family, Cdc42 and Rac. Although Cdc42 and Rac were shown to mediate JNK activation by many growth factor receptors and G protein-coupled receptors (35, 36), they are not involved in all JNK activation pathways (35). For instance, they are not involved in ultraviolet, toxin anisomycin, tyrosine kinase c-Abl and Pyk2, or TNF-induced JNK activation (35). Although mammalian and Drosophila cells are conserved with regard to activation of JNK by Dvl, different signaling pathways are apparently used in JNK activation by Dvl/Dsh. Our data indicate that Rac1 and Cdc42 are not involved in Dvl-mediated JNK activation. Our results as well as others showed that...
RhoA is irrelevant in JNK activation in mammals. The fact that Dvl-1 does not stimulate SRF-dependent transcription (Fig. 1A) further supports the idea that Dvl-1 does not regulate RhoA in mammalian cells. It is certainly of great interest to determine the mechanism by which Dvl regulates JNK in mammalian cells.

Wg signals through Dsh, Arm, and Pan (TCF) in Drosophila (5). Although there is no evidence for the involvement of Wg in the Fz-Dsh-JNK pathway, Wnt-like molecules are speculated to be involved (5, 34). We tested if mammalian Wnts, Wnt-1, and Wnt-5a, can induce JNK activation in a paracrine paradigm by expression of Wnt in COS-7 cells. We found that both Wnt-1 and Wnt-5a gave 2 to 3-fold stimulation of JNK activity (data not shown). However, we were unable to obtain direct evidence to demonstrate that Wnt activates JNK via Dvl. We have tried all of our Dvl mutants, but none of them was able to block JNK activation by Wnt-1 or Wnt-5a (data not shown). We also tested the effect of Fz in JNK activation. A recent report demonstrating the requirement of Fz-5 in Wnt-5a-induced axial duplication in Xenopus implies that Fz-5 may serve as a receptor for Wnt-5a (14). However, coexpression of Fz-5 with Wnt-5a did not potentiate JNK activation by Wnt-5a in COS-7 cells (data not shown). This suggests that Fz-5 alone may not be sufficient for mediating the effect of Wnt-5a on JNK activation. Alternatively, Wnt-5a may use different receptors for JNK activation.

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