Distinct Domains of Mouse Dishevelled Are Responsible for the c-Jun N-terminal Kinase/Stress-activated Protein Kinase Activation and the Axis Formation in Vertebrates*

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Recent studies have shown that Drosophila Dishevelled (Dsh), an essential component of the wingless signaling pathway, is also involved in planar polarity signaling through the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) pathway in Drosophila. Here, we show that expression of a mouse homolog of Dsh (mDvl-1) in NIH3T3 cells activates JNK/SAPK, and its activator MKK7. A C-terminal half of mDvl-1 which contains the DEP domain was sufficient for the activation of JNK/SAPK, whereas an N-terminal half of mDvl-1 as well as the DEP domain is required for stimulation of the TCF/LEF-1-dependent transcriptional activation, a β-catenin-dependent process. A single amino acid substitution (Met for Lys) within the DEP domain (mDvl-1(M)) abolished the JNK/SAPK-activating activity of mDvl-1, but did not affect the activity to activate the LEF-1-dependent transcription. Ectopic expression of mDvl-1(KM) or an N-terminal half of mDvl-1, but not the C-terminal, was able to induce secondary axis in Xenopus embryos. Because the secondary axis formation is dependent on the Wnt/β-catenin signaling pathway, these results suggest that distinct domains of mDvl-1 are responsible for the two downstream signaling pathways, the β-catenin pathway and the JNK/SAPK pathway in vertebrates.

The Wnt proteins constitute a family of secreted glycoproteins, among which Wingless (Wg) in Drosophila is the best characterized member. Genetic evidence reveals that Wg signals through an intracellular cascade that includes Dishevelled (Dsh), Zeste-White3/Shaggy and Armadillo, a β-catenin homolog (13). These exist vertebrate counterparts of these genes, and the corresponding cascade is believed to function in a variety of biological processes (14, 15). Of the known components, Dsh is thought to act most immediately downstream of the receptor. Alignment of members of invertebrate and vertebrate Dsh family proteins (16–19) reveals three conserved domains: a DIX domain, a PDZ domain, and a DEP domain. Recent analysis of the planar polarity-specific dsh1 allele in Drosophila revealed a single amino acid point mutation in the DEP domain and demonstrated requirement of the DEP domain for planar polarity signaling but not for wingless signaling (21, 22). Moreover, it has been shown that Dsh may act through low molecular weight GTPases and the JNK/SAPK pathway in planar polarity signaling and that overexpression of Dsh results in enhanced c-Jun-N-terminal phosphorylation in cultured cells (21). These results suggested the existence of a signaling pathway from Dsh to activation of JNK/SAPK, which has not been directly tested. In this study, we have first demonstrated that a murine homolog of Dsh, mDvl-1, is able to potently activate JNK/SAPK and its direct activator MKK7 probably through Rac or Cdc42. We then produced various mutant forms of mDvl-1 and tested their abilities to activate JNK/SAPK, to regulate the TCF/LEF-1-dependent transcription, a β-catenin-dependent process, in mammalian cells, and to induce the secondary axis in Xenopus embryos. The formation of the secondary axis has been shown to be controlled by the Dsh/β-catenin pathway (14, 15, 23, 24). The results obtained show that distinct domains of mDvl-1 are responsible independently for the two downstream signaling events, the JNK/SAPK pathway and the β-catenin pathway. This mechanism may be important for controlling the bifurcation of signaling pathways from mDvl-1.

EXPERIMENTAL PROCEDURES

Plasmids—To construct CS4-HA and CS4-Myc, the fragment encoding 2 copies of the Myc epitope and the fragment encoding 1 copy of hemagglutinin (HA) epitope were inserted into BamHI site of CS2*, respectively (25). These vectors were used as mammalian expression vectors or templates for mRNA synthesis. To generate mDvl-1 deletion mutant series, the 2.1-, 1.7-, 1.1-, and 0.85-kb fragments encoding full-length mDvl-1, mDvl-1(ΔDEP) (1–370), and mDvl-1(ΔDEP) (414–695) with EcoRI and XhoI sites were synthesized by polymerase chain reaction and subcloned into

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§ The abbreviations used are: JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; HA, hemagglutinin; MBP, myelin basic protein; GST, glutathione S-transferase; Wg, Wingless; Dsh, Dishevelled.
RESULTS AND DISCUSSION

Activation of JNK/SAPK and MKK7 by Expression of mDvl-1—Expression of Drosophila Dsh has been reported to induce c-Jun phosphorylation (21). To investigate a role of Dsh as activators for MAPK pathways, we tested whether four distinct classes of the MAPK superfamily molecules could be stimulated by a mouse homolog of Dsh, mDvl-1. Each of four HA-tagged MAPK superfamily molecules (HA-MAPK, HA-SAPK, HA-p38, and HA-ERK5) was coexpressed with Myc-tagged mDvl-1 in NIH3T3 cells and immunoprecipitated with anti-HA antibody. The immunoprecipitates were subjected to immunoblotting with rabbit anti-HA-antibody (Y-11, Santa Cruz Biotechnology, Inc.). Aliquots of whole cell lysates were subjected to immunoblotting by anti-myec antibody (9E10, Santa Cruz Biotechnology, Inc.) to confirm appropriate expression of Myc-tagged mDvl-1 proteins. For kinase assays of endogenous JNK/SAPK, the lysates (200 µl, 200 µg of NIH3T3 cells expressing each of various mDvl-1 mutants were incubated with 25 µl of protein A-Sepharose beads (Amersham Pharmacia Biotech) and 5 µl (0.5 mg/ml) of mouse anti-JNK1 antibody (Pharmingen) for 2 h at 4°C. Then, the immune complex kinase assay of endogenous JNK/SAPK was performed as described above, and the amounts of immunoprecipitated endogenous JNK/SAPK were determined by immunoblotting with goat anti-JNK1 antibody (Santa Cruz Biotechnology, Inc.).

Subcellular Fractionation—Cells were washed twice with ice-cold Hepes-buffered saline, harvested by scraping from the culture dishes into ice-cold hypotonic buffer consisting of 10 mM 2-glycerophosphate (pH 7.4), 1 mM MgCl₂, 2 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1% aprotinin (200 µl of buffer/60-mm dish), and disrupted by 10 strokes with a homogenizer. The homogenate (200 µl) was loaded onto 200 µl of 2 M sucrose in hypotonic buffer and centrifuged at 15,000 × g for 30 min to pellet nuclei. To prepare the cytosol fraction, the supernatant was taken above the sucrose cushion and centrifuged at 100,000 × g for 30 min. The pellet fraction was designated the postnuclear membrane fraction and solubilized in hypotonic buffer containing 0.5% Triton X-100 and 0.1% deoxycholate. Protein concentration was determined with a protein assay kit (Bio-Rad) with bovine γ-globulins (Sigma) as a standard.

Cell Staining—NIH3T3 cells were cultured on glass coverslips and transiently transfected with mDvl-1 expression plasmids. 15 h after transfection, the coverslips were fixed with 3.7% paraformaldehyde in phosphate-buffered saline for 10 min at 37 °C and treated with 0.5% Triton X-100 in phosphate-buffered saline for 10 min. After blocking with phosphate-buffered saline containing 3% bovine serum albumin (Sigma) and 0.1% γ-globulins (Sigma), the coverslips were incubated with the anti-Myc antibody (9E10, Santa Cruz Biotechnology, Inc.) and then washed three times with phosphate-buffered saline. Reacted proteins were detected by Cy3-conjugated goat anti-mouse secondary antibody (Amersham Pharmacia Biotech).
Drosophila showed that Drosophila RhoA and Rac, but not Cdc42, function downstream of Dsh in planar polarity signaling (21). To investigate potential roles of low molecular weight GTPases in the mDvl-1-induced JNK/SAPK activation in mammalian cells, dominant-negative forms of Ha-Ras, Rac1, Cdc42, and RhoA were coexpressed with HA-SAPKα and Myc-mDvl-1. mDvl-1-induced JNK/SAPK activation was significantly suppressed by dominant-negative Cdc42 or Rac1 (Fig. 1C), indicating that Rac1 and Cdc42 act downstream of mDvl-1 in the JNK/SAPK activation pathway. In contrast, dominant-negative Ha-Ras or RhoA did not interfere with the activation of JNK/SAPK (Fig. 1C). It is possible that functions of the Rho family GTPases in vertebrates are slightly different from those in Drosophila, although it is suggested that the Rho family GTPases lie downstream of Dsh or mDvl-1 in both vertebrates and invertebrates.

A C-terminal Half of mDvl-1 Is Required and Sufficient for JNK/SAPK Activation—It has previously been suggested that the DEP domain of Drosophila Dsh is important for the Dsh-induced phosphorylation of c-Jun, because substitution of a single amino acid (Lys-417 to Met) within the DEP domain or deletion of the DEP domain abolished the c-Jun-phosphorylation-inducing activity of Dsh (21). To investigate the importance of DEP domain in the mDvl-1 induced activation of JNK/SAPK in mammalian cells, NIH3T3 cells were cotransfected with HA-SAPK and various forms of mDvl-1 (see Fig. 2A), and the activity of JNK/SAPK was determined. A mutant mDvl-1 lacking the DIX domain (∆DIX) activated JNK/ SAPK as efficiently as did wild-type mDvl-1 (Fig. 2B, wt and ∆DIX), whereas a mutant lacking the DEP domain (∆DEP) and a mutant in which Lys-438 (which corresponds to Lys-417 of Drosophila Dsh) within the DEP domain was replaced by Met (KM) failed to activate JNK/SAPK (Fig. 2B, ∆DEP and KM). Moreover, the DEP domain-containing C-terminal portion of mDvl-1 (mDvl-1(DEP)) which lacks both the DIX and PDZ domains activated JNK/SAPK as efficiently as did wild-type mDvl-1 (Fig. 2B, DEP). We then measured the activity of endogenous JNK/SAPK by an immune complex kinase assay using anti-JNK1(p46JNK/SAPK) antibody, and found that endogenous JNK/SAPK was activated by overexpression of wild-type mDvl-1, mDvl-1(∆DIX), or mDvl-1(∆DEP), but not by that of mDvl-1(∆DEP) or mDvl-1(KM) (Fig. 2C). These results indicate that the C-terminal portion of mDvl-1, which contains the DEP domain, is required and sufficient for the JNK/SAPK activation.

An N-terminal Half of mDvl-1 Is Required for TCF/LEF-1-mediated Transcription—Expression of Wnt-1 or β-catenin with LEF-1 in NIH3T3 cells is shown to activate TCF/LEF-1-dependent transcription (28). To address whether mDvl-1 is able to activate the Wnt/β-catenin pathway in NIH3T3 cells, we used a luciferase reporter assay. NIH3T3 cells were transfected transiently with pTOPFLASH, a luciferase reporter gene containing multimeric TCF/LEF-1 binding sites (29), together with expression plasmids of LEF-1 and mDvl-1. Expression of wild-type mDvl-1 with LEF-1 strongly increased transcription from pTOPFLASH (Fig. 2D, WT). This transcriptional activation was not observed with pFOFFLASH, a reporter gene containing multimeric mutant TCF/LEF-1 binding sites (data...
**Bifurcate Signaling Pathways from Dishevelled**

**Fig. 2.** The DEP domain of mDvl-1 is essential for JNK/SAPK activation. A, schematic representation of wild-type and mutant mDvl-1 proteins. B, pSRαHA-SAPKα (1.0 μg) was transiently cotransfected into NIH3T3 cells with indicated amounts of an expression plasmid for a Myc-tagged wild-type (wt) or mutant mDvl-1 (∆DIK, ∆DEP, DEP, or KM; see panel A). The expression level of mDvl-1 mutants was determined by immunoblotting of cell lysates (αMyc). Asterisks indicate the position of the mutants. The kinase activity of JNK/SAPK was measured by the immune complex kinase assay (upper panel), and the amounts of immunoprecipitated HA-SAPKα were detected by immunoblotting (αHA). Similar results were obtained in three different experiments. C, NIH3T3 cells were transfected with an expression plasmid for each of various mDvl-1 mutants (3.0 μg of wt, DEP, or KM, 2.4 μg of ∆DIK, and 1.2 μg of ∆DEP). The endogenous JNK/SAPK kinase activity was measured by an immune complex kinase assay (upper panel). The amount of endogenous JNK/SAPK in each cell lysate was determined by immunoblotting using an anti-JNK1 antibody (αJNK1). D, a reporter plasmid pTOPFLASH (0.3 μg) together with an expression plasmid (0.5 μg) for various mDvl-1 proteins (empty vector (control), WT, ∆DIK, ∆DEP, DEP, or KM) was cotransfected into NIH3T3 cells. The total amount of the DNA was adjusted to 1.2 μg. The luciferase activity was measured as described under “Experimental Procedures.” Average relative luciferase activities from three independent experiments were calculated relative to the activity of the control, which was set at 1. Error bars represent mean ± S.D.

not shown), indicating that transcriptional response to mDvl-1 is dependent on the presence of functional TCF/LEF-1 binding sites. Activation of the JNK/SAPK pathway did not increase the TCF/LEF-1-dependent transcription (Fig. 2D, MKK7/JNK).

Although mDvl-1 (∆DIK), mDvl-1(∆DEP), or mDvl-1(DEP) did not significantly increase the luciferase activity, mDvl-1(KM) did increase the activity to the same extent as did wild-type mDvl-1 (Fig. 2D). This result is consistent with the previous reports in Drosophila (21, 30), which demonstrated that the DIK and PDZ domains were required for Armadillo (β-catenin) stabilization, whereas the KM mutation in the DEP domain did not affect the β-catenin pathway. However, our result is different from the result reported by Yanagawa et al. (30) in which the DEP domain was shown to be unnecessary for β-catenin stabilization in Drosophila cells. This discrepancy might result from the differences in cell types, dishevelled proteins used (Drosophila or mouse), or assay systems.

**Effect of the Addition of CAAX Motif on the JNK/SAPK-activating Activity of mDvl-1—**Because the DEP domain has previously been shown to be required for Dsh relocalization to the plasma membrane (22), we considered the possibility that the translocation of Dsh protein might be involved in the activation of the JNK/SAPK pathway. Then, we made several constructs that expressed wild-type and mutant forms of mDvl-1 as fusions to the C terminus of CAAX motif of Ras to obtain membrane-bound forms of mDvl-1. We checked subcellular localization of these mDvl-1 mutants. Transfected NIH3T3 cells were fractionated into postnuclear membrane and cytosol fractions, and the fractions were subjected to immunoblotting. As shown in Fig. 3A, wild-type mDvl-1 protein localized in both the cytosol (lane C) and membrane fraction (lane M), whereas mDvl-1-CAAX protein was only in the membrane fraction. The DEP domain-containing C-terminal portion of mDvl-1 localized predominantly in the membrane fraction (Fig. 3A, DEP). The two mutants, mDvl-1(∆DEP) and mDvl-1(KM), localized in both the cytosol and the membrane fractions, but, unlike wild-type mDvl-1, they were mainly present in the cytosol fraction. In contrast, mDvl-1(KM)-CAAX and mDvl-1(ΔDEP)-CAAX proteins localized predominantly in the membrane fraction (Fig. 3A, DEP-CAAX and KM-CAAX). The cell staining with indirect immunofluorescence showed that wild-type mDvl-1 and mDvl-1(KM) proteins were detected throughout the cytoplasm (Fig. 3B and data not shown), whereas CAAX-fused mDvl-1 proteins were concentrated in the plasma membrane (Fig. 3B and data not shown). Thus, the CAAX motif targeted mDvl-1 proteins to the plasma membrane. It should be noted that CAAX-fused mDvl-1 proteins-expressing cells underwent drastic morphological changes: rounding, shrinkage, and induction of protrusions and processes (see Fig. 3B).

In Drosophila, a mutation in the DEP domain (the KM mutation) impairs both the membrane localization of Dsh and the function of Dsh in planar polarity signaling, suggesting that translocation of Dsh is important for function (22). Con-
mDvl-1(KM) mutant, which was fused to the myristylation motif derived from chick c-Src at its N terminus, could also activate JNK/SAPK (data not shown). These results suggest that mDvl-1(KM) has a potential to activate JNK/SAPK if properly localized to membrane. In contrast, mDvl-1(ΔDEP)-CAAX could not activate JNK/SAPK (Fig. 3C). Thus, the DEP domain may have a role in localizing mDvl-1 to the plasma membrane to activate the JNK/SAPK pathway, but the function of the DEP domain is not solely the targeting of mDvl-1 to the plasma membrane. In the case of the Raf/MAPK pathway, growth factor stimulation recruits Raf-1 to the plasma membrane, and the membrane-bound form of Raf-1 (RafCAAX) is constitutively active (31, 32). However, mDvl-1-CAAX had a lower activity than mDvl-1 to stimulate the activity of JNK/ SAPK (Fig. 3C). These results suggest that the mode of the CAAX-mediated membrane localization of mDvl-1 may be different from that of the DEP domain-mediated membrane localization, the latter being required for optimal activation of the JNK/SAPK pathway.

We then examined the effect of the addition of CAAX-motif to mDvl-1 on the JNK/SAPK activation and the TCF/LEF-1-dependent transcription. A, NIH3T3 cells were transfected with an expression plasmid for Myc-tagged mDvl proteins; wild-type (wt), ΔDEP, KM, DEP, and some of their derivatives fused to CAAX-motif at their C terminus (wt-CAAX, ΔDEP-CAAX, and KM-CAAX). 15 h after transfection, cell lysates were obtained and fractionated into the cytosol (lane C) and membrane (lane M) fractions, and the amounts of Myc-tagged proteins were determined by immunoblotting. B, NIH3T3 cells were transfected with an expression plasmid for Myc-tagged mDvl-1(KM) or mDvl-1(KM)-CAAX. 15 h after transfection, cells were fixed and stained with anti-Myc antibody. Bar, 10 μm. C, pSR-αHA-SAPKα (1.0 μg) was transiently cotransfected into NIH3T3 cells with 3 μg of an expression plasmid for Myc-tagged mDvl-1 proteins indicated. HA-SAPKα was immunoprecipitated with anti-HA antibody and assayed for kinase activity. Similar results were obtained in three different experiments. D, NIH3T3 cells were transfected with pHA-LEF1 (0.03 μg) and the reporter plasmid pTOPFLASH (0.3 μg) together with an expression plasmid (0.5 μg) for various mDvl-1 proteins (empty vector (control), WT, WT-CAAX, ΔDEP, ΔDEP-CAAX, KM, or KM-CAAX) as in Fig. 2D. 10 h after transfection, the cells were harvested and the luciferase activity was measured as described under "Experimental Procedures." Average relative luciferase activities from three independent experiments were calculated relative to the activity of the control, which was set at 1. Error bars represent mean ± S.D.

sistent with this idea, the two mutants, mDvl-1(KM) and mDvl-1(ΔDEP), had no ability to activate JNK/SAPK (see Fig. 2, B and C and Fig. 3C). Interestingly, mDvl-1(KM)-CAAX was able to activate JNK/SAPK to some extent (Fig. 3C). In addition, a...
not known at present. Injection of wild type mDvl-1 mRNA into ventral blastomeres at the four-cell stage embryo triggered the formation of a secondary axis (Fig. 4B, WT). mDvl-1 mRNA injections into dorsal blastomeres at the same stage did not alter normal development (data not shown). Ventral injection of mDvl-1(ΔDEP) (data not shown) or mDvl-1(KM) mRNA, but not that of mDvl-1(ΔDI) or mDvl-1(DEP) mRNA, triggered the secondary axis formation (Fig. 4B). These results demonstrate that in *Xenopus* embryos as well as in mammalian cultured cells, the KM mutation in the DEP domain has no effect on the β-catenin pathway whereas the intact DEP domain is required for JNK/SAPK activation.

In our preliminary experiments, dominant-negative or constitutively active forms of *Xenopus* M KK6 (XMEK3) and MKK7 did not interfere with normal axis formation or cause the axis duplication. These results suggest that JNK/SAPK is not involved in the axis formation in *Xenopus*. Then, it should be elucidated what roles JNK/SAPK plays downstream of Dishevelled in *Xenopus*. The Wnt genes can be classified into at least two subfamily members, Wnt1 and Wnt5α members (33, 34). In *Xenopus* embryos, expression of members of the Wnt1 subfamily induces axis duplication, whereas expression of the other subfamily alters morphogenetic movements during gastrulation. It is tempting to speculate that JNK/SAPK functions downstream of members of the Wnt5α subfamily.

In summary, this study has shown that mDvl-1 is able to induce the activation of both the β-catenin pathway and the JNK/SAPK pathway in both mammalian cultured cells and *Xenopus* embryos. As the KM mutation in the DEP domain did not affect the LEF-1-dependent transcription or the axis duplication, the DEP domain-mediated membrane localization may not be important for the activation of the β-catenin pathway. On the other hand, the KM mutation abolished the ability of mDvl-1 to activate the JNK/SAPK pathway, and the intact DEP domain is sufficient for activation of this pathway in mammalian cells. Therefore, it is suggested that the DEP domain-containing C-terminal half and the DIX and PDZ domain-containing N-terminal half are important for the JNK/SAPK pathway and for β-catenin pathway, respectively. Thus, bifurcation of the Wnt signaling pathway at the point of Dishevelled may be a conserved mechanism in both vertebrates and invertebrates. This study may provide the molecular basis by which the bifurcation is achieved and assured.

After the completion of this study, a paper of Li et al. (35) appeared, which showed the regulation of LEF-1 and JNK/SAPK by Dvl proteins in mammalian cells.

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