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Identification of EPS8 as a Dvl1-Associated Molecule

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Dishevelled (Dsh) is involved in both Wingless (Wg) and Frizzled (Fz) signaling pathways. To further determine the function of Dsh, we have performed yeast two-hybrid screening and isolated several genes encoding the molecules associated with the PDZ domain of Dvl1, one of the murine Dsh homologs. During the screening, we found that EPS8, which is a substrate for activated EGF receptor (EGFR), specifically interacted with Dvl1. This interaction was also confirmed *in vitro*. Through transfection studies, we observed the mutual action between Dvl1 and EPS8. Dvl1 was hyperphosphorylated in the presence of EPS8, whereas the tyrosine phosphorylation of EPS8 by activated EGFR was inhibited in the presence of Dvl1. Immunohistochemistry showed that Dvl1 and EPS8 expression overlap in particular tissues during organogenesis. These results indicate that interaction between Dvl1 and receptor tyrosine kinase signal plays certain roles in developmental events. © 1999 Academic Press

Key Words: **dishevelled; Dvl1; EPS8; Wnt; RTK signal.**

Dishevelled (Dsh) plays a crucial role in the Wingless (Wg) signaling pathway (1–3). Its signaling cassette, which leads to expression of target genes, such as *Engrailed*, has been identified by *Drosophila* genetic epistasis. In the presence of Wg, an orthologue of Wnt1, DFz2 (4) acts through Dsh to prevent the kinase, Zeste-White (Zw3), from phosphorylating the protein, Armadillo (Arm, a homolog of β-catenin), resulting in its stabilization. Stabilizing Arm leads to the formation of the active transcriptional regulator associated with Pangolin (Pan), an HMG-box transcription factor related to TCF/LEF-1. In the absence of Wg, Zw3, a serine/threonine kinase homologous to glycogen synthase kinase 3 (GSK3), phosphorylates Armadillo, resulting in degradation of the protein. It appears that Dsh also participates in the Frizzled (Fz) signaling pathway, which mediates planar cell polarity (5–7). The Fz signal is relayed by RhoA, small GTPase (8), and c-jun N-terminal kinase (JNK) (9, 10). This shows that Dsh may have a dual role in intracellular signaling.

Dsh encodes for a modular protein of unknown function and there are at least three *dsh* homologs in the mouse (11–13). Alignment of the Dsh family members reveals three conserved domains. One is a DIX domain, similar to a domain found in the murine Axin, a regulator of the Wnt pathway (14). There is also a PDZ domain, a domain that works as a protein-protein interaction interface (15). Finally, the Dsh family shares a domain called DEP. This domain is conserved among a series of proteins that regulate various GTPases (16). While it is certain that the regulation of Wg and Fz signals depends upon the disposition of these three distinct domains (9, 17–19), finding the precise regulatory mechanisms will depend on identifying the molecules associated with them.

In this report, we investigate the function of Dsh through the cloning of genes encoding molecules associated with the PDZ domain of Dvl1, one of the murine Dsh homologs. Since one can isolate *eps8*, a cellular substrate for the EGF receptor (EGFR), it is likely that there is interaction between Dsh/Dvl and the receptor tyrosine kinase (RTK) signal. EPS8 enhances the phosphorylation of Dvl1 in 293T cells. On the other hand, the tyrosine phosphorylation of EPS8 by activated EGFR is inhibited in the presence of Dvl1, suggesting a mutual action between Dvl1 and EPS8. Both Dvl1 and EPS8 proteins are co-expressed in restricted re-

Abbreviations used: EGF, epidermal growth factor; RTK, receptor tyrosine kinase; GST, glutathione *S*-transferase; CNS, central nervous system.

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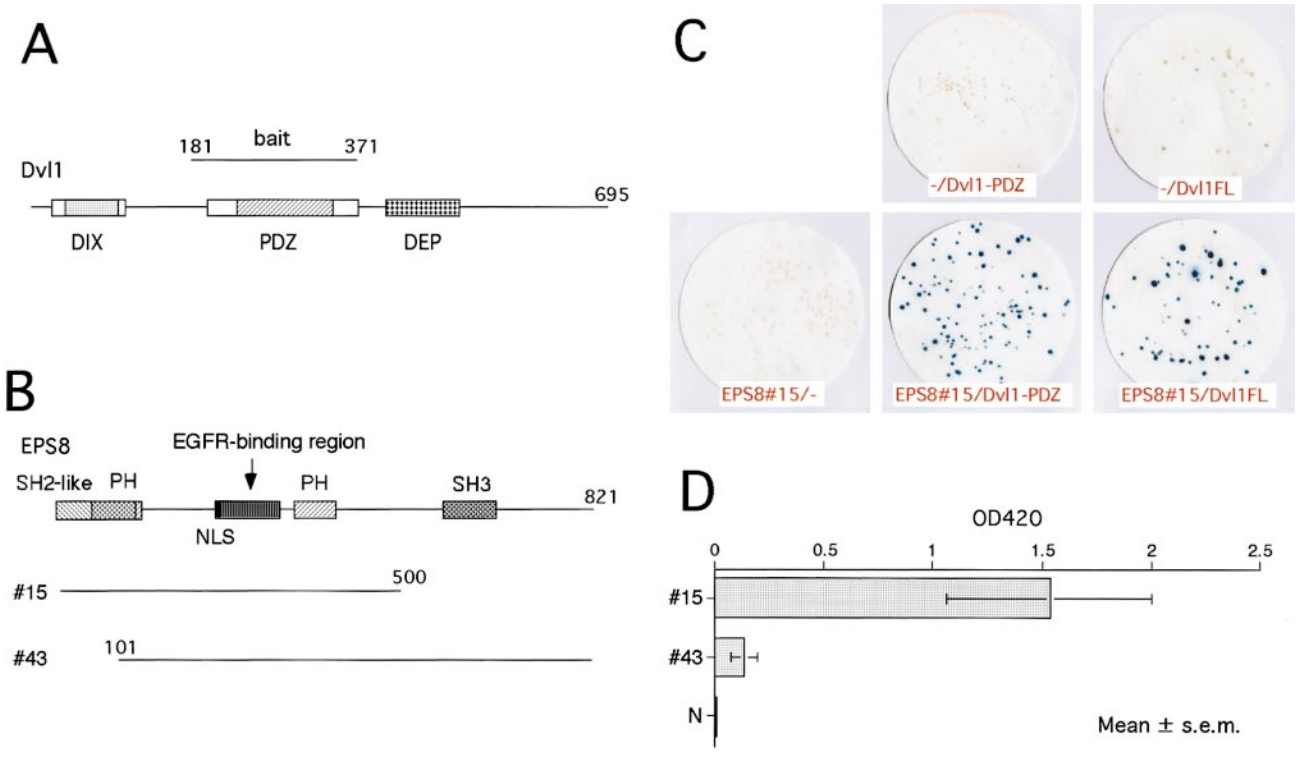

FIG. 1. Detection of the interaction between Dvl1 and EPS8 by yeast two-hybrid assay. (A) Schematic representation of the Dvl1 protein. The amino acid positions are indicated by the flanking numbers. Boxes represent regions conserved among known Dsh/Dvl proteins from other species; the recognized domains are shaded. (B) EPS8 fragments obtained by yeast two hybrid screening. The described structural domains are shown as shaded boxes, and the amino acid positions are indicated as in A. (C) Representative β-galactosidase assay. The Y190 was transformed by various combinations of pGAD10- (left) and pAS2-1-derived plasmid (right). (D) Liquid cultured β-galactosidase assay. Y190 was transformed with both pAS2-1/Dvl1FL and pGAD10/EPS8#15 or pGAD10/EPS8#43. Empty plasmid (N) was used as a control. The β-galactosidase activity was represented by absorbance at 420 nm using ONPG as substrate.

gions during organogenesis. Therefore, a Dvl1/EPS8 association may play a role in developmental events.

MATERIALS AND METHODS

Yeast two-hybrid screening. As bait, we constructed pAS2-1/Dvl1-PDZ containing the PDZ domain of Dvl1 (corresponding to amino acids 181–371). PDZ domain of Dvl1 was amplified by RT-PCR from mouse brain poly(A)$^+$ RNA using specific primer set (forward primer; 5′-GCCATGGCTGTACTGAGCAGTGAG and reverse primer; 5′-GTCGACCGTCAGTGCTGCTGTGTG). The yeast strain Y190, carrying the pAS2-1/Dvl1-PDZ, was transformed with a mouse embryonic day (E) 11 embryo cDNA library constructed in pGAD10 (Clontech, Palo Alto, CA). We also constructed pAS2-1/Dvl1FL which contained full length Dvl1. β-Galactosidase activity was determined using either X-gal (0.6 mg/ml; 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) or ONPG (0.67 mg/ml; *o*-nitrophenyl β-D-galactopyranoside) as substrate.

In vitro pull down assay. To prepare GST-Dvl1-PDZ fusion protein, we constructed pGEX-2T/Dvl1-PDZ. BL21(DE3), carrying this plasmid, was stimulated with 1 mM IPTG and GST-Dvl1-PDZ was then purified by Glutathione Sepharose 4B column chromatography (Pharmacia, Uppsala, Sweden). Truncated *eps8* (EPS8#15; Fig. 1B) was inserted into pET30a(+) (Novagen, Madison, WI). S-tag-EPS8#15 was produced in BL21(DE3) carrying pET30a(+)/EPS8#15. 5 μg GST-Dvl1-PDZ was incubated with 100 μl BL21(DE3) lysate, including S-tag-EPS8#15, for 2 h at 4°C. GST-Dvl1-PDZ was precipitated with Glutathione Sepharose 4B. Co-precipitation of S-tag-EPS8#15 was detected by an S-tag Western detection system (Novagen, Madison, WI).

Cell culture and transfection. *EPS8#15* and *EPS8FL* were cloned into the pFLAG-CMV2 (Eastman Kodak, Rochester, NY) to express the EPS8 molecule, coupled with FLAG epitope, in 293T cells. The *Dvl1FL* and human *EGFR* cDNA were inserted into pcDNA3 (Invitrogen, San Diego, CA) for expression of both proteins in 293T cells. The human *EGFR* cDNA was isolated from pCO12-EGFR (20), obtained from RIKEN DNA Bank (Saitama, Japan). The 293T human embryonic kidney cell line (21) was kindly provided by Dr. D. Baltimore (Massachusetts Institute of Technology, Cambridge, MA). The cells were transfected with various combinations of plasmids using lipofect-AMINE reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. They were then cultured for 60–72 h, at which point the constructs reached their full expression.

Immunoprecipitation and Western blotting. 293T cells were lysed in a lysis buffer containing the following: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP40, 10% glycerol, 2 mM orthovanadate, 10 mM pyrophosphate, 50 mM NaF, protease inhibitor cocktail (Complete, Boehringer Mannheim, Mannheim, Germany). FLAG-EPS8FL and FLAG-EPS8#15 were immunoprecipitated from the lysate using 10 μg anti-FLAG mAb (M2, Eastman Kodak, Rochester, NY) and 10 μl protein A–Sepharose (Pharmacia, Uppsala, Sweden). The immunoprecipitate was then analyzed by Western blot using purified anti-Dvl1 antiserum, regardless of specific Dvl1 co-precipitation. In some cases, immunoprecipitation was performed using 5 μg purified

anti-Dvl1 antiserum, subsequently washing it with PAP buffer (40 mM MOPS (pH 5.5), 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM PMSF) and treating it with 0.3 units potato acid phosphatase (Sigma, St. Louis, MO) for 3 h at 37°C before Western blot analysis. To stimulate the transfectants with recombinant human EGF (rhEGF, Genzyme, Cambridge, MA), the cells were starved for 24 h in ordinary DMEM, then treated with 100 ng/ml rhEGF for 10 min. Tyrosine phosphorylation of FLAG-EPS8FL and FLAG-EPS8#15 was detected using anti-phosphotyrosine (RC20-biotin, Transduction, Lexington, KY).

Immunohistochemistry. To perform immunostaining, anti-EPS8 mAb (clone 15) was purchased from Transduction Laboratories (Lexington, KY). Purified anti-Dvl1 antiserum was prepared as follows: We immunized NZW rabbits with 0.4 mg GST-Dvl1-PDZ with complete freund's adjuvant (FA), then boostered their immunizations 4 times with 0.2 mg GST-Dvl1-PDZ/incomplete FA conjugate at 2-week intervals. Anti-Dvl1 antiserum was then purified from serum by positive and negative affinity chromatography using GST-Dvl1-PDZ and GST coupled Sepharose 4B, respectively. To analyze the expression patterns of Dvl1 and EPS8 during organogenesis, sections prepared from E13.5 mouse embryos were first stained with anti-Dvl1 antiserum or anti-EPS8 mAb, then treated with VECTASTAIN ABC-PO kit (Vector, Burlingame, CA). Finally, Dvl1 and EPS8 were visualized using DAB as substrate.

RESULTS

Identification of the interaction of Dvl1 with EPS8. To clone the cDNA encoding the molecule associated with Dvl1, we performed yeast two-hybrid screening using the PDZ domain of Dvl1 as bait (Fig. 1A). When the cDNA library constructed from mouse E11 embryos was screened, 50 clones were obtained from 1.6×10^6 transformants based on the induction of β-galactosidase. Among them, we focused on two clones that contained *eps8* cDNA (Fig. 1B), because the cointroduction of EPS8#15 with Dvl1FL also resulted in the induction of β-galactosidase (Fig. 1C). Both EPS8#15 and EPS8#43 bound to Dvl1FL according to a liquid cultured β-galactosidase assay (Fig. 1D).

We confirmed the interaction of Dvl1 with EPS8 *in vitro.* GST-Dvl1-PDZ (the PDZ domain of Dvl1 linked to glutathione *S*-transferase) was mixed with S-tag-EPS8#15 (1-500 fragment of EPS8 labeled with S-tag in its N-termini); this combination formed the complex revealed in Fig. 2A (GST alone did not demonstrate any form of binding). Next, we assessed the interaction of the two molecules in cultured cells. Both Dvl1 and EPS8 (tagged with FLAG epitope in its n-termini) expression plasmids were transfected into 293T cells simultaneously. When FLAG-EPS8 was immunoprecipitated using anti-FLAG mAb, the co-precipitation of Dvl1 was detected (Fig. 2B). These results show that Dvl1 is able to form a complex with EPS8 *in vitro* and *in vivo.*

Augmentation of Dvl1 phosphorylation through the interaction with EPS8. To analyze the possible biochemical consequences of the interaction between Dvl1 with EPS8, we examined Dvl1 phosphorylation by gel electrophoresis. When the lysate of 293T cells transfected with Dvl1 expression plasmid alone was electrophoresed, a broad band for Dvl1 was detected, whereas dephosphorylation by potato acid phosphatase treatment resulted in the appearance of a narrow band (Fig. 3A), indicating that Dvl1 is normally highly phosphorylated. When Dvl1 accompanied by FLAG-EPS8 or FLAG-EPS8#15 was introduced into 293T cells, migration of Dvl1 retarded (Fig. 3A). This indicates that EPS8 is able to modify the state of phosphorylation of Dvl1 through their interaction.

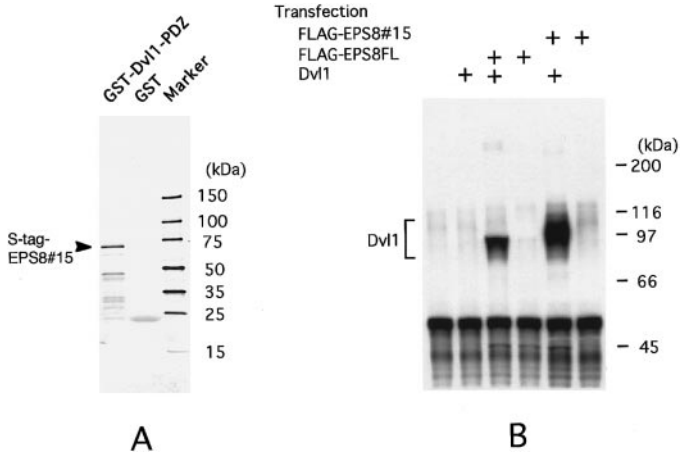

FIG. 2. Physical interaction between Dvl1 and EPS8 *in vitro* and *in vivo.* (A) *In vitro* binding assay using bacterial products. S-tag-EPS8#15 binds to GST-Dvl1-PDZ, but not to control GST (5 μg). (B) Co-immunoprecipitation of Dvl1 with FLAG tagged EPS8. 293T cells were transfected with various combinations of expression plasmids (2.5 μg each) as indicated on top. FLAG-EPS8FL and FLAG-EPS8#15 were immunoprecipitated using anti-FLAG mAb, then probed with anti-Dvl1 antiserum. When Dvl1 is co-precipitated with FLAG-EPS8, it electrophoresed abnormally owing to its co-migration with FLAG-EPS8 (slightly larger than Dvl1 in size).

Effect of Dvl1 on tyrosine phosphorylation of EPS8. It is believed that the tyrosine phosphorylation of EPS8 by activated EGFR is an essential step in signal transduction (22). This led us to evaluate the influence of Dvl1 on tyrosine phosphorylation of EPS8 following EGF stimulation. Consistent with a previous study (22), recombinant human EGF treatment resulted in the phosphorylation on tyrosine residues of FLAG-EPS8 expressed in 293T cells (Fig. 3Ba). When Dvl1 was co-expressed with FLAG-EPS8FL or FLAG-EPS8#15 in 293T cells, tyrosine phosphorylation of EPS8s were markedly inhibited (Fig. 3Bb). This cannot be explained by the amount of EPS8 expressed because this quantity changed little in EGF treated or non-treated 293T cells (Fig. 3Bb). Thus, Dvl1 may function as a negative regulator of EPS8 activation.

Expression of Dvl1 and EPS8 during organogenesis. We examined the spatial relationship between Dvl1 and EPS8 at the protein level using close or adjacent sections prepared from E13.5 mouse embryos. We found a broad expression pattern of Dvl1 (Fig. 4A).

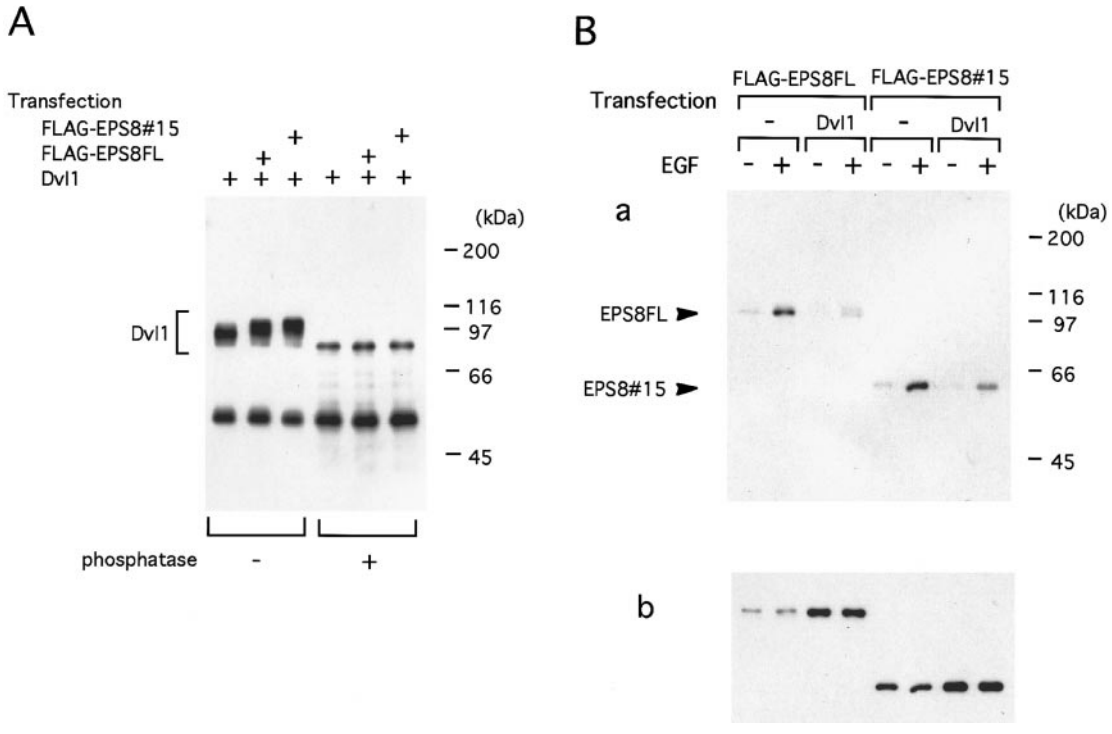

FIG. 3. Mutual action between Dvl1 and EPS8. (A) Dvl1/EPS8 interaction results in hyperphosphorylation of Dvl1. 293T cells were transfected with the various combinations of expression plasmids (2.5 μg each) as indicated on top. Dvl1 was immunoprecipitated from the lysates, then treated with potato acid phosphatase as described under Materials and Methods. Dvl1 was detected using anti-Dvl1 antiserum. (B) Dvl1 inhibits EGFR-induced tyrosine phosphorylation of EPS8. FLAG-EPS8FL and FLAG-EPS8#15 expression plasmid with or without Dvl1 expression plasmid were introduced into 293T cells, then treated with EGF as described under Materials and Methods. FLAG-EPS8 and FLAG-EPS8#15, immunoprecipitated from the lysates, were probed with anti-phosphotyrosine (a). The same blot was re-probed with anti-FLAG mAb as control (b).

By contrast, EPS8 protein appeared only in particular tissues and organs in a relatively restricted manner during organogenesis (Fig. 4D). In the central nervous system (CNS), Dvl1 expression was abundant, especially in the choroid plexus (Fig. 4B). No EPS8 was detected in the CNS except for the mes/metencephalic border (Fig. 4E), ependymal layer of the diencephalon (presumptive tharamus; Fig. 4K). There was a high expression of Dvl1 in the ganglia such as the dorsal root ganglia (drg; Fig. 4A and C). In the trunk, most organs expressed Dvl1 protein, while EPS8 protein was only expressed in restricted organs and tissues. These included the adrenal gland (Fig. 4F), inner mucosal layer of the gut (Fig. 4G), submandibular gland (Fig. 4I), testicular cords (Fig. 4J). An interesting pattern of Dvl1 and EPS8 expression was observed in the adrenal gland. EPS8 was expressed exclusively in the cortex, whereas Dvl1 was highly expressed in medulla in a punctate pattern (Fig. 4C). In this organ, Dvl1 may share function with EPS8. In addition, EPS8 was also expressed in nasal cartilage (Fig. 4D) and the tongue (only the vertical component of intrinsic muscle; Fig. 4H). These observations indicate that an interaction between Dvl1 with EPS8 occurs in restricted tissues and organs, where EPS8 is expressed, and during organogenesis.

DISCUSSION

We isolated several candidate genes encoding the molecules that associate with Dvl1 according to our yeast two hybrid screening. Among these candidates, we took interest in the *eps8* cDNA for three reasons. First, EPS8 fragments screen positively when they are co-introduced into Y190 with either Dvl1-PDZ or Dvl1FL. Second, it has been reported that EPS8 mRNA is expressed specifically in the mes/metencephalic border, where Wnt1 mRNA is also expressed, during neurogenesis (23). This finding raises the possibility of interaction between EPS8 and Wnt signaling. Lastly, EPS8 was first identified as a substrate for EGFR kinase and mediates an EGFR-derived signal in the cytoplasm (22). Similarly, Dsh also localizes in the cytoplasm, and functions as one of the signal transducers (1).

Dsh is implicated in two distinct intracellular signaling pathways that activate Arm and JNK. This dual action is fostered through its three distinct domains. The DIX and PDZ domains are required for Wg signaling (17, 18), while the DEP domain participates in Fz

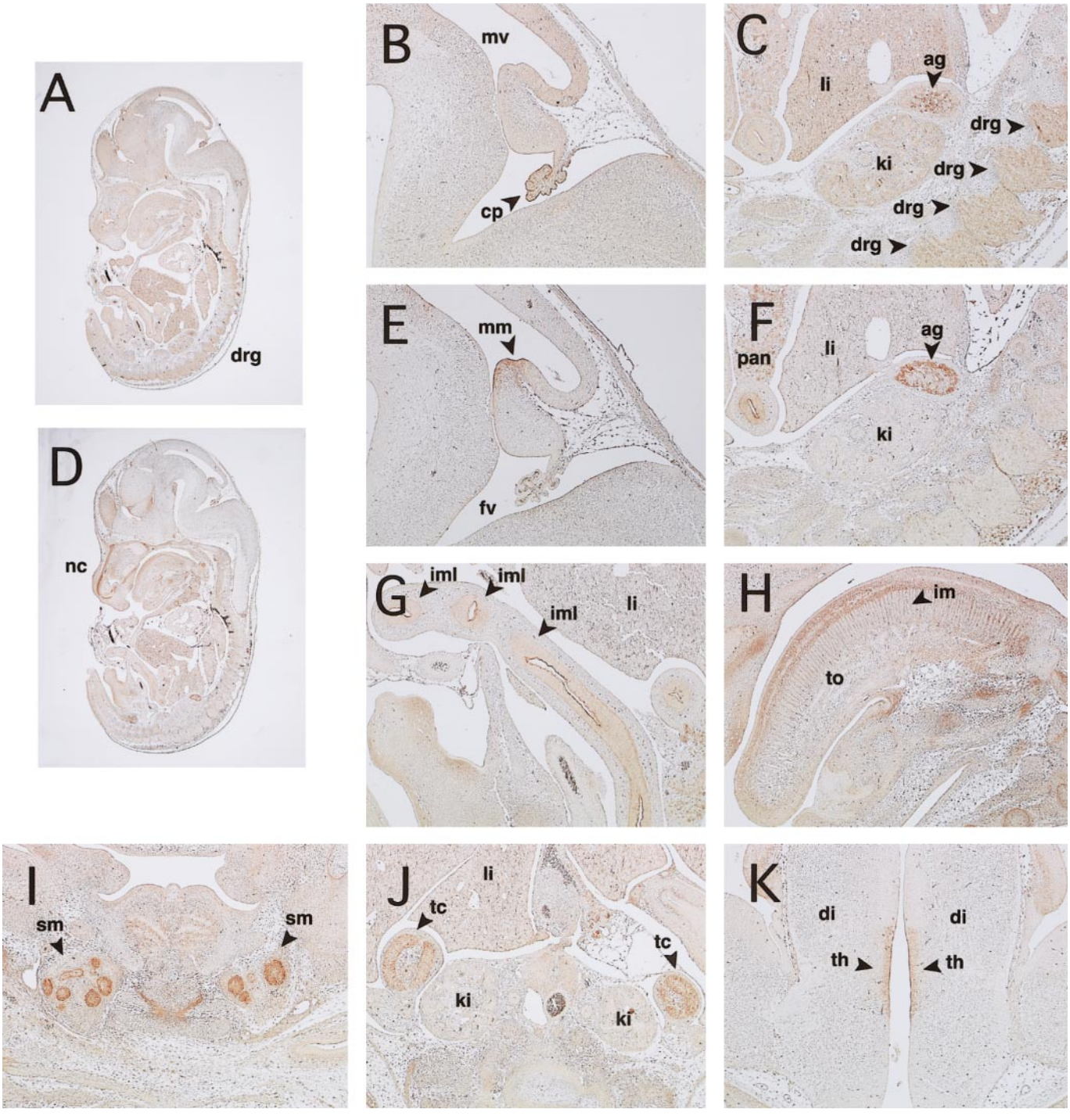

FIG. 4. Expression of Dvl1 and EPS8 proteins in E13.5 mouse embryo. (A–C) Immunostaining of Dvl1 in sagittal section. Low magnification (A) and high magnification (B, C) of the section. (D–K) Immunostaining of EPS8 in sagittal and frontal sections. Low magnification (D) and high magnification (E–H) of the sagittal section. Frontal sections (I–K) are also stained with anti-EPS8 mAb. Recognizable expression is indicated by the arrowheads. Abbreviations: drg, dorsal root ganglion; mv, mesencephalic vesicle; cp, choroid plexus; ag, adrenal gland; ki, kidney; li, liver; nc, nasal cartilage; mm, mes/metencephalic border; fv, fourth ventricle; pan, pancreas; iml, inner mucosal layer of the gut; to, tongue; im, intrinsic muscle of the tongue (vertical); sm, submandibular gland; tc, testicular cord; di, diencephalon; th, ependymal layer of the diencephalon (thalamus).

signaling (9, 19). We found that the phosphorylation of Dvl1 is enhanced by EPS8, when both Dvl1 and FLAG-EPS8 are overexpressed in 293T cells. Yanagawa *et al.* (17) proposed that the hyperphosphorylation of Dsh is essential for Wg signaling. EPS8, which associates with the PDZ domain of Dvl1, may collaborate with

Dvl1 to stabilize β-catenin in Wnt signaling. Coincide with this possibility, EGF leads to suppression of GSK-3 activity as does Wg (24).

Dvl1 inhibits the tyrosine phosphorylation of EPS8 by activated EGFR, suggesting that Dvl1 affects RTK signaling. It is difficult to address the consequences of the inhibition of the tyrosine phosphorylation of EPS8 because the downstream effecters of EPS8 have not yet been identified. Wnt cooperates with FGFR signal to regulate neural cell fate (25–27) and has the potential to promote proliferation (28). These results suggest that Dvl1/EPS8 interaction may affects both proliferation and differentiation.

The expression patterns of *dvl1* and *eps8* during mouse development were previously investigated using *in situ* hybridization. *Dvl1* is broadly expressed and its expression is generally higher in the CNS (11). *Dvl1* expression appears in sections prepared from stages E8.5 to E16.5, demonstrating a range from early somitogenesis through late organogenesis. Furthermore, the embryonic stem cells produce Dvl1 mRNA, indicating the continuous expression of *dvl1* throughout the entire period of embryogenesis. On the other hand, EPS8 mRNA is expressed in tissues and organs in a relatively restricted manner (23). EPS8 mRNA is evident from around E10.5, and increases in certain regions when organogenesis begins. Thus, Dvl1/EPS8 interaction may occur during organogenesis. In E13.5 mouse embryos, the expression patterns of Dvl1 and EPS8 protein are almost identical to the expression patterns of them at the mRNA level, respectively. It should be mentioned that EPS8 expression in the CNS is specific to a few areas. This finding suggests that EPS8 may be essential for the formation of specific structures derived from these regions. Among them, EPS8 expression in the mes/metencephalic border is particularly intriguing. This region acts as an organizer that affects the fate of surrounding cells in ways that prepare for new structures, such as the cerebellum. The secretory factors, Wnt1 and FGF8, are abundantly expressed in this region, and may be critical signaling molecules (26). Furthermore, it is indicated that EPS8 may be a substrate for several RTKs, including FGFR (22). Thus, Dvl1/EPS8 interaction in the mes/metencephalic border region may be important for controlling cellular organization during development.

We have identified EPS8 as a molecule that associates with Dvl1 through a PDZ domain. This finding provides not only clues to how Dsh/Dvl participates in signaling pathways but also the molecular basis of the combinatorial effects between Dsh/Dvl and RTK signals. The mutual action between Dvl1 and EPS8 observed in our transfection studies are noteworthy. Clearly, the function of EPS8 is not yet established, but the investigation of molecules associated with Dsh/Dvl will result in a better understanding of its importance.

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