Regulation of Phosphatidylinositol Kinases and Metabolism by Wnt3a and Dvl

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Wnt signaling plays important roles in various physiological and pathophysiological processes. The pathway that leads to β-catenin stabilization is initiated by Wnt binding to its cell surface receptors, which induces the formation of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) via activation of phosphatidylinositol 4-phosphate 5-kinase (PIP5K) type I. Here, we show that Wnt also stimulated the production of phosphatidylinositol 4-phosphate (PtdIns(4)P), which depended on Frizzled (Fz), Dishevelled (Dvl), and phosphatidylinositol 4-kinase (PI4K) type IIα in HEK293T cells. Dvl directly interacted with and activated PI4KIIα by increasing its Vmax for ATP and PtdIns. In addition, Dvl regulated PI4KIIε and PIP5KI via different domains. Moreover, Dvl, PI4KIIε, and PIP5KI appeared to form a ternary complex upon Wnt3a stimulation. This complex may allow efficient production of PtdIns(4,5)P2 from PtdIns, which is far more abundant than PtdIns(4)P in cells. Therefore, this study provides new insights into the mechanism by which Wnt3a regulates the production of PtdIns(4,5)P2.

The Wnt family of secretory glycoproteins plays important roles in regulation of embryonic development and tumorigenesis. They also regulate many other physiological and pathophysiological processes, including bone development, neurogenesis, adipogenesis, myogenesis, organogenesis, and lipid and glucose metabolism (1–5). Studies using Drosophila and Xenopus embryos as well as mammalian cells have established a canonical Wnt signaling pathway that leads to stabilization of β-catenin. In the absence of Wnt, a number of proteins, including Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1), glycogen synthase kinase-3β (GSK3β), form a complex that facilitates β-catenin phosphorylation by CK1 and GSK3β. This phosphorylation targets β-catenin for ubiquitination and proteasome-mediated proteolytic degradation (3, 6). Some of the Wnt proteins bind to two cell surface receptors Fz and low density lipoprotein receptor-related protein (LRP) 5/6 and initiate a signaling cascade that eventually leads to the suppression of β-catenin phosphorylation by GSK3β and stabilization of β-catenin.

Because the finding that the canonical Wnt proteins transduce signals by inducing the interaction between LRP5/6 and Axin (7), more has been learned about the mechanisms by which this interaction is regulated by Wnt proteins. Studies have indicated that two phosphorylation events at the C-terminal intracellular domain of LRP5/6, the phosphorylation of Thr1479 by CK1γ (8, 9) and of Ser1490 by GSK3 (10, 11), were required for the interaction. We recently showed that Wnt3a stimulated the production of PtdIns(4,5)P2, which in turn regulated the phosphorylation of LRP5/6 at Thr1479 and Ser1490 (12). We also showed that Wnt3a regulated phosphatidylinositol 4-phosphate 5-kinase type I (PIP5KI) activity by inducing the interaction between Dvl and PIP5KI (12). Moreover, Dvl could directly stimulate the lipid kinase activity of PIP5KI (12).

PtdIns(4,5)P2 plays important roles in various cellular functions, including membrane trafficking, cytoskeletal reorganization, migration, ion channel activation, and signal transduction (13). It, however, represents less than 1% of plasma membrane phospholipids and is primarily synthesized in most cells by sequential phosphorylation of PtdIns on the D4 and D5 positions of the inositol ring by two PtdIns kinases, PI4K and PIP5KI, respectively (14, 15). While PtdIns(4)P, the substrate for PIP5KI, is also accounted for around 1% of plasma membrane phospholipids, PtdIns, the substrate for PI4K, is very abundant. Thus, Wnt3a may have to stimulate PI4K activity to provide enough substrate for PIP5KI in PtdIns(4,5)P2 production.

Two types of PI4K (PI4K and PI4KII) have been characterized in mammalian cells. There are two isoforms of PI4KII (PI4KIIα and PI4KIIβ) and two isoforms of PI4K (PI4Kα and PI4Kβ) (16). In our previous study, we demonstrated the involvement of PI4KIIα in Wnt signaling. siRNA-mediated knockdown in mammalian cells and morpholino-mediated suppression in Xenopus embryos of PI4KIIα inhibited LRP6 phosphorylation and Wnt signaling. In this report, we examined whether Wnt3a regulates the lipid kinase activity of PI4KIIα and found that Wnt3a could induce an increase in the level of PtdIns(4)P in a Dvl- and Fz-dependent manner. In addition, the Dvl protein was found to directly interact with and activate PI4KIIα. Moreover, different domains of Dvl appeared to be involved in the regulation of PI4KIIα and PIP5KI, and Wnt3a induced the formation of a complex of Dvl, PI4KIIα, and PIP5KI possibly for more efficient production of PtdIns (4,5)P2 in cells.
MATERIALS AND METHODS

Constructs, siRNAs, Ligands, Chemicals, and Antibodies—Human PI4KIIα and PI5Kβ cDNAs were subcloned into CMV promoter-based mammalian cell expression vector and confirmed by DNA sequencing. Expression plasmids for Dvl and its mutants have previously been described (17). siRNAs were designed using the siRNA Design Program (Dharmacon) and synthesized by Applied Biosystems. The control siRNA was provided by Ambion. The sequences of all the siRNAs have been described (12). Recombinant purified Wnt3a proteins were purchased from R&D Systems.

Mouse anti-Dvl3 antibodies were kindly provided by D. Sussman. Rabbit anti-PI4KIIα antibody has previously been described (18). Rabbit anti-PI5Kβ (Abgent), mouse anti-Flag, mouse anti-HA, mouse anti-Myc, and mouse anti-His (Covance) antibodies were acquired commercially.

Cell Culture and Transfection—HEK293T cells were maintained in Dulbecco’s modified Eagle’s medium in the presence of 10% fetal bovine serum and transfected with DNA using Lipofectamine Plus or with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The LacZ plasmid was added to make the total amount of DNA (0.25 µg/well) in a 24-well plate. For transfection of siRNAs, cells were transfected with 10 pmol/well siRNAs, the total amounts remained same when siRNA mixtures were used.

Lipid Extraction and PtdIns(4)P Content Determination by ELISA—Lipid extraction was carried out as previously described (19). Briefly, cells were washed once with ice-cold PBS and lysed in 3.75 volumes of methanol/chloroform/HCl (40:20:1) mixture on ice, followed with 1 volume of chloroform and 2.25 volumes of water. After vortexing for 1 min, samples were centrifuged at 3000 rpm for 2 min at 4 °C, and the lower organic phase was collected and dried under a nitrogen stream.

The PtdIns(4)P ELISA was carried out as previously described with some modifications (19). Lipid extracts were dissolved directly in ethanol at room temperature, loaded into a microplate, and dried under vacuum. The microplate was incubated with 2% bovine serum albumin in PBS at room temperature for 30 min, followed by a mouse anti-PtdIns(4)P IgM antibody (Echelon Biosciences Inc.) for 1 h and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) for 25 min. The microplate was washed three times with PBS and added with chemiluminescence substrate (SuperSignal West Pico, Pierce). The luminescence intensity was determined by a luminometer.

Immunoprecipitation, Protein Purification, in Vitro Pull-down Assay, and PI4KIIα Kinase Assay—For overexpression assays, HEK293T cells were transiently transfected with the indicated constructs for 24 h. Immunoprecipitation was carried out as described previously (17). Preparation of purified recombinant His-PI4KIIα, GST-PI4KIIα-K(N-terminal 92 amino acid truncation), GST-mDvl1, His-mDvl1, His-hDvl3, GST-DIX, GST-PDZ, GST-DEP, and GST-PI5Kβ proteins was carried out following the procedures described previously (12, 20, 21).

For the in vitro pull-down assay, recombinant proteins were incubated in 250 µl of binding buffer (1× PBS, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 10% glycerol, 1 mM phenylmethyl-
sulfonyl fluoride) for 3 h at 4 °C. After the proteins were captured by glutathione beads, the pull-down complexes were analyzed by Western blotting.

For the kinase assay, proteins were incubated in the kinase buffer (30 mM Heps, pH 7.4, 100 mM NaCl, 2 mM MgCl2, 1 mM EDTA, and 0.2% Triton X-100) at room temperature for 2 h. The samples were then incubated with 10 µCi of [32P]ATP, 50 µM cold ATP, 20 µg of PtdIns (Echelon Biosciences Inc.) vesicle for 15 min at 37 °C in a final volume of 50 µl. The reaction was stopped by adding the lipid extraction solution as described above. Lipids were separated by TLC, visualized, and quantified by a phosphoimager.

RESULTS AND DISCUSSION

Wnt3a Increases PtdIns(4)P Content in HEK293T Cells via Fz and Dvl—We previously demonstrated that treatment of HEK293T cells with Wnt3a resulted in an increase in the cellular PtdIns(4,5)P2 content using an ELISA (12). In the present study, we examined whether Wnt3a treatment could change the level of PtdIns(4)P in cells using an ELISA with an antibody specific to PtdIns(4)P (Fig. 1A). Treatment of HEK293T cells with 50 ng/ml Wnt3a increased PtdIns(4)P contents by more than 70% compared with those in mock-treated cells (Fig. 1A).
Wnt3a and Dvl Stimulate PI4KIIα Activity

There are four PtdIns 4-kinases in mammalian cells that catalyze the production of PtdIns(4)P from PtdIns(4,5)P₂ (22). We have previously shown that knocking down of PI4KIIα, but not any of the other three PtdIns 4-kinases, inhibited Wnt3a-induced β-catenin accumulation, LRP6 phosphorylation, and PtdIns(4,5)P₂ formation in HEK293T cells (12), suggesting that PI4KIIα could be involved in Wnt3a-induced PtdIns(4)P accumulation in these cells. To test this hypothesis, we transfected cells with the PI4KIIα siRNA and found that it abolished Wnt3a-induced formation of PtdIns(4)P (Fig. 1A). This result indicates that Wnt3a-induced PtdIns(4)P formation depends on PI4KIIα in these cells.

Because Fz and Dvl are required for Wnt3a-induced accumulation of PtdIns(4,5)P₂ (12), we examined whether they were also involved in PtdIns(4)P formation. In the previous study we have shown that transfection of a mixture of siRNAs targeting Dvl1–3 or Fz2/4/5 was able to inhibit Wnt3a-induced PtdIns(4,5)P₂ formation (12). We tested the same mixtures of the siRNAs and found that they could also inhibit Wnt3a-induced production of PtdIns(4)P (Fig. 1B). This result suggests that Fz and Dvl are also required for Wnt3a-induced PtdIns(4)P formation in these cells.

**Dvl Directly Interacts with and Activates PI4KIIα**—Because both Dvl and PI4KIIα are required for Wnt3a-induced PtdIns(4)P formation, we tested whether Dvl may interact with and activate PI4KIIα as it did with PIP5K1 (12). We first tested whether Dvl could interact with PI4KIIα using communoprecipitation in HEK293T expressing Flag-Dvl1 and HA-PI4KIIα and found that these two proteins communoprecipitated (Fig. 2, A and B). To determine whether PI4KIIα could directly interact with Dvl, we carried out a pull-down assay using recombinant proteins prepared in *Escherichia coli.,* and found that GST-tagged Dvl1, but not GST, interacted with His-tagged PI4KIIα (Fig. 2C). It seems that N-terminal 92 amino acids of PI4KIIα are not required for the interaction (Fig. 2D).

Knowing that purified recombinant Dvl3 protein could stimulate the PIP5K1 lipid kinase activity (12), we tested whether recombinant Dvl3 protein could also stimulate the lipid kinase activity of recombinant PI4KIIα prepared from *E. coli.* In an in vitro kinase assay, we found that recombinant Dvl3 protein was able to stimulate PI4KIIα activity by ~2.5 fold (Fig. 3A), and recombinant Dvl1 could also stimulate its activity (data not shown). We further characterized the activation of PI4KIIα by Dvl3 by determining the *Kₘ* and *Vₘₐₓ* values of PI4KIIα for its substrates, PtdIns and ATP, in the presence or absence of Dvl3. Using GST protein as a control, Dvl3 increased the *Vₘₐₓ* values for both substrates by at least 2-fold without significantly changing the *Kₘ* values (Fig. 3, B and C and supplemental Fig. S1). These results indicate that Dvl accelerates the rate of phosphorylation reaction, but does not significantly alter the affinities of PI4KIIα for its substrates.

The Dvl proteins are composed of three highly conserved domains: an N-terminal DIX, a PDZ, and a C-terminal DEP domain (Fig. 4A). Communoprecipitation experiments indicated that both PDZ and DEP domains could interact with PI4KIIα (Fig. 4B), and these interactions were direct because purified recombinant proteins interacted in *in vitro* pull-down assays (Fig. 4C). Although the PDZ domain appeared to have a higher affinity than the DEP domain for PI4KIIα (Fig. 4, B and C), the DEP protein, but not PDZ protein, stimulated the lipid kinase activity of PI4KIIα in an *in vitro* kinase assay (Fig. 4D). A titration experiment revealed that close to 8 times more DEP protein than the full-length Dvl protein was needed to achieve the same level of PI4KIIα activation as the full length (Fig. 4E). We interpret these results to suggest that the interaction between DEP and PI4KIIα is involved in activating the kinase, whereas the PDZ and PI4KIIα interaction may contribute to the affinity of Dvl for PI4KIIα.

**Wnt Promotes the Formation of a Dvl, PI4KIIα, and PIP5K1β Complex**—Although Dvl DEP appears to be responsible for PI4KIIα activation, it had little effect on activation of PIP5K1β (Fig. 4F). The N-terminal DIX domain seems to be primarily responsible for activation of PIP5K1β (Fig. 4G). While Dvl PDZ was able to bind to both PI4KIIα and PIP5K1β (12), PI4KIIα and PIP5K1β did not appear to compete for Dvl binding. In a communoprecipitation experiment, more rather than less PI4KIIα

![Image](image_url)
and PIP5KIβ proteins were pulled down with Dvl in cells coexpressing Dvl, PIP5KIβ, and PI4KIIα than those coexpressing Dvl and PI4KIIα or PIP5KIβ (Fig. 5A). One possible explanation to the enhanced interactions is that PI4KIIα may interact with PIP5KIβ. When these two proteins were expressed in HEK293T cells, they coimmunoprecipitated (Fig. 5B and supplemental Fig. S2A). The interaction between these two proteins appeared to be direct, because purified recombinant proteins of PI4KIIα and PIP5KIβ interacted in an in vitro pulldown assay (supplemental Fig. S2, B and C). Consistent with the non-competitive nature of these interactions, expression of Dvl did not reduce, but rather increased, the amount of PI4KIIα pulled down by PIP5KIβ (supplemental Fig. S2D).

Next, we wanted to know how Wnt3a stimulation regulates these interactions. To do this, we needed to perform the interactions using proteins that are expressed at near endogenous levels. Antibodies specific for PI4KIIα, PIP5KIβ, and Dvl were not suitable for immunoprecipitation. To circumvent this difficulty, we established a HEK293 cell line that stably expressed Dvl and PI4KIIα carrying seven HA tags at a level that is lower than that of endogenous PI4KIIα (compare the upper bands with the lower bands in the second left panel of Fig. 5C). The seven HA tags markedly increases immunoprecipitation efficiency. In the immunocomplexes pulled down by anti-HA antibodies, we detected a low level of PIP5KIβ, but not Dvl (Fig. 5C). However, when the cells were treated with Wnt3a, Dvl3 was readily detected (Fig. 5C). In addition, an increased amount of PIP5KIβ was detected (Fig. 5C). All of these results together indicate that Wnt3a is able to induce the binding of Dvl to the PIP5KIβ and PI4KIIα complex, leading to more stable interaction between PIP5KIβ and PI4KIIα.

In this report, we describe our findings that Wnt3a stimulates the production of PtdIns(4)P via one of the PI4Ks, namely PI4KIIα in HEK293 cells. It is likely that the same PI4K is also regulated by Wnt in Xenopus embryos because its suppression by morpholino oligos inhibited Wnt signaling in the embryos (12). In overexpression systems, we found that Dvl could also interact with PIP5KIβ (data not shown). In our previous studies, we presented evidence to indicate that Dvl regulates all of the three PIP5K isoforms even though PIP5KIβ appeared to be the major isoform in HEK293T cells (12). The lack of significant effects of siRNAs to other PIP5KI or PI4K isoforms on Wnt signaling in these cells may be due to their expression levels or subcellular localization. In HEK293 cells, Wnt3a appears to regulate PI4KIIα via Fz and Dvl, probably through its activation by Dvl. It is intriguing and logical for Dvl to directly stimulate both PI4K and PIP5K, both of which constitute the major pathway for PtdIns(4,5)P2 synthesis in cells. Given that PtdIns(4)P

![FIGURE 3. Regulation of PI4KIIα lipid kinase activity by Dvl protein.](image)

![FIGURE 4. Characterization of Dvl domains in interaction and activation of PI4KIIα.](image)
Wnt3a and Dvl Stimulate PI4KIIα Activity

![Diagram of Wnt3a and Dvl Stimulate PI4KIIα Activity](image)

**A** Transfection
- 4KIIα-HA: + + - +
- 5KIIβ-HA: + + - +
- Dvl-Flag: - + - +

**B** Transfection
- 4KIIα-HA: - + - +
- ΔN-β-catenin-HA: - + - +
- 5KIIβ-Flag: - + - +

**C** Input
- IP: anti-HA

**D** IP: anti-Dvl

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