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Axin and Frat1 interact with Dvl and GSK, bridging Dvl to GSK in Wnt-mediated regulation of LEF-1

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Wnt proteins transduce their signals through dishevelled (Dvl) proteins to inhibit glycogen synthase kinase 3β (GSK), leading to the accumulation of cytosolic β-catenin and activation of TCF/LEF-1 transcription factors. To understand the mechanism by which Dvl acts through GSK to regulate LEF-1, we investigated the roles of Axin and Frat1 in Wnt-mediated activation of LEF-1 in mammalian cells. We found that Dvl interacts with Axin and with Frat1, both of which interact with GSK. Similarly, the Frat1 homolog GBP binds *Xenopus* Dishevelled in an interaction that requires GSK. We also found that Dvl, Axin and GSK can form a ternary complex bridged by Axin, and that Frat1 can be recruited into this complex probably by Dvl. The observation that the Dvl-binding domain of either Frat1 or Axin was able to inhibit Wnt-1-induced LEF-1 activation suggests that the interactions between Dvl and Axin and between Dvl and Frat may be important for this signaling pathway. Furthermore, Wnt-1 appeared to promote the disintegration of the Frat1–Dvl–GSK–Axin complex, resulting in the dissociation of GSK from Axin. Thus, formation of the quaternary complex may be an important step in Wnt signaling, by which Dvl recruits Frat1, leading to Frat1-mediated dissociation of GSK from Axin.
Keywords: Axin/dishevelled/Frat1/GSK/Wnt signalling

Introduction

The Wnt family of secretory glycoproteins plays an important role in embryonic induction, generation of cell polarity and specification of cell fate (see reviews by Dickinson and McMahon, 1992; Nusse and Varmus, 1992; Klingensmith and Nusse, 1994; Perrimon, 1994; Cadigan and Nusse, 1997; Dale, 1998; Gumbiner, 1998). Genetic studies in *Drosophila* revealed the involvement of *Dishevelled* (Dsh), *Zeste-white-3* (Zw-3), *Armadillo* (Arm) and *Pangolin* (Pan) in *Wingless* (Wg) signaling. The genetic order of these signal transducers has been established, in which Wg acts through Dsh to inhibit Zw-3, thus relieving the repression of Arm by Zw-3, resulting in the up-regulation of Arm (Dickinson and McMahon, 1992; Nusse and Varmus, 1992; Klingensmith and Nusse, 1994; Perrimon, 1994; Cadigan and Nusse, 1997; Dale, 1998). The Wnt signaling pathway appears to be largely conserved in mammals. In addition to the existence of a large number of Wg homologs, there are mammalian homologs for Dsh, Zw-3, Arm and Pan.

Molecular cloning has revealed several mammalian Dsh homologs (Dvl), including three from the mouse (Sussman *et al*., 1994; Klingensmith *et al*., 1996; Tsang *et al*., 1996). 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 (Ponting *et al*., 1997). Studies have shown that the PDZ domain in PSD-95 can bind to a C-terminal motif of four amino acids (X-Thr/Ser-X-Val) (Doyle *et al*., 1996). However, ligands for the PDZ domain of Dsh/Dvl remain unknown. At the C-terminal side of the PDZ domain is located a DEP (dishevelled, egl-10 and pleckstrin) domain. Similar DEP motifs are also found in a number of other proteins (Ponting and Bork, 1996). The N-terminal conserved domain shares homology with a newly identified protein, Axin, which was shown to antagonize Wnt signaling (Zeng *et al*., 1997; Behrens *et al*., 1998; Ikeda *et al*., 1998; Sakanaka *et al*., 1998; Hamada *et al*., 1999). This N-terminal domain is referred to as the DIX (dishevelled and axin) domain. Dvl as well as its *Drosophila* homolog Dsh recently have been shown to regulate two independent signaling pathways: one leads to LEF-1/TCF-dependent transcription via glycogen synthase kinase 3β (GSK) and β-catenin, and the other to c-Jun-dependent transcription via activation of JNK kinases (Strutt *et al*., 1997; Boutros *et al*., 1998; Li *et al*., 1999).

The mammalian homologs of Zw-3 and Arm are GSK and β-catenin, respectively. GSK was found to form a complex with β-catenin and the product of the adenomatous polyposis coli (APC) gene (Rubinfeld *et al*., 1993, 1996). Recent evidence indicates that Axin and its homologs, Conductin and Axil, may also be part of the complex (Behrens *et al*., 1998; Ikeda *et al*., 1998; Sakanaka *et al*., 1998; Yamamoto *et al*., 1998). GSK was shown to phosphorylate APC and β-catenin, which is thought to result in the destabilization of β-catenin via ubiquitination pathways (Rubinfeld *et al*., 1996; Aberle *et al*., 1997; Cadigan and Nusse, 1997; Behrens *et al*., 1998). APC was identified originally as a tumor suppressor gene (Polakis, 1997), and mutations in APC that are associated

with human colorectal cancers appear to lose the ability to destabilize β-catenin (Rubinfeld *et al*., 1996; Gumbiner, 1997). β-Catenin has been shown to bind HMG box transcription factors of the TCF/LEF-1 family (Huber *et al*., 1996). Complexes between β-catenin and TCF/LEF-1 have strong transcriptional activity on reporter gene constructs containing the TCF/LEF-1 recognition sequences (Molenaar *et al*., 1996; Brannon *et al*., 1997; Korinek *et al*., 1997; Morin *et al*., 1997; Li *et al*., 1999).

Despite great interest and significant recent advances, major gaps remain in our understanding of the pathway. Among these gaps is the molecular mechanism by which Dsh/Dvl is involved in signal transduction of Wnt. By using co-transfection, co-immunoprecipitation and a LEF-1-dependent transcription assay, we present evidence for the involvement of both Axin and Frat1, a GSK-binding protein (Yost *et al*., 1998), in Wnt-1-mediated LEF-1 activation.

Results

Axin DIX domain inhibits Wnt-1 signaling

Axin has been shown to interact with the Wnt signaling components GSK, APC and β-catenin. It was proposed that Axin may function as a scaffold protein and that the binding of GSK and β-catenin to Axin may facilitate the phosphorylation of β-catenin by GSK (Ikeda *et al*., 1998). The phosphorylation is thought to contribute to the destabilization of β-catenin (Yost *et al*., 1996). To investigate the effects of Axin and its various domains on Wnt-1-induced activation of LEF-1, we co-expressed Axin or its mutants with Wnt-1 and LEF-1 reporter plasmids in NIH 3T3 cells. Wnt-1, when co-expressed with LEF-1 in NIH 3T3 cells, led to marked increases in transcription of the luciferase reporter gene under the control of a LEF-1 regulatory sequence (Figure 1A) as previously reported (Hsu *et al*., 1998; Sakanaka *et al*., 1998; Li *et al*., 1999). These results suggest that Wnt-1 is able to activate LEF-1 through NIH 3T3 cell endogenous signaling components. The expression of Axin inhibited Wnt-1-stimulated luciferase activity (Figure 1A), which is consistent with previous findings that Axin antagonizes Wnt-1's effects (Zeng *et al*., 1997; Sakanaka *et al*., 1998). Two Axin mutants were generated to assess the roles of specific Axin domains in inhibiting Wnt-1 action (Figure 1C). The mutants are AxinC, containing the C-terminal DIX domain and AxinN, a deletion of the DIX domain retaining the APC, GSK and β-catenin-binding domains. As predicted based on previous findings by Ikeda *et al*. (1998), AxinN inhibited Wnt-1-mediated LEF-1 activation (Figure 1A). However, AxinC was also able to inhibit Wnt-1-induced increases in luciferase activity in co-transfected 3T3 cells (Figure 1A). Similar inhibitory effects of AxinC on Wnt-1-induced LEF-1 activation were also observed in COS-7 and HEK cells (data not shown).

To confirm that AxinC blocked the activation of the reporter system by secreted Wnt proteins, a paracrine paradigm was used where the Wnt-1 cDNA and LEF-1 reporter gene were transfected into two separate groups of COS-7 cells. These two groups of cells were combined after transfection, and luciferase activities were determined after the cells were co-cultured for 24 h. Since Wnt-1 and the reporter gene are produced in different cells, the effect of Wnt-1 can be attributed only to the interaction of secreted Wnt-1 proteins with cell surface receptors on cells containing the reporter gene. The effects of Axin and its two mutants were tested in the paracrine paradigm. When Axin or its mutants were co-transfected with the LEF reporter plasmid, they were able to inhibit the effect of Wnt-1 produced by separate cells (Figure 1B). This result confirms that AxinC as well as Axin and AxinN can inhibit the effect of secreted Wnt.

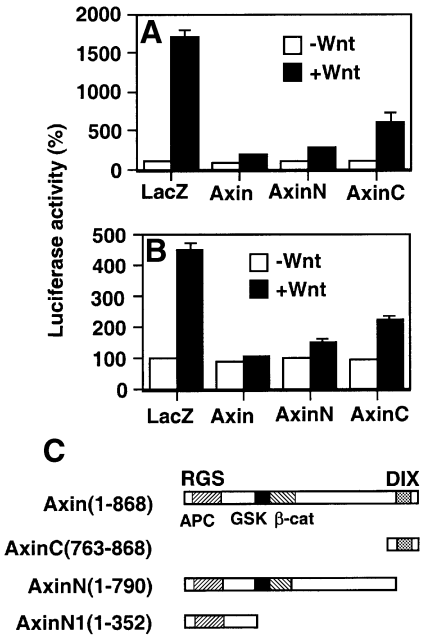

Fig. 1. Effects of Axin on Wnt-induced LEF-1-dependent transcription. (**A**) Effects on co-expressed Wnt-1. Cells in 24-well plates were transfected with 0.025 μg of LEF-1 expression plasmid, 0.075 μg of LEF-1 luciferase reporter plasmid, 0.15 μg of GFP expression plasmid and 0.15 μg of LacZ, Axin, AxinC or AxinN in the presence or absence of 0.1 μg of Wnt-1. LacZ plasmid was added to make the total amount of DNA equal (0.5 μg/transfection). After 1 day, cells were lysed, and GFP levels and luciferase activities were determined. The luciferase activities presented are normalized against the levels of GFP expression. Each experiment was carried out in triplicate, and error bars represent the standard deviation. (**B**) Effects in a paracrine paradigm. Cells in plate A were transfected with 0.1 μg of LEF-1 expression plasmid, 0.15 μg of LEF reporter plasmid or 0.1 μg of GFP expression plasmid, along with 0.15 μg of LacZ, Axin, AxinC or AxinN, while cells in plate B were transfected with 0.5 μg of LacZ or Wnt-1. At 3 h post-transfection, cells from both plates were trypsinized. An equal number of cells from plate A were mixed with cells from plate B, and reseeded into a new 24-well plate. Luciferase activity was determined after 24 h co-culture. (**C**) Schematic representation of Axin and its mutants. Binding sites on Axin for APC, GSK and β-catenin are shown.

Axin binds to Dvl

The inhibitory effect of AxinC suggests that it may interact with proteins that are involved in Wnt-1 signaling. Since the binding sites for β-catenin, GSK and APC are not included in AxinC (Ikeda *et al*., 1998; Kishida *et al*., 1998; Yamamoto *et al*., 1998), we tested whether AxinC interacts with Dvl using an immunoprecipitation approach. COS-7 cells were co-transfected with cDNAs encoding hemagglutinin (HA)-tagged AxinC and Flag-tagged Dvl. The next day, Dvl-Flag was immunoprecipitated with an anti-Flag antibody, and the immunocomplexes were detected by Western blot analysis using an anti-HA anti-

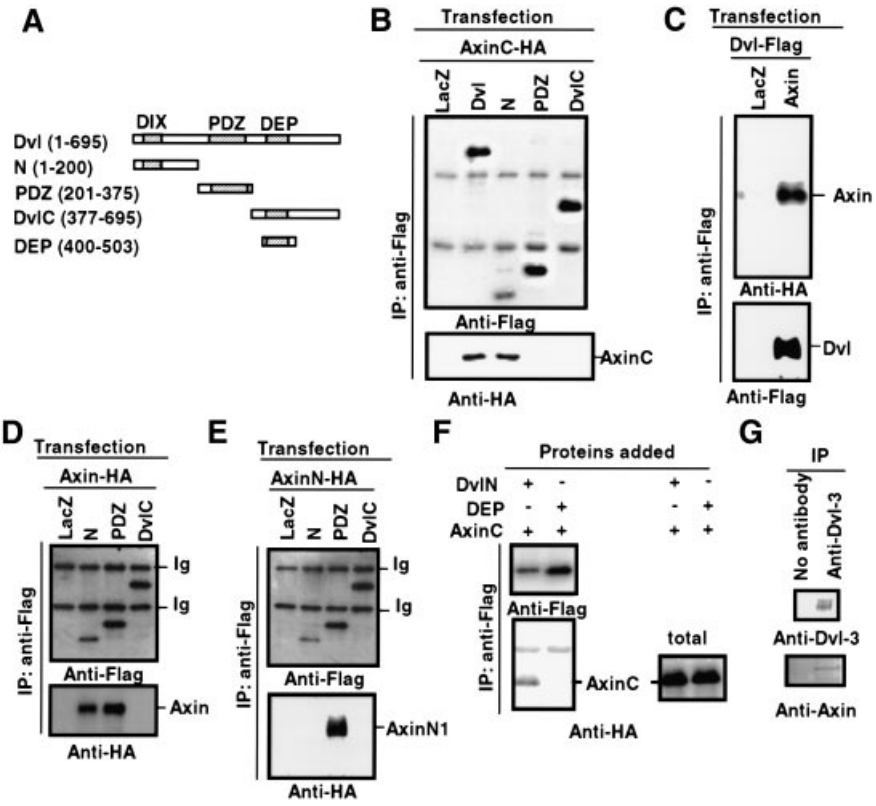

Fig. 2. Interaction of Dvl with Axin. (**A**) Schematic representation of Dvl and its mutants. Domains that are highly conserved among Dsh/Dvl molecules from different species are shaded. (B–E) Interactions between Dvl and Axin. COS-7 cells in 6-well plates were co-transfected with the expression plasmid (0.5 μg) LacZ, Dvl-Flag, DvlN-Flag (N), DvlPDZ-Flag (PDZ) or DvlC-Flag (DvlC) together with that (0.5 μg) of AxinC-HA (**B**), Axin-HA (**D**) or AxinN1-HA (**E**). Cells in (**C**) were transfected with the expression plasmid (0.5 μg) of Axin-HA or LacZ and that (0.5 μg) of Dvl-Flag. One day after transfection, cells were lysed, and Flag-tagged proteins (B, D and E) were immunoprecipitated with the anti-Flag antibody conjugated on agarose beads (IBI, CT). HA-tagged proteins in (C) were precipitated with the anti-HA antibody followed by protein A/G beads. (**F**) Interaction between recombinant DvlN (1–105) and AxinC. Recombinant DvlN-Flag, DvlDEP-Flag and AxinC-HA were purified from *E.coli*. AxinC was incubated with DvlDEP and DvlN (1–105), respectively. DvlDEP and DvlN were immunoprecipitated with anti-Flag antibodies, and the immunocomplexes were detected with the anti-HA antibodies. (**G**) Interaction between endogenous Dvl-3 and Axin. Cells were lysed and immunoprecipitated using the monoclonal anti-Dvl-3 antibody. The immunocomplexes were detected by Western blot using a polyclonal anti-Axin antibody.

body. AxinC was detected in the immunocomplexes (Figure 2B), demonstrating that AxinC binds to Dvl. To determine to which portions of the Dvl molecule AxinC binds, AxinC-HA was co-transfected with a number of Dvl mutants—DvlN-Flag, DvlPDZ-Flag or DvlC-Flag (Figure 2A). Only DvlN-Flag was co-immunoprecipitated with AxinC-HA (Figure 2B), indicating that DvlN, which contains a DIX domain, interacts with AxinC. The interaction between AxinC and DvlN was also confirmed using purified recombinant proteins from *Escherichia coli* (Figure 2F). Since there is no Wnt signaling pathway in *E.coli*, the interaction between AxinC and DvlN should be direct.

The interaction between AxinC and Dvl suggests that full-length Axin may also bind to Dvl. As shown in Figure 2C, Dvl can interact with Axin in the co-immunoprecipitation experiment. The interactions between endogenous Dvl and Axin proteins were also detected; Axin was detected in immunocomplexes pulled down by the anti-Dvl-3 monoclonal antibody (Figure 2G). To test whether Axin binds only to DvlN, Axin was co-expressed with DvlPDZ or DvlC. Interestingly, Axin binds not only to DvlN but also to DvlPDZ (Figure 2D). Axin does not bind to DvlC (Figure 2D). We also found that the Axin mutants, AxinN (data not shown) and AxinN1 (Figure 2A), could be co-immunoprecipitated with DvlPDZ but not with DvlC or DvlN (Figure 2E). All of these results indicate that the binding of Axin to Dvl is probably mediated by two interactions; one between DvlN and AxinC and the other between DvlPDZ and an N-terminal sequence of Axin.

Dvl, GSK and Axin form a complex

Axin has been shown to bind to GSK (Behrens *et al*., 1998; Ikeda *et al*., 1998; Sakanaka *et al*., 1998). Because Axin sequences involved in interactions with GSK and Dvl do not overlap (Figures 1C and 2), it is possible that Axin, Dvl and GSK form a ternary complex. When Myc-tagged GSK was co-expressed with Dvl-HA, GSK and Dvl were not co-immunoprecipitated (Figure 3), suggesting that GSK does not interact directly with Dvl. However, co-expression of Axin allows GSK and Dvl to be co-immunoprecipitated (Figure 3). Thus, GSK, Dvl and Axin can form a complex, which is probably bridged by Axin. In addition, we also detected endogenous β-catenin in the immunocomplexes pulled down via Dvl. This is probably due to the interaction between Axin and β-catenin (Behrens *et al*., 1998; Ikeda *et al*., 1998; Sakanaka *et al*., 1998).

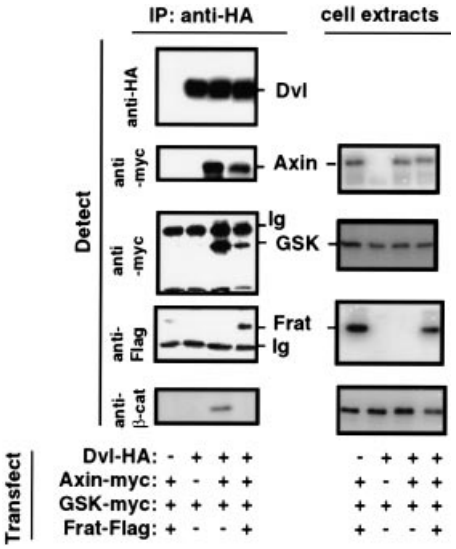

Fig. 3. Dvl, Axin, β-catenin and GSK form a complex. COS-7 cells in 6-well plates were co-transfected with a combination of expression plasmids (0.25 μg) LacZ, Dvl-HA, Axin-Myc, GSK-Myc and Frat1-Flag as indicated in the figures. LacZ plasmid was added to make the total amount of DNA equal (1 μg/well). At 1 day post-transfection, cells were lysed, and immunoprecipitation was carried out with the antibodies indicated in the figure. Equal amounts of cell extracts were saved before the immunoprecipitation and analyzed by Western blotting.

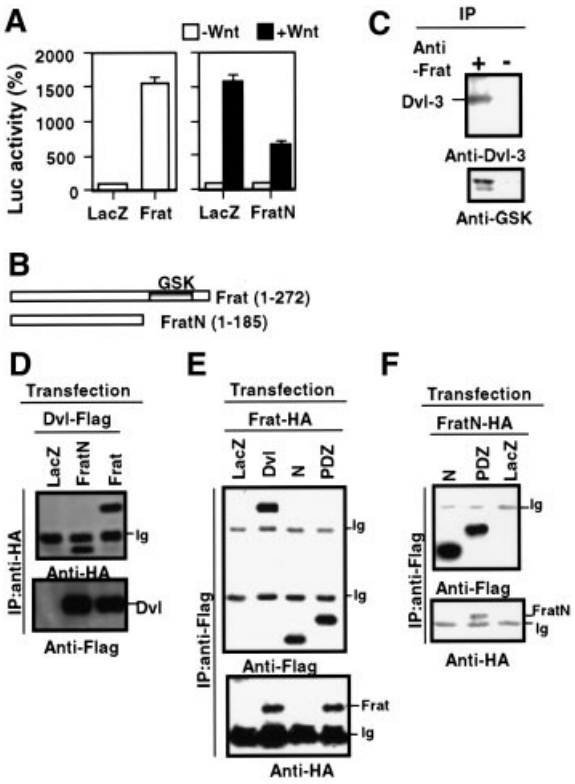

Fig. 4. Involvement of Frat1 in Wnt-1 signaling. (**A**) Effect of Frat1 on Wnt-1-induced LEF-1-dependent transcription. Transfection, luciferase assays, data processing and presentation were as described in Figure 1A, except that Frat1 or FratN was used instead of Axin in the transfection. (**B**) Schematic representation of Frat1 and FratN. The Frat1 GSK-binding site is shown. (**C**) Interaction between endogenous Frat and Dvl-3. Endogenous Frat proteins were immunoprecipitated using a polyclonal antibody raised against mouse Frat1, and the immunocomplex was detected with a monoclonal antibody against Dvl-3 and one against GSK-3β. (**D**–**F**) Frat1 binds to Dvl in COS-7 cells. Cells in 6-well plates were co-transfected with 0.5 μg of LacZ, Dvl-Flag, DvlN-Flag (N) or DvlPDZ-Flag (PDZ) together with Frat1-HA or FratN-HA as indicated. Immunoprecipitation was carried out 1 day after transfection.

Involvement of Frat1 in Wnt signaling

A GSK inhibitor, GBP, was identified recently in *Xenopus* based on its ability to bind to GSK and inhibit GSK-mediated phosphorylation of a protein substrate (Yost *et al*., 1998). The mammalian homolog of GBP, named Frat1, was cloned independently for its tumor-promoting activity in lymphocytes (Jonkers *et al*., 1997). Ectopic overexpression of GBP or the C-terminal GSK-binding domain of GBP or human FRAT2 can mimic Wnt's effects in *Xenopus* (Yost *et al*., 1998). As shown in Figure 4C, we also confirmed that endogenous GSK is bound to endogenous Frat in mammalian cells. In addition, expression of Frat1 stimulated LEF-1-dependent transcriptional activity in COS-7 cells (Figure 4A), indicating that Frat1 can also mimic Wnt's effect in mammalian cells. The observation that the GSK-interacting site deletion mutant of Frat1 (FratN; Figure 4B) could inhibit Wnt-1-mediated LEF-1 activation (Figure 4A) suggests that Frat1 may be a mediator of Wnt-1 signaling.

Frat1 binds to Dvl-1

The dominant-negative effect of FratN implies that FratN may interact with intracellular factors in the Wnt signaling pathway. We investigated whether Frat1 interacts with Dvl and Axin. We found that Frat1 and FratN could be co-immunoprecipitated with Dvl (Figure 4D), but not with Axin (Figure 6; data not shown). Co-immunoprecipitation of Frat1 and Dvl mutants was performed to delineate the Dvl sequences involved in Frat1 binding. DvlPDZ, but not DvlN, was found to bind to Frat1 (Figure 4E) and FratN (Figure 4F). Thus, the N-terminal half of Frat1 interacts with Dvl via binding to the PDZ domain of Dvl. The interaction between FratN and DvlPDZ was also confirmed using recombinant proteins purified from *E.coli* (data not shown). Importantly, we showed that the endogenous GSK and Dvl proteins were bound to the endogenous Frat1 (Figure 4C), demonstrating that these interactions occur even without overexpression.

GBP binds to Xdsh in Xenopus

To confirm the observation that Frat1 and Dvl interact directly and to investigate whether the interaction might play a role in Wnt signaling in an intact vertebrate system, we examined whether the *Xenopus* Frat1 ortholog, GBP, can bind to *Xenopus* Dsh (Xdsh) in embryos using epitope-tagged constructs in immunoprecipitation assays. GBP-Flag immunoprecipitated Xdsh-Myc only in the presence of Xgsk-3-Myc (Figure 5, lanes 1 and 2). To determine if binding of GBP to Xgsk-3 is required for the GBP–Xdsh interaction, a GBP mutant that does not bind Xgsk-3 (Yost *et al*., 1998) was immunoprecipitated after co-expression with Xdsh-Myc and Xgsk-3-Myc. Xdsh-Myc was precipitated by the mutant GBP-Flag in a manner dependent upon Xgsk-3-Myc (Figure 5, lanes 4 and 5), even though no binding of the mutant GBP-Flag and Xgsk-3-Myc was observed (lanes 3 and 5). We conclude that the direct

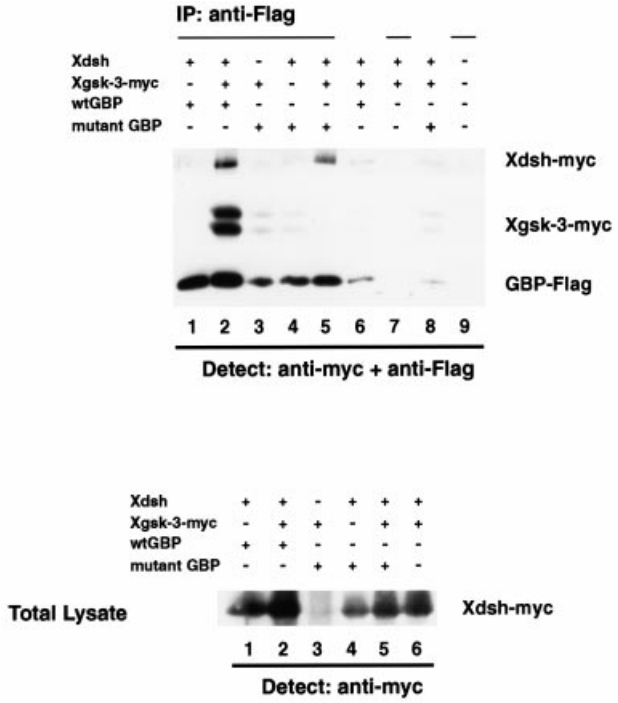

Fig. 5. GBP immunoprecipitates Xdsh in the presence of Xgsk-3. *Xenopus* embryos were injected in the animal pole at the 2–4 cell stage with the indicated RNAs at the following doses: Xdsh, 1 ng; Xgsk-3-Myc, 0.5 ng; wild-type and mutant GBP, 2 ng. Extracts were prepared after 4–5 h, immunoprecipitated with anti-Flag antibody (lanes 1–5 and lanes 7 and 9), and immunocomplexes collected on protein G beads. A portion of the lysates shown in lanes 2 and 5 was precipitated in the absence of antibody as negative controls (lanes 6 and 8, respectively). Xgsk-3 was produced at comparable levels among those samples in which it was injected (not shown).

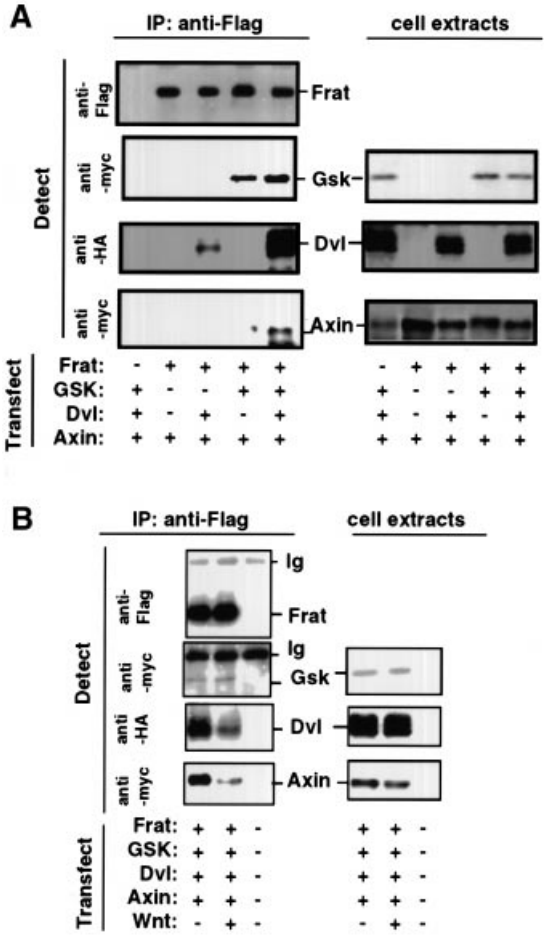

Fig. 6. Frat1 forms a complex with Dvl, Axin and GSK. COS-7 cells in 6-well plates were co-transfected with: (**A**) a combination of LacZ, Dvl-HA, Axin-Myc, GSK-Myc and Frat1-Flag (0.25 μg each); and (**C**) 0.1 μg of Dvl-HA, GSK-Myc and Frat1-Flag, 0.2 μg of Axin-Myc and 0.5 μg of Wnt-1 as indicated. LacZ plasmid was added to make the total amount of DNA equal (1 μg/well). At 1 day post-transfection, immunoprecipitation was carried out with the antibodies indicated in the figure. Equal amounts of cell extracts were saved before the immunoprecipitation and analyzed by Western blotting to demonstrate that similar levels of recombinant proteins were expressed in different transfections. (**B**) The model depicts the interactions in the quaternary complex of Dvl, Axin, GSK and Frat1.

interaction of Dvl and Frat is conserved in *Xenopus* and that this interaction may require a GSK-dependent phosphorylation event.

Dvl, Axin, GSK and Frat1 form a quaternary complex

The inhibition of Wnt-1-mediated LEF-1 activation by Axin C (Figure 1A and B) and FratN (Figure 4A) implies that the interactions between Dvl and Axin and between Dvl and Frat1 may be required for Wnt-1 signaling. A possible model to explain the involvement of these interactions in this signaling pathway is that Dvl, Axin, Frat1 and GSK may form a complex. Although Axin may compete with Frat1 for the Dvl PDZ domain, Axin and Frat1 may still be able simultaneously to bind to Dvl, because Axin can still bind to Dvl via the DIX domain interaction. Additionally, Dvl and GSK bind to different portions of Frat1 (Figure 4), and Dvl, Axin and GSK were shown to form a complex (Figure 3). Therefore, it is theoretically possible that Frat1, Dvl, GSK and Axin form a bigger complex. To test this possibility, we expressed combinations of Axin-Myc, GSK-Myc and Dvl-HA along with Frat1-Flag as shown in Figure 6A. We found that when Frat1-Flag was co-expressed with Axin-HA even in the presence of GSK or Dvl, little Axin was detected in the immunocomplexes precipitated with the anti-Flag antibody (Figure 6A). This indicates that Frat1 has little affinity for Axin and that Frat1 and Axin may not form a ternary complex with Dvl or GSK. We also co-expressed Frat1 with Dvl and GSK, and found that Dvl could not precipitate GSK in the presence of Frat1, or vice versa (data not shown). Thus, unlike Axin, Frat1 could not bridge the formation of a stable complex of GSK–Frat1–Dvl in our assay system. However, when all four proteins were expressed together, Axin was able to be immunoprecipitated by Frat1-Flag, and increased levels of Dvl and GSK proteins were also detected in the immunocomplexes (Figure 6A). These results indicate that Frat1 may form a quaternary complex with GSK, Dvl and Axin. However, when Dvl was used to pull down the complexes, co-expression of Frat1 reduced the levels of the Axin and GSK in the immunocomplexes (Figure 3). We interpret these results to suggest that GSK, Dvl and Axin may form a more stable complex than the quaternary complex that contains Frat1.

Wnt-1 promotes dissociation of the quaternary complex

Having established that Frat1 forms a complex with Dvl–Axin–GSK, we wanted to know the effect of Wnt on the formation of the complex. Wnt-1 was co-expressed with Frat1, Dvl, Axin and GSK in COS-7 cells. As shown in Figure 6B, the expression of Wnt-1 significantly decreased the levels of Axin and Dvl in the immunocomplexes pulled down via Frat1, while the levels of GSK in the immunocomplexes did not seem to change when compared with the absence of Wnt-1. The simplest explanation of this observation is that Wnt may promote the disintegration of the quaternary complex. Because the level of GSK associated with Frat1 remained the same in the absence and presence of Wnt-1 (Figure 6B), GSK maintains its association with Frat1 after the disintegration of the complex.

Discussion

Despite the progress in understanding the protein domains and downstream effects of Dvl in several systems, the biochemical interactions between Dvl and the proteins that regulate and mediate its activity have remained elusive. In this report, we show that Dvl binds to Axin via two interaction sites. The DIX domain of Dvl binds the C-terminus of Axin, which also contains a DIX domain. As the interaction was observed both in cell culture and using recombinant proteins purified from *E.coli*, we conclude that this is likely to be a direct interaction. We also observed an interaction between the Dvl PDZ domain and the N-terminus of Axin, indicating that Dvl can interact with Axin at multiple sites.

We also show here that Frat1 interacts with Dvl by examining both ectopically expressed and endogenous proteins in mammalian cells. The interaction appears to be mediated by the Dvl PDZ domain and the N-terminal portion of Frat1. Similarly, the related *Xenopus* GSK-binding protein GBP binds the *Xenopus* Dvl homolog Xdsh. Frat1 and GBP share three highly conserved domains. The most C-terminal domain interacts with GSK, and we had earlier speculated that the other two domains might interact with additional proteins (Yost *et al*., 1998). Since both the Frat1 mutant FratN lacking the GSK-binding region and a GBP mutant that does not bind GSK bind Dvl/Xdsh, it is likely that one or both of the other two conserved regions are involved in interacting with dishevelled. In *Xenopus*, we have shown that the interaction between GBP and Xdsh requires GSK activity, but does not require GBP binding to GSK, since Xdsh binding was observed with both wild-type GBP and with the GBP mutant that does not bind GSK. In mammalian cells, exogenous GSK did not need to be added in order to observe the Dvl–Frat1 interaction, although addition of exogenous GSK enhanced the interaction (data not shown). While this might reflect species-specific differences, it is also likely that the NIH 3T3 cells have a sufficiently high endogenous level of GSK that allows the detection of the Frat1–Dvl complex.

In this report, we also demonstrate that GSK, Axin and Dvl can form a stable ternary complex, which appears to be bridged by Axin, in the absence of Wnt signaling. Previous studies have shown that Axin binds β-catenin and APC (Behrens *et al*., 1998; Ikeda *et al*., 1998; Sakanaka *et al*., 1998), so we expect that the complex of GSK, Axin and Dvl would normally include APC and β-catenin as well. In the absence of Wnt signaling, this complex results in the phosphorylation of β-catenin by GSK-3 (Yost *et al*., 1996), causing β-catenin to bind βTrCP (Winston *et al*., 1999), and be degraded by the ubiquitin pathway (Aberle *et al*., 1997). While Dvl appears to be part of the complex in the absence of Wnt signaling, Dvl is not likely to be required for β-catenin degradation since experiments in cultured *Drosophila* cells indicate that β-catenin is maintained at a low level in the absence of dishevelled (Yanagawa *et al*., 1995).

A quaternary complex of GSK, Axin, Dvl and Frat1 was also detected by immunoprecipitation in overexpressed mammalian cells. This quaternary complex appears to be less stable than the ternary complex of GSK, Axin and Dvl. The unstable nature of the quaternary complex suggests that it may either represent a transition state or is normally stabilized by other proteins that are not co-expressed in the experiments. In the presence of Wnt signaling, this quaternary complex disassociates, although Frat1 and GSK remain tightly bound to each other. Together with the observation that expression of FratN and AxinC inhibited Wnt signaling, most likely by acting as a dominant-negative mutant that interferes with the interaction of the endogenous Dsh and Axin/Frat1 proteins, this result suggests that the complex is potentially important for Wnt signaling. In studies in *Xenopus*, we have shown that GBP and Axin compete for binding to GSK, and that both proteins can not be bound to GSK at the same time (G.Farr, D.Ferkey, C.Yost, S.Pierce and D.Kimelman, submitted). A similar result was also observed in mammalian cells (data not shown). Thus, these results suggest a role for dishevelled in the Wnt signaling pathway, i.e. recruiting Frat/GBP. In this model, in the absence of Wnt signaling, Axin binds GSK, β-catenin and APC, permitting GSK to phosphorylate β-catenin. Dishevelled binds to this complex by interacting with Axin, but does not appear to have an important role in the absence of Wnt signaling. Dishevelled also binds Frat1/GBP, and this interaction may be stabilized either by other proteins or by Wnt signaling. How exactly Wnt signaling leads to the stabilization of β-catenin remains unclear, although our results indicate that a critical step could be the dissociation of GSK from Axin with the help of Dvl and Frat/GBP. We suggest that post-translational modification of dishevelled (Yanagawa *et al*., 1995) and/or other components in the complex causes a conformational change in the complex that potentiates the interaction between Frat1/GBP and GSK, disrupting the binding of Axin to GSK (G.Farr III *et al*., submitted). With GSK no longer bound to Axin, it can not phosphorylate β-catenin, leading to an elevation in β-catenin levels and the activation of downstream genes. This model suggests that an important role for dishevelled is to recruit Frat1/GBP to the Axin complex where it can interact with GSK upon Wnt signaling. This is not its only role, however, since overexpression of dishevelled leads to an activation of the Wnt pathway, which presumably reflects an additional role for this protein.

How Wnt signaling causes these effects still remains a major question. Inhibition of GSK by PKC has been

suggested to be important (Cook *et al*., 1996), although we have found recently that inhibition of GSK kinase activity by an activated protein kinase, Akt, is not sufficient for activation of LEF-mediated transcription (H.Yuan and D.Wu, unpublished results). However, inhibition of GSK activity, together with additional events, could be critical for the disassembly of the complex. The results presented here elucidate an additional part of the regulatory network that comprises the increasingly important Wnt signaling pathway.

Materials and methods

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% CO_2. For transfection in 24-well plates, cells (5×10^4 cells/well) were seeded the day before transfection. Cells were transfected with 0.25 μg of DNA/well for COS-7 cells and 0.5 μg of DNA/well for NIH 3T3 cells using Lipofectamine Plus (Life Technologies, MD), as suggested by the manufacturer. For transfection in the 6-well plate, the number of cells and amount of DNA are increased in proportion. The transfection was stopped by switching to normal growth medium after 3 h. Cell extracts were collected 24 h later for luciferase assays, immunoprecipitation and Western analysis.

Luciferase assays were performed using a Boehringer Mannheim luciferase assay kit as instructed. Cell lysates were first taken for determining fluorescence intensity emitted by co-expressed green fluorescence protein (GFP) fusion proteins in a Wallac multi-label counter, which is capable of measuring 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 mutants of mouse Dvl1, Axin and Frat1-1 were generated by PCR using the high fidelity thermostable DNA polymerase *Pfu* (Stratagene, CA). HA or Flag epitope tags were introduced to the C-termini of the full-length and mutant molecules. The expression of these molecules was driven by a cytomegalovirus promoter. All constructs were verified by DNA sequencing. The LEF-1 reporter gene constructs were kindly provided by Dr Grosschedl (UCSF).

Immunoprecipitation assays

Cells were lysed with the lysis buffer containing 1% NP-40, 137 mM sodium chloride, 20 mM Tris, pH 7.4, 1 mM dithiothreitol (DTT), 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 pre-cleared 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 anti-tag antibody (Berkeley Antibody Company, CA) and 20 μl of protein A/G–Sepharose beads for 3.5 h on ice. The immunocomplexes were pelleted and washed three times with cold lysis buffer in the absence of protease and phosphatase inhibitors. The proteins were released from beads by boiling in SDS sample buffer. The samples were loaded on SDS–PAGE gels. Proteins were electroblotted to nitrocellulose membranes. The results were visualized using a Raytest imaging system with a cooled CCD camera.

Xenopus methods

The Flag-epitope tagged GBP mutant that does not bind Xgsk-3 (BP139), Myc epitope-tagged Xgsk-3 (XG134) and Flag-epitope tagged wild-type GBP (BP20) have been described previously (Yost *et al*., 1998). Myc epitope-tagged *Xenopus* dishevelled was the kind gift of J.Miller and R.Moon. Embryos were obtained as previously described (Newport and Kirschner, 1982). Embryos were microinjected (Moon and Christian, 1989) with RNA synthesized from CS2+- (Turner and Weintraub, 1994), derived constructs linearized with *Not*I or *Asp*718 using the mMessage mMachine kit (Ambion) according to the manufacturer's instructions. Immunoprecipitations and Western blotting were performed as described (Yost *et al*., 1998).

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