Phosphorylation of Frizzled-3*

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Wang A. Yanfeng1, Chang Tan1,2, Robert J. Fagan1, and Peter S. Klein1,3

From the 1Cell and Molecular Biology Graduate Program and the 2Hematology-Oncology Division, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Frizzleds are secreted proteins important to many biological processes. frizzled genes encode a family of Wnt receptors that signal to the extracellular compartment through the cytosolic protein Disheveled. Limited information is available concerning the regulation of Frizzleds at a biochemical level. We report here that Xenopus Frizzled-3 is phosphorylated in a Disheveled-dependent manner that appears to require the DEP domain of Disheveled. Phosphorylation of serine 576 causes a decrease in electrophoretic mobility and accounts for a significant fraction of receptor phosphorylation, although additional residues in the C-terminal tail are also phosphorylated. In addition, mutations that interfere with Frizzled-3 function also interfere with phosphorylation, but these inactive mutants can be phosphorylated when an active form of Frizzled-3 is co-expressed. Mutation of C-terminal serines including serine 576 significantly enhances Frizzled-3-mediated induction of neural crest markers, suggesting that C-terminal phosphorylation plays a role in down-regulating Frizzled signaling.

Wnts are secreted glycoproteins involved in cell-cell communication in diverse settings. Over 90% of colorectal cancers are caused by mutations that activate the canonical Wnt signaling pathway. Activation of this pathway is also observed in solid childhood tumors, gastric carcinoma, ovarian carcinoma, hepatoma, medulloblastoma, and pancreatic tumors (reviewed in Ref. 1). During embryonic development, the Wnt pathway is required in dorsal axis specification, segmentation, neural crest formation, and organogenesis. Wnt signaling also regulates cell fate determination, cell adhesion and separation, cell movement, and cell polarity. During adult life, Wnts are essential in maintenance and differentiation of stem cells or early progenitor cells in skin, muscle, intestinal crypts, and hematopoietic tissues (reviewed in Ref. 2).

The Wnt/Frizzled cassette signals through several branches including the canonical Wnt/β-catenin, Wnt/Ca2+7, and planar cell polarity (PCP)8 pathways (reviewed in Ref. 3). The shared components of these branches so far identified are the receptors (Frizzleds), the phosphoprotein disheveled (Dsh), and in some cases the ligand (Wnts). wnt and frizzled genes are encoded by large multigene families, whereas the number of dsh genes is more limited. In mouse, there are 19 wnt genes, 10 frizzled genes, and three dsh genes.

Frizzleds contain multiple hydrophobic domains predicted to form seven transmembrane helices. Conserved sequences are found in the extracellular cysteine-rich domain (CRD), the putative transmembrane domains, and parts of the intracellular loops (4), whereas the C-terminal tails are highly divergent. The mechanisms by which Frizzleds transduce signals remain unclear. The highly conserved juxtamembrane motif, KT.XXXW, found in the membrane-proximal C-terminal region of all Frizzleds, is essential for canonical signaling (5) and has been proposed to interact with Disheveled (6, 7). Recent work also identified residues in the intracellular loops that are crucial to signaling (6).

The predicted topology of Frizzleds, with seven proposed transmembrane domains, is reminiscent of G-protein-coupled receptors (GPCRs), and recent evidence supports a role for G-proteins in canonical and noncanonical Frizzled signaling (8–11). Early data suggested that canonical Wnt signaling is sensitive to pertussis toxin and that chimeric receptors incorporating adrenergic ligand binding domains with Frizzled-derived intracellular sequences could activate both canonical and Wnt/Ca2+8 pathways when overexpressed in cultured cells and stimulated with adrenergic ligands. More recently, epistasis experiments in Drosophila suggested that Goα is required downstream of frizzled and upstream of dsh for canonical Wnt and PCP signaling (10). These observations strengthen the proposal that Frizzleds signal through G-proteins and suggest that Frizzleds may share other aspects of GPCR regulation. A common feature of GPCR regulation is the phosphorylation of the receptors by a family of GPCR-associated protein kinases (GRKs (12)), which is associated with attenuation of signaling, receptor internalization, protein turnover, and, in some cases, novel signaling events (13, 14). In support of the parallel between Frizzleds and GPCRs, Frizzled-4 can be internalized in cultured cells in the presence of Wnt5A, Disheveled, and activated protein kinase C (PKC (15)). Wang and Malbon (16) have pointed out that consensus phosphorylation sites for protein kinase A, protein kinase C, and casein kinase II are commonly found in Frizzled C-terminal regions. Furthermore, Smoothened, a receptor for Hedgehog that also contains seven putative transmembrane domains and is distantly related to Frizzleds, is also phosphorylated and internalized (11, 17). In a recent publication, in vitro phosphorylation of a C-terminal fragment of Drosophila Frizzled-1 (Fz1) by PKC was reported (18). Phosphorylation of intact Frizzleds in a cellular context has not been reported, however.

Frizzled-3 (Fz3) has been studied extensively in different model systems. In Xenopus, Fz3 is required for neural crest induction (19). Overexpression of Fz3 disrupts convergent extension movements during gastrulation in Xenopus embryos and can induce complete ectopic eyes (20, 21). In mouse, Fz3 is required for the development of major axonal tracts in forebrain-derived structures and for axon guidance of commissural neurons within the spinal cord (22, 23).

In this work, we show that Fz3 becomes highly phosphorylated in the presence of Disheveled. The DEP domain of Dsh is necessary and sufficient for this phosphorylation. Disheveled-dependent phosphorylation of Fz3 requires serine 576 in the C terminus. Mutation of serine 576 to alanine does not inhibit Fz3 activity, and rather appears to enhance the signaling activity of Fz3, suggesting that Frizzled signaling is down-

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1 These authors contributed equally to this work.

2 Current address: Division of Biological Sciences, University of Missouri, Columbia, MO 65211.

3 To whom correspondence should be addressed: 364 CRB, 415 Curie Blvd., Philadelphia, PA 19104. Tel: 215-898-2179; E-mail: pklein@mail.med.upenn.edu.

4 The abbreviations used are: PCP, planar cell polarity; Fz, Frizzled; Dsh, disheveled; GPCR, G-protein-coupled receptor; GRK, GPCR-associated protein kinase; PKC, protein kinase C; HEK, human embryonic kidney; PP1, protein phosphatase 1; CK, casein kinase; Dfz1, Drosophila Fz1.
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regulated through phosphorylation, which is an additional parallel with GPCR regulation.

EXPERIMENTAL PROCEDURES

Materials—frizzled-3 (fz3) and disheveled (dsh) cDNAs used in these studies were from Xenopus and were subcloned into pCS2, as described previously (19, 24). Mutations in fz3 and dsh were generated by site-directed mutagenesis using the QuickChange kit (Stratagene) and confirmed by sequencing. Fz338 is a chimeric construct that replaces the C-terminal tail of fz3 with that of Xenopus fzh. MycDshAC and myc-Dep+ were from J. C. Smith (25). Phosphate-free Dulbecco’s modified Eagle’s medium was from Invitrogen, dialyzed fetal bovine serum was from Amersham Biosciences (PBS13), protein phosphatase 1 (PP1) was from New England Biolabs, and protein G-agarose was from Invitrogen.

Phosphorylation of Fz3—Xenopus embryos were injected with mRNAs encoding wild-type fz3 or fz3 mutants (0.5 ng), dsh or dsh mutants (0.5 ng), and β-galactosidase with a nuclear localization signal (nBGal; 0.5 ng) into the animal pole at the one-cell stage, developed to the gastrula stage (stage 10.5, unless specified), lysed in embryo lysis buffer (20 mM Tris, pH 7.5, 140 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 2 mM sodium vanadate, 25 mM sodium fluoride, 1% Nonidet P-40, and a premixed protease inhibitor mixture (Sigma)), and analyzed by Western blot using a monoclonal anti-Fz3 antibody (26). For in vitro dephosphorylation of Fz3, lysates from fz3 and dsh mRNA-injected embryos were treated with PP1 (for a 20-μl reaction: 10 μl of lysate, 2 μl of PP1 buffer, 2 μl of MnCl2 (10 mM), 1 μl (2.5 units) of PP1) in the presence or absence of the protein phosphatase inhibitor microcyst (1 μl, 0.5 mM) at 37 °C for 20 min and then analyzed by Western blotting as described above.

Metabolic Labeling and Immunoprecipitation of Fz3—HEK293T cells were plated at 10% confluence in 6-well plates and transfected with plasmids as indicated in Fig. 1B. Fresh medium was added after 24 h. After an additional 12 h, cells were washed twice with phosphate-free Dulbecco’s modified Eagle’s medium and then incubated in phosphate-free Dulbecco’s modified Eagle’s medium with 10% dialyzed fetal bovine serum and 0.5 mCi of carrier-free [32P]orthophosphate for 2 h. Cells were washed and harvested in phosphate-buffered saline and resuspended in cold embryo lysis buffer (as described above). Lysate was centrifuged at 20,000 × g for 3 min at 4 °C; supernatant was recovered, and anti-Fz3 antibody was added at a 1:100 dilution on ice. After 2 h, protein G-agarose beads were added, and suspension was incubated with rotation at 4 °C for 4 h. Protein G was centrifuged briefly to pellet beads, which were washed three times with lysis buffer. Immunoprecipitation was eluted with SDS sample buffer and analyzed by SDS-PAGE and autoradiography as well as Western blotting.

Neural Crest Induction—Neural crest induction assays were performed in animal pole ectodermal explants as described previously (19) with fz3wt and fz3S576A mRNAs at the concentrations indicated in Figs. 6 and 7. Expression of the neural crest markers Slug and Twist, as well as the EF-1α loading control, was assessed by reverse transcription-PCR as described previously (19).

RESULTS

Phosphorylation of Fz3 in Xenopus Embryos—Phosphorylation of GPCRs has been implicated in receptor desensitization, internalization, and degradation. Although Frizzled proteins have a predicted membrane topology similar to GPCRs and have been proposed to function through G-proteins, phosphorylation of Frizzled proteins has not been described (however, while this manuscript was in preparation, the in vitro phosphorylation of a C-terminal fragment of Drosophila Fz1 by PKC was reported (18)). In the course of our studies on the regulation of Fz3 by downstream modulators, including Disheveled and the Fz3-binding protein Kermit/GIPC, we observed multiple immunoreactive species in Western blots for Fz3 (Fig. 1). In the absence of Dsh, a predominant doublet migrates at ~63–66 kDa, and a faint, slower mobility species is present at 73 kDa (Fig. 1A, lane 1). However, when Fz3 is co-expressed with Dsh, the intensity of the 73-kDa species is markedly increased (Fig. 1A, lane 2), whereas the 66-kDa species is reduced to an almost undetectable level (the 63-kDa species is unchanged). This apparent shift in mobility from 66 to 73 kDa suggests a post-translational modification of Fz3, such as protein phosphorylation. We therefore tested whether the 73-kDa form is sensitive to protein phosphatase treatment. When cell lysates containing the 73-kDa form were treated with PP1 (lanes 3 and 4) or without the PP1 inhibitor microcyst (lanes 1 and 2), a predominant doublet migrates at ~63–66 kDa, and a faint, slower mobility species is present at 73 kDa (Fig. 1A, lane 1). However, when Fz3 is co-expressed with Dsh, the intensity of the 73-kDa species is markedly increased (Fig. 1A, lane 2), whereas the 66-kDa species is reduced to an almost undetectable level (the 63-kDa species is unchanged). This apparent shift in mobility from 66 to 73 kDa suggests a post-translational modification of Fz3, such as protein phosphorylation. We therefore tested whether the 73-kDa form is sensitive to protein phosphatase treatment. When cell lysates containing the 73-kDa form were treated with PP1 (lanes 3 and 4) or without the PP1 inhibitor microcyst (lanes 1 and 2), a predominant doublet migrates at ~63–66 kDa, and a faint, slower mobility species is present at 73 kDa (Fig. 1A, lane 1). However, when Fz3 is co-expressed with Dsh, the intensity of the 73-kDa species is markedly increased (Fig. 1A, lane 2), whereas the 66-kDa species is reduced to an almost undetectable level (the 63-kDa species is unchanged). This apparent shift in mobility from 66 to 73 kDa suggests a post-translational modification of Fz3, such as protein phosphorylation. We therefore tested whether the 73-kDa form is sensitive to protein phosphatase treatment. When cell lysates containing the 73-kDa form were treated.
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**FIGURE 2. Enhancement of Fz3 phosphorylation by the Dsh DEP domain.** A, schematic representation of Dsh constructs. Dsh represents wild-type construct with conserved N-terminal DIX, central PDZ, and C-terminal DEP domains. DshΔC contains the DIX and PDZ domains but lacks the DEP domain; Dsh-DEP+ includes the DEP domain and lacks the DIX and PDZ domains (25). DshK441M is full-length Xenopus Dsh with a point mutation corresponding to the dsh1 mutation in Drosophila. A Myc epitope tag was added to the N terminus of each construct. B, mRNAs encoding Fz3 and Dsh or Dsh mutants were injected as indicated. Embryos were harvested at the gastrula stage (stage 10.5) and analyzed by Western blotting (as described in the legend for Fig. 1) with antibodies (Ab) to Fz3 (upper panel) or the Myc epitope (lower panel). uninj, uninjected sample.

with PP1, the 73-kDa band disappeared and the 66-kDa form reappeared (Fig. 1A, lane 3). This mobility shift is dependent on phosphatase activity per se, as it is blocked by inclusion of microcystin, a phosphatase inhibitor (Fig. 1A lane 4). The observations that the 66-kDa form shifts almost completely to the 73-kDa form with Dsh, and the 73-kDa form is reduced completely to the 66-kDa mobility form with PP1 suggest that the phosphorylation is stoichiometric, with at least one phosphate added per molecule of the 66-kDa form of Fz3. The 63-kDa form did not change in mobility with expression of Dsh or after phosphatase treatment (Fig. 1A, lanes 1–4).

These observations strongly suggest that Fz3 is phosphorylated in a Dsh-dependent manner. However, the sensitivity of an electrophoretic mobility shift to phosphatase treatment is an indirect measure of phosphorylation. To demonstrate phosphorylation of Fz3 directly, we labeled HEK293T cells metabolically with 32P orthophosphate and immunoprecipitated Fz3. The cells had been transfected previously with Fz3 with or without Dsh. As shown in Fig. 1B (upper panel), Fz3 alone incorporates a low level of 32P, and this increases markedly upon co-expression of Dsh, accompanied by a shift in electrophoretic mobility to the 73-kDa species. PhosphorImager analysis of 32P-labeled proteins indicates an 8–10-fold increase in Fz3 phosphorylation (normalized to total Fz3 protein in the immunoprecipitated sample) when Dsh is co-expressed. Thus, phosphorylation of Fz3 is markedly enhanced in a Dsh-dependent manner in mammalian cells and Xenopus embryos.

To test whether the phosphorylation of Fz3 changes during embryonic development, we harvested embryos expressing Fz3 with or without Dsh at four stages. Fz3 phosphorylation was first detected weakly at the mid-blastula stage (stage 8.5) and increased significantly during gastrulation (stages 10 and 12; Fig. 1C). At later stages, the overall level of Fz3 expression declined. Fz3 did not undergo Dsh-dependent phosphorylation in oocytes, even after a 26-h incubation, indicating that the capacity to phosphorylate Fz3 is developmentally regulated (data not shown).

Dsh is a downstream cytosolic component of both canonical and noncanonical Wnt signaling pathways. Dsh contains at least three functional domains (the DIX domain, the central PDZ domain, and the C-terminal DEP domain (Fig. 2A)), which have been implicated in distinct pathways downstream of Frizzleds (27, 29). To test whether a specific domain of Dsh is required for Fz3 phosphorylation, we used Dsh deletion and point mutants. As shown in Fig. 2B, a deletion construct lacking the DEP domain (dshΔC) fails to induce the 73-kDa phosphorylated form of Fz3, whereas expression of a C-terminal fragment containing the DEP domain without the DIX or PDZ domains (dshDEP+) strongly enhances Fz3 phosphorylation. In addition, we tested a mutation (DshK441M) corresponding to the Drosophila mutant dsh1 (30), which disrupts PCP signaling and interferes with translocation of Dsh to the plasma membrane. DshK441M did not stimulate Fz3 phosphorylation (Fig. 2B), similar to dshΔC. This analysis shows that the DEP domain is necessary and sufficient for Dsh-stimulated Fz3 phosphorylation.

**Fz3 Mutations That Disrupt Phosphorylation**—To identify potential phosphorylation sites in Fz3, we mutated all of the serines and threonines on the predicted cytoplasmic surface of Fz3 to alanine. This included each of the 41 serines and threonines in the C-terminal tail of Fz3 and two within the predicted intracellular loops. The C-terminal tail also contains several regions of multiple, adjacent serines, and several of these were also mutated as a group to alanines. Each of these mutants was expressed in Xenopus embryos with or without Dsh and examined by Western blot. A representative Western blot showing a subset of the Fz3 mutations analyzed is shown in Fig. 3A (additional point mutants are shown in Fig. 4). This analysis identified two point mutations, serine 576 to alanine (S576A) and S641A, that markedly reduced the Dsh-dependent electrophoretic mobility shift (Figs. 3A and 4). (The electrophoretic mobility shift of S579A was also modestly reduced). The reduction in the 73-kDa form of Fz3 could indicate that these serines are required for phosphorylation or, alternatively, that the mutation disrupts the folding and/or trafficking of Fz3 to the subcellular compartment where phosphorylation occurs, such as the plasma membrane. We therefore addressed whether the serine to alanine mutants were present on the plasma membrane by expressing each mutant and wild-type Fz3 in Xenopus embryos and performing immunohistochemical staining on ectodermal explants using the Fz3 antibody. We found that S576A is expressed predominantly on the plasma membrane, similar to wild-type Fz3, but S641A appears to accumulate within cells rather than at the cell surface (data not shown). We therefore did not pursue further analysis of the S641A mutant. Furthermore, the effect of the S579A mutation on electrophoretic mobility was modest (and did not appear to affect activity in the functional assays described below; data not shown); we therefore focused on the S576A mutation for further analysis.

To confirm that Ser576 is required for Fz3 phosphorylation, Fz3S576A was expressed in 293T cells that were then metabolically labeled with...
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**FIGURE 3.** Phosphorylation of Fz3 point mutants. A, mRNAs encoding wild-type Fz3 alone (lane 1), Fz3 with myc-Dsh (lane 2), or mutated Fz3 with myc-Dsh (lanes 3–13) were injected into one-cell embryos as described for Fig. 1. Embryos were harvested at the gastrula stage (stage 10.5) and analyzed by Western blotting with antibodies to Fz3. The numbers above lanes 3–13 indicate the positions of serine or threonine residues replaced with alanines. In lanes 10–13, all three indicated residues were mutated to alanines. B, reduced 32P labeling of Fz3S576A and Fz37A, in which Ser508, Thr541, Thr562, Ser576, Ser587, Ser624, and Ser636 were mutated to alanines; HEK293T cells were transfected with plasmids encoding Fz3wt, Fz3S576A, or Fz37A with or without myc-Dsh (0.5 μg each), labeled with 32PO4, and harvested for immunoprecipitation (IP) with Fz3 antibody as described in the legend for Fig. 1B. Immunoprecipitated samples were analyzed by autoradiography (upper panel) and by Western blotting (lower panel) to control for loading of immunoprecipitated protein. NT, non-transfected. C, the intensity of 32P-labeled Fz3 in B was measured using a Typhoon PhosphorImager and, after background subtraction, was normalized to the relative amount of total Fz3 protein as measured by Western blotting (B, lower panel). Relative incorporation of 32P on total Fz3 protein is shown in histogram form with open bars representing basal Fz3 phosphorylation and filled bars representing phosphorylation after transfection of Dsh. The experiment was repeated three times with similar results.

\[ ^{32}\text{P}O_4 \] as described above for wild-type Fz3. In the absence of Dsh, Fz3S576A is 32P-labeled at a low level (Fig. 3B). However, Fz3S576A fails to shift to the 73-kDa form and shows markedly reduced incorporation of 32P when Dsh is co-expressed (Fig. 3B). These observations confirm that Ser576 is required for the Dsh-induced mobility shift and for maximal phosphorylation of Fz3. However, Fz3S576A can still be phosphorylated (2.5-fold increase) in the presence of Dsh, indicating that phosphorylation of additional sites can occur, although at a lower level and without a change in electrophoretic mobility. As pointed out by Wang and Malbon (16), the C-terminal regions of Frizzleds contain consensus phosphorylation motifs; we therefore examined the Fz3 C terminus for additional phosphorylation potential phosphorylation sites. Analysis of the Fz3 sequence for known protein kinase recognition motifs using the Scansite algorithm at medium stringency identified seven consensus sequences for potential phosphorylation in the C-terminal tail: in addition to Ser576, the program identified Ser508, Thr541, Thr562, Ser576, Ser587, Ser624, and Ser636. As discussed above, with the exception of Ser576, single point mutations in these sites did not affect the Dsh-induced mobility shift (Fig. 3A and data not shown). However, mutation of all seven sites to alanines (termed Fz37A) further attenuated Dsh-induced phosphorylation compared with Fz3S576A (Fig. 3, B and C). Thus, in the presence of Dsh, Fz3 appears to be phosphorylated at multiple sites in the C-terminal tail.

Receptor phosphorylation is associated with either activation, as in the case of receptor tyrosine kinases and the Wnt co-receptor Arrow/ LRPs 5/6, or desensitization, as is typically observed with GPCRs. We have thus far not identified a Wnt that can induce Fz3 phosphorylation (data not shown). Therefore, to begin to test whether Frizzled activation may play a role in subsequent phosphorylation, we examined previously described inactive Fz3 mutants. Several mutations in frizzled genes have been described that interfere with Frizzled signaling, including mutations in the highly conserved KTXXXW motif (K501M, T502V, and W506G in Fz3) and mutation of a conserved arginine in the first intracellular loop (R229A in Fz3) (5, 6) (all of these mutants are expressed on the plasma membrane (Ref. 5 and data not shown). We found that none of these signaling-defective forms of Fz3 demonstrates a Dsh-dependent electrophoretic mobility shift (Fig. 4). These data indicate that mutations that inactivate Fz3 also interfere with phosphorylation. This corollation could indicate that phosphorylation is either required for Frizzled signaling or is a consequence of Frizzled signaling. As a control for equal expression of Dsh, we assessed Dsh protein levels by Western blot (Fig. 4, bottom panel); aside from confirming equal expression of Dsh protein in each sample, these Western blots revealed an electrophoretic mobility shift in Dsh when Fz3 WT was co-expressed (see also Fig. 5) consistent with prior publications demonstrating Dsh phosphorylation in response to Wnt/Frizzled signaling (31). Furthermore, the R229A, K501M, T502V, and W506G forms of Fz3 failed to cause the electrophoretic mobility shift/phosphorylation in Dsh, as expected, because these mutated forms are defective in signaling. However, Fz3S576A, which is not phosphorylated itself, nevertheless induces a mobility shift/phosphorylation of Dsh (Figs. 4 and 5), suggesting that this mutant could be active; if the S576A mutant is active, as addressed in more detail below; this would imply that Ser576 phosphorylation is not required for signaling.

Phosphorylation of Fz3 Mutants—KTXXXW mutants and R229A cannot activate Wnt signaling (5, 6). For these mutants, it is possible that the failure to activate Dsh results in the deficiency in Fz3 phosphorylation. Alternatively, these mutations could alter Fz3 conformation in a manner that interferes with phosphorylation. To test whether the inac-
tive Fz3 mutants can still be phosphorylated, we co-expressed inactive Fz3 mutants with an active form of Frizzled; because the antibody recognizes the C terminus of Fz3, we used an active chimera (Fz338) in which the Fz3 C-terminal tail was replaced with the C-terminal tail from Fz8, similar to frizzled chimeras described by others (32). Co-expression of Fz338 with Dsh and the inactive Fz3 mutants led to phosphorylation of K501M (Fig. 5, lane 8), T502V, W506G, and R229A (data not shown). These data show that the inactive Fz3 mutants are phosphorylated as long as an active Fz is present. In contrast, S576A is not phosphorylated under these conditions (Fig. 5, lane 12), indicating that Ser576 is required for Fz3 phosphorylation.

**Signaling by a Fz3 Phosphorylation-defective Mutant**—Co-expression of Dsh with Fz3 in animal pole explants induces neural crest, providing a robust functional assay for Fz3 activity. We therefore expressed Fz3 and phosphorylation-defective mutants in animal pole explants and assayed for induction of the neural crest markers Twist and Slug, as described previously (19). Fz3 with mutations in Arg229 or in the KTXXXW motif was inactive in neural crest induction assays (data not shown), consistent with previous reports on these Fz3 mutations (5, 6). However, S576A was active in this assay, demonstrating that phosphorylation association with this residue is not required for activity (Fig. 6). Furthermore, Fz3S576A showed modestly increased activity in inducing neural crest markers when compared with Fz3wt (Fig. 6), raising the additional possibility that phosphorylation of a glutathione S-transferase fusion protein containing the C terminus of Dfz1 was reported recently (18), but to our knowledge this is the first evidence for phosphorylation of intact Frizzled proteins and the first direct demonstration of Frizzled signaling. Furthermore, Fz3S576A, and to a lesser extent Fz3S576A, is more active in inducing neural crest markers than the wild-type Fz3, raising the additional possibility that C-terminal phosphorylation reduces Fz3 activity.

**DISCUSSION**

The data presented here demonstrate phosphorylation of Fz3 in a Dsh-dependent manner that also requires an active form of Fz3. Evidence for Fz3 phosphorylation includes an electrophoretic mobility shift that is reversed by exposure to a serine-threonine phosphatase and direct evidence showing markedly increased incorporation of 32P into Fz3 upon co-expression of Dsh. Furthermore, we have identified a specific site, Ser576, that is required for the Dsh-dependent electrophoretic mobility shift and for maximal incorporation of 32P into Fz3. Mutation of this site modestly enhances Fz3 signaling without altering steady state protein levels. Mutation of multiple predicted phosphorylation sites further reduces Fz3 phosphorylation and demonstrates a clear increase in Fz3 activity in the induction of neural crest markers. In vitro phosphorylation of a glutathione S-transferase fusion protein containing the C terminus of Dfz1 was reported recently (18), but to our knowledge this is the first evidence for phosphorylation of intact Frizzled proteins and the first direct demonstration of Frizzled phosphorylation within a cellular context.

Phosphorylation of transmembrane receptors can be required for activation, as in the case of receptor tyrosine kinases and receptor serine kinases (e.g. TGF-β receptors), or for desensitization, as with GPCRs (13, 33). Although several mutations that interfere with Fz function also interfere with the Dsh-induced phosphorylation of Fz3, inactive Fz3 mutants can be phosphorylated, as inferred from electrophoretic mobility shift and from direct incorporation of 32P, if an active form of Fz is co-expressed with the inactive Fz3 mutant. Furthermore, Fz3S576A and Fz3S576A (which includes the S576A mutation) do not undergo the Dsh-dependent electrophoretic mobility shift, and the incorporation of 32P is

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**FIGURE 5.** Phosphorylation of inactive Fz3 mutants. mRNAs (0.5 ng) encoding wild-type (Fz3) and mutated (K501M and S576A) Fz3, Myc-tagged Dsh (Dsh), and the Fz3/8 chimera (Fz338), which contains the Fz8 C terminus and is not recognized by the Fz3 antibody, were co-expressed in the neural crest induction assay as in Fig. 6. The neural crest markers Slug and Twist were induced more potently by Fz3S576A than by Fz3wt (Fig. 7) Thus, the markers were barely detectable with 10 pg of Fz3wt mRNA but were induced by more than 20-fold with the same dose of Fz3S576A mRNA; the potency of this lowest dose of Fz3S576A mRNA (10 pg) was similar to or more than 50 pg of Fz3wt mRNA. The observations that nonfunctional Fz3 mutants can be phosphorylated if an active Frizzled is co-expressed (Fig. 5) and that mutants that cannot be phosphorylated can nevertheless activate the pathway suggest that phosphorylation of Fz3 at these sites is not a requirement for but is a consequence of Frizzled signaling. Furthermore, Fz3S576A, and to a lesser extent Fz3S576A, is more active in inducing neural crest markers than the wild-type Fz3, raising the additional possibility that C-terminal phosphorylation reduces Fz3 activity.
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Frizzled receptors (Fz) are critical components of the planar cell polarity (PCP) pathway. Recent findings suggest that Fz proteins bind to G protein-coupled receptors (GPCRs) similar to GRK-mediated phosphorylation of GPCRs. Phosphorylated Fz receptors have been implicated in the regulation of ommatidial chirality in Drosophila. In contrast to wild-type and serine to alanine mutations, which potently disrupt ommatidial organization, phosphorylated Fz3 in Drosophila is both necessary and sufficient to induce Fz3 phosphorylation.

Phosphorylation is likely to be a general feature of Frizzleds, as we observed incorporation of 32P into other Xenopus Fz proteins (Fz7 and Fz8), and the data from Djiane et al. (18) are consistent with phosphorylation of endogenous Fz proteins in Drosophila. Furthermore, Smothened (which is involved in the response to Hedgehog, contains seven putative transmembrane domains, and is distantly related to Frizzled proteins) is also phosphorylated (11, 17). Future work will be necessary to establish whether these are also phosphorylated in a Dsh-dependent or ligand dependent manner.

Fz proteins appear to be glycosylated as well as phosphorylated. Under basal conditions, two bands of Fz3 are observed at 63–66 kDa, both of which are sensitive to treatment with the glycosidase PNGase F (data not shown). These observations are consistent with recent evidence demonstrating glycosylation of Fz7 and Fz8 in Xenopus (28). Glycosylation and maturation of Fz proteins appears to be inhibited by a
novel gene product named Shisa, which is highly expressed in the ante-rior neural ectoderm of *Xenopus* embryos, similar to the pattern of Fz3 expression. Yamamoto et al. (28) have proposed that Shisa retains Fz proteins in the endoplasmic reticulum in order to antagonize the caudalizing activity of Wnt/Fz signaling in this region.

In summary, we have shown that full-length Fz3 is phosphorylated in *Xenopus* embryos and in mammalian cells. Phosphorylation is markedly enhanced by co-expression of the downstream Wnt/Fz signaling com-
ponent Dsh and specifically requires the DEP domain of Dsh, a region previously implicated in PCP signaling. Furthermore, phosphorylation appears to modulate Fz3 activity, supporting and extending recent work suggesting a role for Frizzled phosphorylation in modulating the PCP pathway in *Drosophila*. Future work will include the identification of the endogenous protein kinase(s) that mediate signal dependent phospho-
rylation of Frizzleds and identification of phosphorylation sites within other Frizzled proteins. Acknowledgments—We thank Drs. Dave Man-
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