Regulation of the Protein Kinase Activity of Shaggy\textsuperscript{Zeste-white3} by Components of the Wingless Pathway in \textit{Drosophila} Cells and Embryos\textsuperscript{*}

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(Received for publication, April 1, 1999, and in revised form, May 14, 1999)

The protein-serine kinase Shaggy\textsuperscript{Zeste-white3} (Sgg\textsuperscript{Zw3}) is the \textit{Drosophila} homolog of mammalian glycogen synthase kinase-3 and has been genetically implicated in signal transduction pathways necessary for the establishment of patterning. Sgg\textsuperscript{Zw3} is a putative component of the Wingless (Wg) pathway, and epistasis analyses suggest that Sgg\textsuperscript{Zw3} function is repressed by Wg signaling. Here, we have investigated the biochemical consequences of Wg signaling with respect to the Sgg\textsuperscript{Zw3} protein kinase in two types of \textit{Drosophila} cell lines and in embryos. Our results demonstrate that Sgg\textsuperscript{Zw3} activity is inhibited following exposure of cells to Wg protein and by expression of downstream components of Wg signaling, \textit{Drosophila} frizzled 2 and dishevelled. Wg-dependent inactivation of Sgg\textsuperscript{Zw3} is accompanied by serine phosphorylation. We also show that the level of Sgg\textsuperscript{Zw3} activity regulates the stability of Armadillo protein and modulates the level of phosphorylation of D-Axin and Armadillo. Together, these results provide direct biochemical evidence in support of the genetic model of Wg signaling and provide a model for dissecting the molecular interactions between the signaling proteins.

The product of the \textit{Drosophila} wingless (\textit{wg}) gene is a secreted protein homologous to vertebrate Wnts (1). Genetic analysis of \textit{wg} has revealed roles in processes controlling embryonic segmentation, gut formation, and imaginal disc patterning (2–4). Additional genes have been implicated in the secretion, reception, or interpretation of the Wg \textsuperscript{1} signal: dishevelled (\textit{dsh}) (5) and armadillo (\textit{arm}) (6). Dsh protein is a novel protein with a discs-large homology region, whereas the \textit{arm} gene encodes the \textit{Drosophila} homolog of \textit{β}-catenin, a component of vertebrate adherens junctions. \textit{Drosophila} frizzled 2 (\textit{Dfz2}) was recently identified as a protein with an amino-terminal cysteine-rich extracellular domain followed by seven transmembrane domains (7). The \textit{Dfz2} protein functions as a Wg receptor in cultured cells, and as yet, there are no known \textit{Dfz2} mutants. Whereas the above-mentioned genes act positively in Wg signaling, an additional gene called \textit{shaggy} or \textit{zeste-white3} (Sgg\textsuperscript{Zw3}) plays an inhibitory role in this pathway (1, 4, 8). Sgg\textsuperscript{Zw3} encodes a protein-serine kinase that has been highly conserved throughout the eukaryotic kingdoms (4, 9, 10). The mammalian homolog of Sgg\textsuperscript{Zw3} is glycogen synthase kinase-3 (GSK-3), which is encoded by two independent genes, GSK-3\textalpha and GSK-3\textbeta (11).

By a combination of clonal analysis, genetic epistasis, and biochemical experiments, \textit{wg} class genes have been ordered within the same pathway (12–15). \textit{armadillo} and \textit{dishevelled} embryonic phenotypes are similar to the \textit{wg} embryonic phenotype (12–14), whereas Sgg\textsuperscript{Zw3} has a mutant phenotype very similar to that of embryos in which \textit{wg} has been expressed in all cells (12, 16, 17). Genetic data in \textit{Drosophila} suggest that the functions of Sgg\textsuperscript{Zw3} are antagonized by Wg signaling (4). In fact, mutations in \textit{wg} and Sgg\textsuperscript{Zw3} have opposite effects on cell fate determination, and each mutation has an opposite effect on Arm protein levels (17, 18). In embryos, the normal segmental accumulation of Arm protein is absent in \textit{wg}, whereas Sgg\textsuperscript{Zw3} mutants have uniformly high levels of Arm protein.

Recently, an additional protein called Axin has been implicated in the regulation of \textit{β}-catenin/Arm (19). Axin and its \textit{Drosophila} homolog (D-Axin) act as scaffold proteins and bind GSK-3/Sgg\textsuperscript{Zw3}, \textit{β}-catenin/Arm, and APC (adenomatous polyposis coli protein) in a complex (20). In \textit{Drosophila} cells, the overexpression of D-Axin results in Arm destabilization.\textsuperscript{2} The presence of Axin is necessary for GSK-3 to efficiently phosphorylate \textit{β}-catenin (19) and to inhibit \textit{β}-catenin-mediated LEF-1 activation (22).

These data have been assembled into a model in which Wg protein is secreted and received by neighboring cells, where a signal transduction cascade is initiated (1). The Wg signal, at least in embryos and cultured cells, is transduced through Dsh and induces hyperphosphorylation of Dsh protein, possibly via casein kinase-2 (15, 23). Through an unknown mechanism, activation of Dsh blocks the function of Sgg\textsuperscript{Zw3} and D-Axin, resulting in decreased phosphorylation of Arm. Unphosphorylated Arm has increased stability and accumulates in the cytoplasm (15, 24), where it interacts with an high mobility group-like factor, LEF-1/pangolin (25, 26).

Recently, the mammalian homolog of Sgg\textsuperscript{Zw3}, GSK-3, has been shown to be regulated by \textit{Drosophila} Wg protein in fibroblasts (27), but direct biochemical evidence for inhibition of Sgg\textsuperscript{Zw3} by Wg signaling has yet to be demonstrated. To address the mechanism by which Wg signals via Sgg\textsuperscript{Zw3}, the effect of the known components of \textit{Drosophila} Wg signaling (\textit{Wg}, \textit{Dfz2},

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\textsuperscript{*} This work was supported in part by grants from the Medical Research Council of Canada (to J. R. W. and A. S. M.), The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{‡} Supported by a European Molecular Biology Organization long-term fellowship.

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\textsuperscript{1} The abbreviations used are: Wg, Wingless; Dsh, Dishevelled; Arm, Armadillo; \textit{Dfz2}, \textit{Drosophila} frizzled 2; Sgg\textsuperscript{Zw3}, Shaggy\textsuperscript{Zeste-white3}; GSK-3, glycogen synthase kinase-3; GST, glutathione S-transferase; PAG, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1, 1-bis(hydroxymethyl)ethyl]glycine.

\textsuperscript{2} L. Ruel, N. Anthopoulos, J. Goncalves, A. S. Manoukian, and J. R. Woodgett, submitted for publication.
and Dsh) on Sgg<sup>Zw3</sup> activity was investigated in cultured cells and embryos. We used an imaginal disc cell line (cl-8 (clone 8)) that responds to Wg signals and Schneider (S2) cells, which are unresponsive to Wg (15, 24). Using Wg-conditioned medium, we show that the activity of Sgg<sup>Zw3</sup> protein kinase is inhibited by Wg in cl-8 cells and that overexpression of Dfs2 or Dsh in cells reconstitutes Wg signaling in the absence of Wg as judged by inhibition of the kinase and accumulation of Arm protein. We also demonstrate that the regulation of Sgg<sup>Zw3</sup> activity, in turn, controls the stability of Arm protein by modulating the level of phosphorylation of D-Axin and Arm. These results provide direct biochemical evidence in support of previous genetic analyses.

**EXPERIMENTAL PROCEDURES**

**Antiserum**—Rabbit antisera to Arm and Dsh were raised against glutathione S-transferase (GST) fusion proteins. GST-Dsh was constructed by cloning a 1256-base pair XhoI-NotI fragment of the dishevelled coding region, corresponding to amino acids 395–624, into XhoI-NotI sites in pGEX-4T-1 (Amersham Pharmacia Biotech). cDNA fragments encoding amino acids 1–367 of Arm protein and 1–314 of Sgg<sup>Zw3</sup> protein were cloned into pGEX-4T-1 and pET15b (Novagen), respectively. Fusion proteins were produced in Escherichia coli strain BL21(DE3) and purified from bacterial lysates before immunization.

**Transfections and Cell Culture**—Drosophila Schneider line-2 and wing imaginal disc cl-8 cells were maintained as described (24). Wg protein assays were performed essentially as published (24, 28). Selection of stably transformed cl-8 cell lines was performed using methotrexate (29). The expression vector pRmHa-1 is designed to express proteins under control of the metallothionein promoter. The 2.8-kilobase pair BamHI-HindIII fragment of dsh cDNA in pBluescript SK<sup>+</sup> (Stratagene) corresponding to the entire coding region was cloned into the BamHI-HindIII sites of pRmHa-1. The dsh/pRmHa-1 or sgg<sup>Zw3</sup>/HApRmHa-1 vector was introduced into cl-8 cells by cotransfection with a second vector, pHGC<sub>0</sub>, carrying a selectable dhfr gene, which confers resistance to methotrexate (0.5 μg/ml). Transformed cells were maintained between 1 × 10<sup>6</sup> and 1 × 10<sup>7</sup> cells/ml and examined for metal-inducible gene expression (by addition of 0.5 mM CuSO<sub>4</sub>) by immunoblotting.

For expression in cl-8 cells, the D-axin-(332–642) fragment (amplified by polymerase chain) was subcloned into the pA5.1V5-His<sub>6</sub> vector (Invitrogen) in frame with the His epitope. Transfected cells were washed with phosphate-buffered saline and lysed in 20 mM Tris-HCl (pH 8) and 100 mM NaCl. For purification of D-Axin-(330–642)-His<sub>6</sub>, 10 μl of nickel-Sepharose beads were added in lysates. The complexes were washed four times with 20 mM Tris-HCl (pH 8), 100 mM NaCl, and 10 mM imidazole and resolved by SDS-PAGE or incubated with [γ-<sup>32</sup>P]ATP for 30 min.

**Metabolic Labeling of S2 Cell Lines**—Transfected Dsh S2 cells were treated with CuSO<sub>4</sub> to induce Dsh expression and labeled overnight with 1 μCi of [<sup>32</sup>P]orthophosphate/ml of S2 phosphate-free medium + 10% dialyzed fetal calf serum. Radioimmunoprecipitation assay buffer cell lysates were normalized for incorporation by Cerenkov counting (30). After immunoprecipitation of Sgg Zw3 protein and separation by SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes. <sup>32</sup>P-Labeled Sgg Zw3 was subjected to partial acid hydrolysis and the phosphoamino acids were separated in two dimensions by thin-layer electrophoresis (31).

**Preparation of Embryo Lysates**—For overexpression of Sgg<sup>Zw3</sup>, homozygous HS-Sgg<sup>Zw3</sup>-Drosophila eggs were collected 3 h after laying, heat-shocked for 8 min at 37 °C, and allowed to recover for an additional 1.5 h at 25 °C. To generate sgg<sup>Zw3</sup>-cl-8 mutant embryos, germ line mosaics were produced using the yeast recombinase-base flipase-dominant female sterile system as described by Chou and Perrimon (32). Homozygous mutant embryos can be recognized morphologically by a lack of segmentation. For overexpression of Wg, Drosophila males homozygous for arm-Gal4 were crossed to virgin Drosophila females harboring pUAS-Wg, and their progeny embryos were collected at 3–6 h. Wild-type embryos of the same stage were used as controls. Embryos were used in Gentle Soft buffer (28) and were subjected to immunoprecipitation analysis as described below.

**Immunoprecipitation and Sgg<sup>Zw3</sup> Kinase Assays**—Cells lines were washed with phosphate-buffered saline and lysed in Gentle Soft buffer (28). For Sgg<sup>Zw3</sup> immunoprecipitation, 20 μl of protein A-Sepharose or 20 μl of protein G-Sepharose were pre-bound to rabbit polyclonal antigen or to monoclonal antibodies (anti-Sgg<sup>Zw3</sup>, 2G2C5), respectively, and were added to the clarified cell lysates at 4 °C for 2 h. Immunocomplexes were washed four times with Gentle Soft buffer (28). In vitro Sgg<sup>Zw3</sup> kinase assays were performed for 30 min as described previously (33, 34). Phosphorylated peptide was separated from unincorporated [γ-<sup>32</sup>P]ATP by Tricine/SDS-PAGE and quantified using a PhosphorImager.

**RESULTS**

**Wingless Protein Represses Sgg<sup>Zw3</sup> Activity and Induces Accumulation of Cytoplasmic Armadillo**—To analyze the biochemical consequences of Wg signaling, we exploited an imaginal disc cell line (cl-8) that is responsive to Wg (24). To determine the biological effects of Wg, cl-8 cells were exposed to the serum-free conditioned medium from either heat-shocked Schneider HS-wg (Wg-conditioned medium) or Schneider control cells (S2 control medium), and cytoplasmic extracts were prepared and immunoblotted with antibodies to Wg, Arm, and Dsh (Fig. 1A) (15). Wg-containing medium increased Arm levels within 2 h, reaching a maximum after 6 h. By contrast, cellular levels of Dsh did not change in this time period. How-
ever, Wg induced the formation of electrophoretically retarded forms of Dsh. These modifications have been previously observed by Yanagawa et al. (15) and Willert et al. (23) and correspond to hyperphosphorylation of Dsh protein. Exposure of cells to medium conditioned by control S2 cells affected neither Arm levels nor the Dsh electrophoretic mobility.

To determine whether Wg modulates Sgg\textsuperscript{Zw3} activity, Sgg\textsuperscript{Zw3} was immunoprecipitated from lysates of cl-8 cells treated with Wg-conditioned medium or S2 control medium. Protein kinase activity was measured using a peptide substrate specific for the GSK-3 family of protein kinases (GS-1 peptide (33)). Incubation of cl-8 cells with Wg-conditioned medium caused a time-dependent inhibition of Sgg\textsuperscript{Zw3} protein kinase activity (Fig. 1B). After 2–4 h of treatment with Wg-conditioned medium, total GS-1 peptide kinase activity was suppressed by 40–50% compared with the activity observed in cells treated with S2 control medium. Wg did not affect the expression of Sgg\textsuperscript{Zw3} as judged by immunoblotting (Fig. 1A).

To confirm the effect of Wg protein on the activity of Sgg\textsuperscript{Zw3}, we investigated how Sgg\textsuperscript{Zw3} functions in Wg signaling during embryogenesis, analyzing Sgg\textsuperscript{Zw3} activity in embryos with a wild-type or sgg\textsuperscript{null} mutant genotype, embryos overexpressing sgg\textsuperscript{Zw3}, and embryos expressing wg ubiquitously. sgg\textsuperscript{null} embryos were made homozygous for the sgg\textsuperscript{null} M11-1 allele, and Sgg\textsuperscript{Zw3} immunoprecipitates from these mutant embryos contained no detectable Sgg\textsuperscript{Zw3} activity, which verified the specificity of the assay (Fig. 2B). Furthermore, Sgg\textsuperscript{Zw3} immunoprecipitates from embryos overexpressing Sgg\textsuperscript{Zw3} from a heat shock-inducible transgene (HS-\textit{Sgg} \textsuperscript{Zw3}) exhibited 2.5-fold higher activity than immunoprecipitates from wild-type embryos (Fig. 2B).

To determine the effect of Wg overexpression on Sgg\textsuperscript{Zw3} activity, Wg was ectopically expressed in early embryos using a line that carries a GAL4-driven \textit{wg} transgene (\textit{pUAS-Wg}) crossed to a line that ubiquitously expresses GAL4 (arm-GAL4). The activity of Sgg\textsuperscript{Zw3} from these embryos was determined to be ~30% lower than that from wild-type control lyses (Fig. 2B). Immunoblotting of the embryonic extracts revealed equivalent Sgg\textsuperscript{Zw3} levels in the wild-type sgg\textsuperscript{null} M11-1 allele and in the pUAS-Wg-expressing embryos, as expected (Fig. 2A). Armadillo immunoblots revealed accumulation of Arm protein in the sgg\textsuperscript{null} M11-1 and pUAS-Wg extracts.

**Overexpression of Dsh Represses Sgg\textsuperscript{Zw3} Protein Kinase Activity**—Overexpression of Dsh protein in cl-8 and S2 cells bypasses the need for Wg and mimics Wg signaling (15). To investigate the effect of overexpression of Dsh on Sgg\textsuperscript{Zw3} activity, we used S2 and cl-8 cell lines expressing Dsh under the control of an inducible metallothionein promoter. Treatment of these cell lines with CuSO\textsubscript{4} led to a time-dependent increase in Dsh protein levels, as well as induction of forms of the protein with reduced electrophoretic mobility similar to the forms observed in untransfected cl-8 cells exposed to Wg protein (Fig. 3, A and C). Concomitant with the increase in Dsh protein levels was an increase in Arm levels (Fig. 3, A and C), indicating that overexpression of Dsh in S2 and cl-8 cells mimics Wg signaling.

To determine whether Dsh protein inhibits Sgg\textsuperscript{Zw3} activity, we examined Sgg\textsuperscript{Zw3} protein kinase activity in the Dsh-inducible cl-8 and S2 cell lines (Fig. 3, B and D). Dsh overexpression in cl-8 and S2 cells revealed similar inhibition curves in both lines and induced a rapid decrease in Sgg\textsuperscript{Zw3} activity that was detectable after 2 h and reached a maximum (70%) after 4–6 h, whereas Sgg\textsuperscript{Zw3} expression levels were unaffected (Fig. 3, A and C). The decrease in Sgg\textsuperscript{Zw3} activity observed in the Dsh experiments in cl-8 cells coincided with the effects of Wg on Sgg\textsuperscript{Zw3} activity in cl-8 cells and supports the genetic model in which Wg repression of Sgg\textsuperscript{Zw3} is mediated via Dsh.

**Overexpression of Drosophila Frizzled 2, a Putative Wg Receptor, Mimics Wg Signaling**—Unlike cl-8 cells, S2 cells do not respond to extracellular Wg as judged by Dsh modification and Arm stabilization (data not shown) (15, 24). Transfection of the transmembrane protein \textit{Drosophila} Frizzled 2 (Dfz2) into S2 cells enables the cells to accumulate Arm in response to Wg, suggesting that Dfz2 acts as a receptor for Wg and that the reason for the lack of responsiveness of these cells to Wg is simply due to lack of Dfz2 expression (7). To investigate whether Dfz2 expression affected Sgg\textsuperscript{Zw3} activity, we used S2 cell lines expressing Dfz2 under the control of an inducible metallothionein promoter. Addition of CuSO\textsubscript{4} to the medium of these cells induced an increase in the levels of Dfz2 RNA (Fig. 4A), leading to the appearance of slower migrating forms of Dsh and an increase in cytoplasmic Arm levels within 2 h, whereas Sgg\textsuperscript{Zw3} protein levels were unaffected (Fig. 4A). However, immunoprecipitates of Sgg\textsuperscript{Zw3} exhibited a time-dependent decrease in protein kinase activity upon induction of Dfz2 expression, similar to the effects of overexpression of Dsh in S2 cells (Fig. 4B). Together, these data demonstrate that overexpression of Dfz2 in S2 cells is sufficient to trigger the Wg pathway, including modification of Dsh, repression of Sgg\textsuperscript{Zw3}, and stabilization of Arm.

**Dishevelled Induces Serine Phosphorylation of Sgg\textsuperscript{Zw3}**—To probe the mechanism via which Wg, Dfz2, and Dsh inactivate...
SggZw3, S2 cell lines harboring inducible Dsh were metabolically labeled with \([32P]\)phosphate, and SggZw3 was immunoprecipitated and resolved by SDS-PAGE. Induction of Dsh expression caused a 2–2.5-fold increase in \([32P]\)phosphate associated with SggZw3 (Fig. 5A). Subsequent phosphoamino acid analysis revealed the presence of only phosphoserine in the S2 cell sample (Fig. 5B). These data suggest that Dsh induces a specific increase in serine phosphorylation of Sgg Zw3, which may mediate the reduction in protein kinase activity. Surprisingly, SggZw3 in S2 cells does not contain detectable phosphotyrosine (34). SggZw3 contained both phosphotyrosine and phosphoserine in cl-8 cells. Since induction of the Wg pathway resulted in equal -fold inhibition in both S2 and cl-8 cells, we conclude that Wg-mediated regulation of SggZw3 is independent of tyrosine phosphorylation.

**Phosphorylation of Arm and D-Axin by SggZw3**—We have shown that negative regulation of SggZw3 activity leads to Arm accumulation in *Drosophila* embryos and cells. Biochemical analysis has indicated that D-Axin/Axin negatively regulates \(\beta\)-catenin/Arm by interacting with GSK-3\(\beta\)/SggZw3 (19). D-Axin is structurally related to vertebrate Axins, with the regions of highest identity corresponding to previously defined binding domains of Axin.2 Armadillo contains “consensus” phosphorylation site sequences for GSK-3/SggZw3 (35). D-Axin also contains such sequences (19). However, it has been reported that mammalian GSK-3 phosphorylates \(\beta\)-catenin significantly only in the presence of the Axin protein (19). Therefore, we examined whether SggZw3 could phosphorylate Arm and D-Axin under conditions in which these proteins formed a complex. To determine whether D-Axin and Arm are substrates for SggZw3, we purified D-Axin or various deletion mutants of D-Axin and Arm from *E. coli* as histidine fusion proteins (Fig. 6). Baculovirus-expressed GST-SggZw3 (36) phosphorylated D-Axin, D-Axin(302–746), D-Axin(356–565), and D-Axin(356–746), but not D-Axin(383–565) and D-Axin(34–356) (Fig. 6). In the absence of D-Axin, no significant phosphorylation of Armadillo was observed, whereas in its presence, the phosphorylation was greatly increased (Fig. 6). These data indicate that Sgg phosphorylation of Armadillo is directed via D-Axin.

**Inhibition of SggZw3 Activity by Wg Affects Its Phosphorylation and Interaction with D-Axin Protein**—We therefore examined whether the inhibition of SggZw3 activity by Wg affects its interaction with D-Axin. To test this possibility, *in vitro* binding and phosphorylation assays were carried out using a D-Axin-(330–642) fusion protein containing SggZw3-binding sites and consensus sites of phosphorylation for SggZw3. D-Axin-(330–642)-His\(_6\) was transfected as a histidine fusion protein into cl-8 cells, cl-8 cells treated with Wg, and cl-8 cells expressing SggZw3. The histidine-tagged complexes from
the cl-8 cell lysates were purified using nickel-Sepharose beads, and the amount of Sgg Zw3 captured on the beads was determined by immunoblotting. In addition, the phosphorylation of D-Axin-(330–642)-His6 by SggZw3 was determined by addition of \([\gamma-32P]\)ATP.

In the lysates from cells treated with Wg, Sgg Zw3 was found in association with D-Axin-(330–642)-His6. However, the degree of binding was reduced; 2-fold compared with the amount of SggZw3 associated with Axin in lysates of untreated cl-8 cells (Fig. 7). The negative effect of Wg signal on the binding of Sgg Zw3 correlated with a decrease in phosphorylation of D-Axin. By contrast, Axin complexes within lysates expressing Sgg Zw3 contained more Sgg Zw3 protein as well as higher Axin kinase activity (Fig. 7). These results indicate that D-Axin physically interacts with SggZw3 and that Wg signaling leads to a reduction of both Sgg Zw3 activity and its interaction with D-Axin.

**DISCUSSION**

Previous studies have shown that treatment of cl-8 cell lines with Wg leads to hyperphosphorylation of Dsh protein and to cytoplasmic accumulation of Armadillo (15, 23, 24). Here, we report that Wg signaling as initiated by Wg, Dfz2, or Dsh expression causes enzymatic inactivation of SggZw3, suggesting post-translational modification of this protein kinase activity. In support of this, induction of Dsh expression increased the levels of SggZw3 phosphorylation 2-fold (Fig. 6), and the presence of phosphoserine in SggZw3 protein from S2 cells suggested that the mechanism of repression of SggZw3 activity is mediated by serine phosphorylation. Previous studies have shown that members of the GSK-3 family are inhibited by phosphorylation at an amino-terminal serine residue (serine 9 in GSK-3\(b\) and serine 21 in GSK-3\(a\)) (33, 37). Phosphorylation of the Sgg Zw3 residue equivalent to serine 9 does not appear to be the mechanism via which the Wg pathway inhibits SggZw3 for several reasons. In mammals, this site is targeted by agents acting via phosphatidylinositol 3-kinase, and the residue can be phosphorylated in vitro and in transfected cells by protein kinase B/AKT (38). However, Wg inhibition of GSK-3 in 10-T1/2 cells is not sensitive to inhibitors of phosphatidylinositol 3-kinase, nor is *Drosophila* protein
activity. However, Arm is a poor in vitro target of Sgg\textsubscript{zw3}. Phosphorylation of Arm is enormously increased in the presence of D-Axin. We have demonstrated that D-Axin is phosphorylated by Sgg\textsubscript{zw3} and that the binding of Sgg\textsubscript{zw3} to D-Axin is dependent upon the level of Sgg\textsubscript{zw3} activity. Repression of Sgg\textsubscript{zw3} activity by Wg signaling induced dissociation of the Sgg\textsubscript{zw3}/D-Axin-Arm complex, leading to an accumulation of Arm protein. Together, these data suggest that Sgg binding is dependent upon or stimulated by its phosphorylation of Axin. Once bound to Axin, it can access the Arm molecule that is associated with Axin and phosphorylate it. Inactivation of Sgg results in dephosphorylation of Axin and release of the kinase, compartmentalizing it away from Arm.

Mammalian studies have suggested that a more complex mechanism for the regulation of β-catenin levels by GSK-3 involved another player, APC. In this case, Axin forms a complex with GSK-3, β-catenin, and APC (19, 20). APC is directly phosphorylated by GSK-3 via Axin, which increases binding of APC to β-catenin and its subsequent degradation (40, 41). Mutation of a Drosophila APC homolog did not affect Wg function, suggesting either divergence of the molecular mechanisms of Arm stabilization or the existence of additional APC-like molecules in flies (21). Resolution of these mechanisms will require identification of the serine kinase acting to inhibit Sgg\textsubscript{zw3} and the means by which it is, in turn, controlled by Dsh.

Acknowledgments—We thank R. Nusse for kindly providing cl-8 cells and Drosophila frizzled 2 cDNA. We thank A. Martinez Arias for Wg antibodies, L. Cherbas and P. Cherbas for pHGCO and pRmHa-1 vectors, and M. Barber for animal assistance.

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