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Serpentine Proteins Slither into the Wingless and Hedgehog Fields

Minireview

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Three recent papers (Bhanot et al., 1996; van den Heuvel and Ingham, 1996; Alcedo et al., 1996) report the discovery of putative receptors for both the Wingless (Wg) and Hedgehog (Hh) proteins. These reports are welcome news as progress towards an understanding of the regulation of the signaling pathways activated by Wnt and Hh signals has been hampered by the lack of identified receptors. Wnt and Hh proteins have been implicated in numerous patterning events during development of both invertebrates and vertebrates. In addition, as is the case for most cell fate determination pathways, abnormal expression of components of either signaling system leads to oncogenesis. In this review, the most recent findings and the current working models for these signaling pathways are discussed.

Dfz2, a Candidate Wingless Receptor

Bhanot et al. (1996) have identified the Drosophila gene, *Dfz2*, as a putative receptor for Wg. *Dfz2* encodes a 624 amino acid protein that belongs to the Frizzled (Fz) family of serpentine proteins (Vinson et al., 1989; Wang et al., 1996), which to date includes ten family members from both vertebrates and invertebrates. Fz proteins possess a putative signal sequence, an extracellular domain composed of a conserved region of 120 amino acids with an invariant pattern of ten cysteine residues (the cysteine rich domain, CRD), a highly divergent hydrophylic region of 40–100 amino acids, 7 putative transmembrane domains, and a short cytoplasmic domain.

The first identified member of this family, *frizzled* (hereafter referred to as *Dfz1*; Vinson et al., 1989), was identified as a Drosophila gene required for planar polarity of epithelia. *Dfz1* mutations exhibit a "tissue polarity" phenotype which is visualized by aberrant orientation of the single hair secreted by each epidermal cell. A connection between the Fz and Wnt families of proteins was indicated by the observation that Dishevelled (Dsh), a cytoplasmic protein required in receiving cells to transduce the Wg signal (Klingensmith et al., 1994), is also necessary for Dfz1 signaling (Krasnow et al., 1995). Dfz1 is probably not a Wg receptor in vivo since *Dfz1* mutants, unlike *wg*, do not have a segment polarity phenotype. The discovery of a second Drosophila *fz*-like gene, *Dfz2*, prompted Bhanot and colleagues (1996) to investigate whether Dfz2 and Wg were functionally connected.

Previously, van Leeuwen et al. (1994) had developed a tissue culture assay for Wg in vitro. In this assay, Wg protein produced and secreted by Drosophila S2 cells is added in soluble form to the imaginal disc–derived cell line, clone 8. Clone 8 cells respond to Wg by specifically increasing the level of hyperphosphorylated Armadillo protein (Arm), a Drosophila homolog of β-catenin. Unlike clone 8 cells, S2 cells are unresponsive to soluble Wg, presumably because they do not express a Wg receptor. When Bhanot and colleagues (1996) transfect Dfz2 into S2 cells, which do not express endogenous Dfz2, the level of hyperphosphorylated Arm is increased, suggesting that Wg acts through Dfz2. Furthermore, immunostaining reveals that Wg protein binds to the surface of S2 cells expressing either full length Dfz2 or the Dfz2 extracellular region. These results provide compelling evidence that Wg and Dfz2 interact either directly or together with a co-receptor, as has been observed for other growth factor receptors. Is Dfz2 the Wg receptor in vivo? Preliminary characterization of the expression pattern of *Dfz2* is consistent with this proposed function. However, the answer to this question will have to await the isolation and characterization of *Dfz2* mutations.

Wg is a member of the large family of Wnt secreted glycoproteins. To begin addressing the question of whether specific Wnts bind to specific Fz proteins, Bhanot et al. (1996) examined the ability of Wg to bind to an array of other Fz receptors. They showed that Wg can bind to some of them, but not all, suggesting that Wnt and Fz proteins are involved in specific ligand–receptor interactions.

Downstream of Dfz2

If Fz proteins encode Wnt receptors, what are the molecular events triggered by the activation of these transmembrane receptors? Many serpentine receptors are G protein–coupled; however, the primary sequences of Fz proteins suggest no such connection. Nevertheless, the binding of Wnts to the Fz CRD extracellular domains could result in a rearrangement of transmembrane domains and activation of downstream cytoplasmic proteins (Bhanot et al., 1996).

Genetic analysis of Wg signaling in Drosophila has identified a number of proteins that have been proposed to act downstream of the activated Wg receptor (reviewed by Perrimon, 1994). In particular, Dsh, a cytoplasmic phosphoprotein absolutely essential for transduction of the Wg signal, is the best candidate for a target of Dfz2. Yanagawa et al. (1995) have proposed that, in clone 8 cells, Wg signaling generates a hyperphosphorylated form of Dsh that is membrane associated. It is unclear whether either of these changes is significant for Dsh signaling function; however, they suggest a commonly used molecular mechanism whereby activation of a transducing receptor leads to recruitment and activation of cytoplasmic molecules.

Dsh contains a PDZ domain that could mediate direct association with Dfz2. The structure of the third PDZ domain from the synaptic protein PSD-95 has recently been solved and has revealed that it interacts with a four residue C-terminal stretch (X-Ser/Thr-X-Val-COO^-) (Doyle et al., 1996). Because this consensus is present at the C-terminus of Dfz2, it is possible that activation of Dfz2 by Wg promotes the recruitment of Dsh to Dfz2. This event may lead to Dsh activation, perhaps by a kinase located at the membrane. Alternatively, the mere increase in the level of Dsh at the membrane may lead to activation or inhibition of other signaling molecules.

Two other proteins, Arm and Zw3 (or Shaggy), which encodes the fly homolog of the mammalian serine/threonine protein kinase GSK3, have been implicated in Wg signaling (see review by Perrimon, 1994). In response to the Wg signal, the level of intracellular Arm is stabilized and correlates with a decrease in the level of Arm phosphorylation. Arm can be detected in the nucleus where it may control gene expression in cooperation with other factor(s), suggesting that, as is observed for other signaling pathways, Wg signaling regulates the nuclear translocation of a cytoplasmic molecule (Orsulic and Peifer, 1996; Yost et al., 1996).

Changes in Arm phosphorylation have been proposed to be a direct consequence of Wg signaling. In one model Wg signaling inhibits, through Dsh, Zw3 kinase activity whose function is to destabilize intracellular Arm and increase the average levels of Arm phosphorylation (see review by Perrimon, 1995). One problem with this model is that it is not clear whether GSK3 activity is downregulated by Wg signaling. Possibly GSK3 is constitutively active and Wg signaling acts in a parallel pathway counteracting GSK3 negative effects. Another problem is that the biochemical relationship between GSK3 and β-catenin is not clear. A number of results have been suggested that β-catenin is a direct target of GSK3. For example, Yost et al. (1996) provided evidence that phosphorylation of the Xenopus β-catenin in vivo requires an *in vitro* amino-terminal GSK3 phosphorylation site. However, Orsulic and Peifer (1996) have proposed that the altered phosphorylation state of Arm in response to Wg signaling may be indirect and simply reflect the function of β-catenin in assembly of adherens junction. In addition to Wg signaling, Arm is also involved in an independent signaling event that involves cadherin. Membrane-associated Arm is more highly phosphorylated than intracellular Arm, and Orsulic and Peifer speculate that the kinase that phosphorylates Arm is localized to the adherens junctions. Thus, the modulation of Arm phosphorylation in response to Wg signaling could be indirect because when the levels of cytoplasmic Arm are raised in response to Wg signaling, the overall average of phosphorylated Arm drops.

Recently, Rubinfeld et al. (1996) have reported that GSK3 and β-catenin are in a complex with the mammalian tumor suppressor protein APC, the product of the adenomatous polyposis coli gene. They showed that GSK3 phosphorylates APC, regulating its interaction with β-catenin. Thus, GSK3 may downregulate the level of intracellular β-catenin by preventing its association with APC. Further studies will be necessary to determine whether a Drosophila APC protein is involved in Wg signaling. Perhaps APC acts as an active effector in Wg signaling, doing more than just acting as a negative regulator of Arm.

If APC is involved in Wg signaling, it will be important to examine how Dfz2 and Dsh regulate the activity of the APC–GSK3–β-catenin complex. Remarkably, APC–β-catenin binds to the human homolog of the Drosophila Disc-large (Dlg) protein and this interaction requires the C-terminal region of APC and the PDZ domain of human Dlg (Matsumine et al., 1996). It is tantilizing to propose that the Dsh PDZ domain interacts with APC and thereby provides a molecular link between Dsh and the APC–GSK3–β-catenin complex. Further support for this model may come from the characterization of the role of Dsh in Dfz1 signaling. Unlike Dfz2, there is no canonical PDZ consensus binding site in the C-terminus of Dfz1, making it unclear whether Dsh and Dfz1 associate directly. Since there is no genetic evidence that either Zw3 or Arm are involved in Dfz1 signaling, the function of Dsh PDZ domain may be specific to Wg signaling in regulating the activity of the APC–GSK3–β-catenin complex.

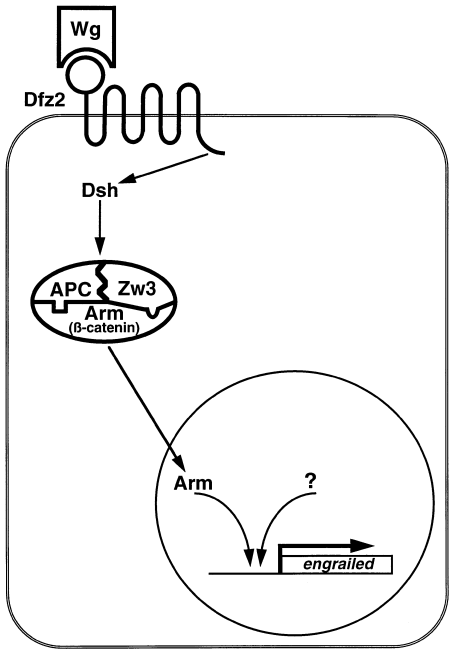

Figure 1. Wg Signaling Pathway
See text for details.

Smoothened, a Candidate Hedgehog Receptor

Two groups (Alcedo et al., 1996; van den Heuvel and Ingham, 1996) report that the Smoothened (Smo) gene encodes a second, more distantly Fz-related serpentine protein that may act as a Hh receptor. Molecular characterization of *smo* reveals that it encodes a 1024 amino acid protein with seven predicted transmembrane domains. The N-terminal region of Smo shows extensive homology to Dfz1 (29% identity and 46% similarity) while the C-terminal cytoplasmic region, which is not present in Fz proteins, contains several consensus target sites for cAMP-dependent protein kinase (PKA, a protein involved in Hh signaling) and G proteins. The presence of these motifs suggest that Smo is a G protein–coupled receptor (Alcedo et al., 1996; van den Heuvel and Ingham, 1996).

Embryos that lack zygotic *smo* gene activity exhibit a weak "segment polarity" phenotype similar to that of weak alleles of *hh* or *wg*. However, van den Heuvel and Ingham (1996) showed that when both maternal and zygotic *smo* gene activity is removed, *smo* embryos have a strong segmentation phenotype similar to that exhibited by mutant embryos of both *wg* and *hh*. In the embryonic epidermis Wg is required for maintenance of *en* and *hh* transcription, and Hh, in turn, is required for maintenance of *wg* expression (reviewed by Perrimon, 1994). Examination of *wg*, *en*, and *hh* expression in *smo*

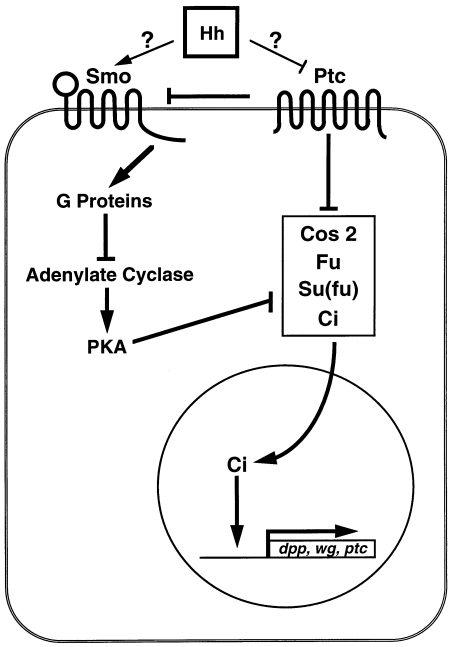

Figure 2. Hh Signaling Pathway

See text for details, as well as reviews by Perrimon (1995) and Ingham (1995).

mutants reveals that Smo is involved in this regulatory network. Furthermore, both groups examined the ability of ectopically expressed Wg or Hh signals to function in the absence of *smo* activity. They found that Wg, but not Hh, was able to signal in a *smo* mutant background, indicating that Smo is necessary for Hh signaling.

Similar conclusions were reached when the function of Smo was examined during imaginal disc development (van den Heuvel and Ingham, 1996). Expression of Hh in the posterior compartment of the wing disc triggers *decapentapegic* (*dpp*) expression along the antero-posterior (A/P) border (reviewed by Perrimon, 1995). Clones of *smo* mutant cells at the A/P border lose *dpp* expression, indicating that Smo is required to transduce the Hh signal.

The results described above provide strong genetic evidence that Smo could encode a receptor for Hh. However, further experiments that use functional in vitro assays, as was used for the characterization of Dfz2, or biochemistry, are clearly needed to establish that Smo acts as a Hh receptor. Hh is a rather unusual protein in that it cleaves itself (reviewed by Perrimon, 1995). It is synthesized as a precursor that undergoes an autocatalytic internal cleavage to generate two products, HhN (19kDa), the signaling activity, and HhC (25KDa), the processing activity. Interestingly, the crystal structure of Sonic Hedgehog reveals that the HhN domain might hydrolyze a peptide substrate (Tanaka Hall et al., 1995). This putative hydrolytic activity of HhN raises the possibility that Hh may activate a receptor by cleavage.

Function of Patched in Hedgehog Signaling and Downstream Events

Previously, the Patched (Ptc) protein, which contains multiple transmembrane domains, was proposed to act as a constitutively active receptor that becomes inactivated by binding to Hh (reviewed by Perrimon, 1995). According to this model, only cells receiving the Hh signal are able to overcome this repression. However, Bejsovec and Wieschaus (1993) reported that the level of *wg* expression is lower in *ptc*; *hh* double mutant embryos than in *ptc* single mutant embryos, suggesting that Hh is able to signal in the absence of Ptc. Although it is unclear whether protein null mutations were used in this study, these results are consistent with a model whereby Hh directly activates Smo, which transduces the signal, while Ptc acts as a protein that downregulates Smo activity. Genetic epistasis experiments between *ptc* and *smo* suggest that Ptc acts upstream of or in parallel to Smo (Alcedo et al., 1996). Assuming that these two integral transmembrane proteins interact, a plausible scenario is that upon binding of Hh to the Smo–Ptc complex, dissociation occurs and Smo is released. Furthermore, the finding that *wg* expression is maintained in the *hh*; *ptc* double mutant suggests that Smo or a downstream component has constitutive activity. To sort out whether it is Smo that is constitutively active, it will be critical to compare the level of *wg* expression between *smo*; *ptc* and *hh*; *ptc* double mutants.

The model that Smo is activated in the absence of its postulated Hh ligand is at odds with the activation mechanism of other known G protein–coupled receptors and may suggest a novel mechanism by which Smo regulates downstream events (Alcedo et al., 1996). Alternatively, it is possible that the activation of Smo is more complicated. In addition to Wg and Hh paracrine pathways, a number of results suggest that Wg also acts in an autocrine manner to maintain its own expression (Alcedo et al., 1996, and references therein). Because Wg binds Dfz2, it is also possible that Wg binds Smo and regulates its activity. Indeed, many of the cysteine residues found in the extracellular CRD domains of Fz proteins are conserved in Smo, suggesting that the topology of the ligand-binding domains may be similar. Alcedo et al. (1996) suggest that it is unlikely that Wg can signal through Smo because ectopic Wg, in the absence of Smo, activates *gooseberry*, a known target of the Wg autocrine pathway in the embryo. However, it is unclear whether this experiment was conducted in the absence of both maternal and zygotic *smo* activity or simply in the absence of zygotic *smo* activity. If Wg is able to contribute to Smo activation, then it would explain why Smo is activated in the absence of both Hh and Ptc. Clearly, this issue needs clarification.

Genetic analyses have identified a number of proteins, PKA, fused (Fu), Suppressor of Fused (Su(fu)), and Cubitus Interruptus (Ci/Gli), whose activity might be directly regulated by Hh signaling (reviewed by Perrimon, 1995; Ingham, 1995). In particular, increased levels of the Zn-finger transcription factor Ci/Gli is able to activate the Hh-target genes *dpp* and *ptc* in imaginal discs (Dominguez et al., 1996). In light of the new results on Smo, additional putative components and regulatory interactions can be added to this pathway. In response to the extracellular Hh signal, Smo may regulate the activity of a heterotrimeric G protein complex. According to this model, in the absence of Hh signal, adenylate cyclase activity results in the production of cyclic AMP and activation of PKA. Repression of adenylate cyclase activity in the presence of Hh may in turn downregulate the level of PKA. Results from genetic epistasis experiments

between Smo and PKA are consistent with this model. In *smo*; *PKA* double mutant clones, *dpp* is expressed, as it is in *PKA* but not in *smo* mutant clones, which is consistent with the model that Smo acts upstream of PKA (van den Heuvel and Ingham, 1996). An obvious prediction of this model is that mutations in both adenylate cyclase and G proteins will interfere with Hh signaling.

Conclusions

Recent progress in our understanding of both Wg and Hh signaling has led to sophisticated working models of their molecular mechanisms of signaling (see Figures 1 and 2). These new findings are the result of the fruitful combination of information gained from both genetic and biochemical approaches. The next steps to be taken should test whether the predictions made from the genetics hold true following biochemical analyses. Reciprocally, biochemical interactions identified between specific molecules need to be validated in vivo.

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