Frizzled and DFrizzled-2 function as redundant receptors for Wingless during *Drosophila* embryonic development

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SUMMARY

In cell culture assays, Frizzled and Dfrizzled2, two members of the Frizzled family of integral membrane proteins, are able to bind Wingless and transduce the Wingless signal. To address the role of these proteins in the intact organism and to explore the question of specificity of ligand-receptor interactions in vivo, we have conducted a genetic analysis of *frizzled* and *Dfz2* in the embryo. These experiments utilize a small gamma-ray-induced deficiency that uncovers *Dfz2*. Mutants lacking maternal *frizzled* and zygotic *frizzled* and *Dfz2* exhibit defects in the embryonic epidermis, CNS, heart and midgut that are indistinguishable from those observed in *wingless* mutants. Epidermal patterning defects in the *frizzled*, *Dfz2* double-mutant embryos can be rescued by ectopic expression of either gene. In *frizzled*, *Dfz2* mutant embryos, ectopic production of Wingless does not detectably alter the epidermal patterning defect, but ectopic production of an activated form of Armadillo produces a naked cuticle phenotype indistinguishable from that produced by ectopic production of activated Armadillo in wild-type embryos. These experiments indicate that *frizzled* and *Dfz2* function downstream of *wingless* and upstream of *armadillo*, consistent with their proposed roles as Wingless receptors. The lack of an effect on epidermal patterning of ectopic Wingless in a *frizzled*, *Dfz2* double mutant argues against the existence of additional Wingless receptors in the embryo or a model in which Frizzled and Dfrizzled2 act simply to present the ligand to its bona fide receptor. These data lead to the conclusion that Frizzled and Dfrizzled2 function as redundant Wingless receptors in multiple embryonic tissues and that this role is accurately reflected in tissue culture experiments. The redundancy of Frizzled and Dfrizzled2 explains why Wingless receptors were not identified in earlier genetic screens for mutants defective in embryonic patterning.

Key words: Wingless, Frizzled, Wnt, *Drosophila*, Segmentation

INTRODUCTION

Wnts are secreted cysteine-rich glycoproteins with diverse roles in animal development (reviewed in Cadigan and Nusse, 1997). Frizzled proteins are cell surface Wnt receptors characterized by an extracellular cysteine-rich domain (CRD) followed by seven transmembrane segments (Vinson et al., 1989; Wang et al., 1996). Members of both protein families have been conserved in species as evolutionarily distant as nematodes and humans (Wang et al., 1996).

The prototypic member of the Frizzled family is the *Drosophila* tissue-polarity gene *frizzled* (*fz*), which was identified because of its phenotype in the adult cuticle (Adler et al., 1987; Gubb and Garcia, 1982). Null alleles of *fz* are viable as adults but their bristles, hairs and ommatidia lose their characteristic polarities (Adler et al., 1987; Gubb and Garcia, 1982; Vinson and Adler, 1987), a condition that is referred to as a tissue or planar polarity phenotype. Historically, tissue polarity signaling has been studied without reference to Wnt signaling, although both signaling pathways are known to be affected by mutations in the *dishevelled* gene (Theisen et al., 1994; Krasnow et al., 1995). A second member of the Frizzled family in *Drosophila*, *Drosophila frizzled* 2 (*Dfz2*), was identified by sequence homology (Bhanot et al., 1996). Both *fz* and *Dfz2* are expressed during embryonic and larval life (Adler et al., 1990; Bhanot et al., 1996), with *fz* expression in the embryo also being maternally derived (Park et al., 1994). Mutations affecting only the *Dfz2* gene have not been described.

The most intensively studied member of the Wnt family is the *Drosophila* segment polarity gene *wingless* (*wg*). *wg* has multiple essential functions during *Drosophila* development (reviewed in Klingensmith and Nusse, 1994). During embryogenesis, it is required for epidermal patterning...
The Wg signal is transduced from the cell surface to the nucleus via the actions of Dishevelled (Dsh; Klingensmith et al., 1994) as noted above, Zeste-white 3 (Zw3), a serine-threonine kinase (Siegfried et al., 1994) and Armadillo (Arm), a β-catenin homolog (Peifer et al., 1994; reviewed in Cadigan and Nusse, 1997). Wg signalling stabilizes cytoplasmic Arm, which translocates to the nucleus where it forms a complex with dTCF/pangolin, an HMG-box protein (Brunner et al., 1994; Riese et al., 1997; van de Wetering et al., 1997). This complex then activates target genes (Brunner et al., 1997; Riese et al., 1997; van de Wetering et al., 1997). Work by several investigators has shown that these same components are utilized to transduce the Wg signal in different tissues or cell types but the identity of the Wg receptor(s) in these tissues had, until recently, been unknown.

Several lines of evidence indicate that the Fz family encodes receptors for the Wnt family of signaling molecules. In tissue-culture experiments, Drosophila S2 cells transfected with either fz or Dfz2 bind exogenous Wg on the cell surface and transduce the Wg signal as assayed by the stabilization of Arm (Bhanot et al., 1996). Similar binding results have been obtained using Wg or Xenopus Wnt8 (XWnt8) and mammalian cells transfected with various Frizzled proteins (Hsieh et al., 1999). In Xenopus embryos, Xwnt-8 synergizes with rat Frizzled-1 (Rfz1) to activate transcription of the Wnt target, Siamois (Yang-Snyder et al., 1996) and XWnt-5A synergizes with human Frizzled-5 (Hfz5) to induce axis duplication (He et al., 1997). Finally, experiments in Drosophila show that ectopic expression of Dfz2 transgenes in wing imaginal discs expands the domain of Wg signaling and ectopic expression of the extracellular domain of fz or Dfz2 inhibits Wg signaling (Zhang and Carthew, 1998; Cadigan et al., 1998).

In addition to the cell culture and ectopic expression studies described above, loss-of-function mutations in Caenorhabditis elegans have also implicated particular Frizzled and Wnt proteins as potential receptor-ligand pairs. These experiments define the roles of lin-17 (Fz) and lin-44 (Wnt) in various asymmetric cell divisions (Herman et al., 1995; Sawa et al., 1996), mom-5 (Fz) and mom-2 (Wnt) in endoderm induction (Rocheleau et al., 1997), and lin-17 and egl-20 in neuronal migration (Harris et al., 1996; Malof et al., 1999). However, in each of these cases, the phenotypes of the respective Wnt and Fz genes are qualitatively similar but are not identical, possibly due to additional factors (Sawa et al., 1996), lack of null mutations (Harris et al., 1996) or, in the case of mom-5, a possible role in antagonizing mom-2 signaling (Rocheleau et al., 1997).

In this report, we focus on the role of Fz and Dfz2 in Drosophila embryogenesis. fz is widely expressed in the embryo and Dfz2 is expressed in the CNS, midgut and dorsal vessel, and in a segmental pattern in the embryonic epidermis (Adler et al., 1990; Park et al., 1994; Bhanot et al., 1996). Our point of departure was the hypothesis, based on the tissue-culture experiments described above and the pattern of embryonic expression, that Dfz2 and/or Fz might act in vivo as receptors for Wg. This model is superficially at odds with the observation that fz mutants do not display any defects in segment polarity as would be expected from a loss of Wg signaling during embryogenesis. If Fz functions as a Wg receptor during embryogenesis, its role must be redundant.

During the course of this study, several groups reported experiments that point to a requirement for both Fz and Dfz2 in Wg signalling in the embryo. These experiments used RNA interference (Kennerdell and Carthew, 1998) or large synthetic deficiencies (Bhat, 1998; Muller et al., 1999) to demonstrate that the simultaneous loss of both fz and Dfz2 mimics the loss of wg on the embryonic epidermis and CNS. However, the low efficiency of the wg-like phenotype in the RNA interference experiments and the developmental arrest associated with elimination of approximately 5% of the genome in the synthetic deficiency experiments limit the conclusions that can be drawn from these experiments. Thus, a number of questions regarding the in vivo relationship between wg, fz and Dfz2 remain to be addressed, in particular the question of whether loss of fz and Dfz2 fully recapitulates the wg phenotype.

In the present study, we have generated a small deficiency that encompasses the Dfz2 locus. We show that embryonic development in general, and Wg signaling in particular, is nearly normal in deletion homozygotes. However, embryos lacking both maternal fz and zygotic fz and Dfz2 display defects in epidermal patterning, RP2 neuron specification, midgut morphogenesis and heart formation, which are extremely similar to the defects exhibited by wg mutants. We further show that the defects in epidermal patterning in the double mutants are rescued by ectopic expression of either fz or Dfz2, are unaffected by ectopic expression of wg and are over-ridden by ectopic expression of an activated form of arm. These results indicate that Dfz2 and Fz are the principal embryonic receptors for Wg and that they function redundantly in multiple tissues to transduce the Wg signal. Moreover, since the fz, Dfz2 double mutants do not display defects in addition to those seen in wg mutants, it is likely that Wg serves as the predominant or only ligand for Dfz2 and Fz during embryogenesis.

MATERIALS AND METHODS

Cloning of Dfz2 cDNA and genomic DNA

To isolate Dfz2 cDNA clones, we screened a 0-9 hour embryo cDNA library (a gift from K. Zinn) with a PCR product corresponding to the first 237 nucleotides of the coding exon and sequenced three clones with the longest inserts 5' of that point. To isolate genomic clones corresponding to exons 1 and 2, a Drosophila genomic library (a gift from T. Maniatis) was screened with a PCR product corresponding to exons 1 and 2. Sequences from the cDNA and genomic clones were compared to identify the splice sites between exons 1/2 and exons 2/3. Two P1 clones encompassing the Dfz2 locus were obtained by PCR screening (Genome Systems) and the extended restriction map shown in Fig. 1 was assembled by construction of a lambda phage library from one of these P1 clones.

Drosophila stocks and recombinant chromosomes

The creation of the 469-2 deletion (hereafter referred to as Df(3L)Dfz2) and the various fz alleles used are described in the text. Df(3L)Dfz2 was recombined with fz alleles in the following way. From a cross with Df(3L)fz (at 76A) over a Dichaete (D), P[FRT24, w*]
A chromosome (D is at map position 70D1 and the w+ at map position 79F), 20% of the w+ chromosomes that had lost the D marker had acquired Df(3L)Dfz2. A chromosome containing Df(3L)Dfz2, P[FRT2A, w+] was then created by screening for w+, D progeny and used to recombine various fz alleles (at map position 70D5) with Df(3L)Dfz2, P[w+] by selecting for the loss of D. The wg alleles used were wg\text{IN}, wg\text{CX4}, and wg\text{CN}. The wg\text{IN} is a molecular null and wg\text{CN} encodes a nonsecreted Wg protein (van den Heuvel et al., 1993).

**Rescue and ectopic overexpression experiments**

For the rescue experiments described in the text, where the Da-Gal4 driver (Georgias et al., 1997) is used to drive either a fz (provided by P. Adler), Dfz2 (Cadigan et al., 1998) wg (provided by I. Levine-Bar and H. Krause) or an activated arm (arm\text{null}; Pae et al., 1997) transgene. P[Da-Gal4], fz recombinants were used to create P[Da-Gal4], fz, Df(3L)Dfz2 P[FRT2A, w+] recombinants (P[Da-Gal4] and fz are closely linked). These could be crossed with P[Da-Gal4], fz flies to create the P[Da-Gal4], fz, Df(3L)Dfz2 P[FRT2A, w+]/Da-Gal4, fz mothers for the experiments. For the ectopic Dfz2 experiments, P[UAS-Dfz2\text{FRT2A, w+}] was recombined onto a fz, Df(3L)Dfz2 P[FRT2A, w+] chromosome in the following way. Both P[w+] minigenes have orange pigmentation when present in one copy, so recombinants containing both P[w+] were identified. The presence of fz and Df(3L)Dfz2 were then tested by complementation. For UAS lines of fz, wg and arm\text{null} (all of which are on the second chromosome), stocks with these transgenes and fz\text{R52}, Df(3L)Dfz2 P[FRT2A, w+] were created over the 2-3 compound chromosome SM5a-TM6B. The compound chromosome ensured that the UAS chromosome and the fz, Dfz2 recombinant co-segregates. Thus, one-quarter of the progeny will be UAS-X+, P[Da-Gal4], fz, Df(3L)Dfz2/fz, Df(3L)Dfz2.

An unforeseen problem with the crosses involving SM5-TM6 chromosomes is that a large number of poorly developed cuticles are observed (see Fig. 6D). Control crosses indicated that these occur in any cross with fz\text{D21}, Df(3L)Dfz2 mothers and SM5-TM6 fathers. It may be that the combination of the fz\text{D21} and Df(3L)Dfz2 deficiencies over this compound chromosome results in non-specific lethality of some (but not all) of these animals.

**Cuticle preparation**

Croses were set up with the specific phenotypes indicated in Tables 1 and 2. Flies were allowed to lay eggs on grape juice plates for 20-24 hours. Flies were removed and the plates were incubated for an additional 24-36 hours at 25°C. During this time, moist yeast was placed in the center of each plate to attract hatching larvae. In this way, the majority of hatched animals could easily be removed. Unhatched eggs could then be removed with a wet brush and cuticles prepared as previously described (Cadigan et al., 1994), using a heptane/methanol treatment to remove the vitelline membrane.

In these experiments, there is usually a background of hatched wild-type larvae carried along in the cuticle preparation. As indicated in Tables 1 and 3, these individuals were typically between 1 and 8% of the total in crosses where one-quarter of the progeny did not hatch. In a few cases (crosses D in Table 1 and F in Table 3), the percentage of wild type is higher due to a lower number of unhatched larvae.

**Immunohistochemistry**

Antibody staining was performed as described (Reuter and Scott, 1990). Embryos were dechorionated, devitellinized in methanol/heptane and fixed in 4% formaldehyde in PBS. Antibody dilutions were as follows: monoclonal anti-Engrailed 4D9 at 1:1000 (Iowa Developmental Studies Hybridoma Bank; IDSHB), monoclonal anti-Eve skipped 3C10 at 1:20 (IDSHB), monoclonal anti-Wingless at 1:20 (IDSHB).

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**Table 1. Distribution of cuticle phenotypes seen with various fz, Dfz2 chromosomes**

<table>
<thead>
<tr>
<th>Cross</th>
<th>Maternal</th>
<th>Paternal</th>
<th>Most severe genotype</th>
<th>n</th>
<th>Cuticle phenotype of unatched embryos (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>75</td>
<td>100%</td>
</tr>
<tr>
<td>B</td>
<td>Df(3L)Dfz2</td>
<td>Df(3L)Dfz2</td>
<td>Df(3L)Dfz2/Df(3L)Dfz2+</td>
<td>93</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>fzR52, Df(3L)Dfz2</td>
<td>Df(3L)Dfz2</td>
<td>Df(3L)Dfz2</td>
<td>103</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Df(3L)Dfz2</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>fzR52, +</td>
<td>Df(3L)Dfz2</td>
<td>+</td>
<td>32</td>
<td>72</td>
</tr>
<tr>
<td>E</td>
<td>fzR52, +</td>
<td>Df(3L)Dfz2</td>
<td>+</td>
<td>117</td>
<td>3</td>
</tr>
<tr>
<td>F</td>
<td>fzR52, +</td>
<td>Df(3L)Dfz2</td>
<td>+</td>
<td>118</td>
<td>3</td>
</tr>
<tr>
<td>G</td>
<td>Df(3L)Dfz2</td>
<td>+</td>
<td>+</td>
<td>146</td>
<td>3</td>
</tr>
<tr>
<td>H</td>
<td>Df(3L)Dfz2</td>
<td>+</td>
<td>+</td>
<td>86</td>
<td>5</td>
</tr>
<tr>
<td>I</td>
<td>Df(3L)Dfz2</td>
<td>+</td>
<td>+</td>
<td>102</td>
<td>4</td>
</tr>
</tbody>
</table>

Cuticle preparations were made and analyzed as described in Materials and Methods. The classes of cuticle phenotype (1-5) are defined as follows: (1) trapezoidal arrays are slightly disorganized; distinct from wild type; phenotype seen in crosses B and C distinct from D. The n value for D is low because there are very few unhatched embryos. (2) Extra denticles observed in posterior compartment of at least two segments. (3) Denticles connecting at least 2 trapezoids. (4) Entire lawn of denticles. (5) Similar to wg mutants. See Fig. 2 for examples of these classes.

#These are hatched larvae.
1:10 (a gift from S. Cohen), rat anti-Titin at 1:1000 (a gift from D. Andrew), and polyclonal anti-lab at 1:1000 (a gift from D. Andrew), and polyclonal antimouse anti-Teashirt (a gift from M. P. Scott) at 1:2000, rat anti-Titin at 1:10000 (a gift from C. Machado), rat anti-Teashirt (a gift from M. P. Scott) at 1:2000, rat anti-Titin at 1:10000 (a gift from C. Machado), rat anti-Teashirt (a gift from M. P. Scott) at 1:2000, and polyclonal antimouse anti-Teashirt (a gift from M. P. Scott) at 1:2000, rat anti-Titin at 1:10000 (a gift from C. Machado), rat anti-Teashirt (a gift from M. P. Scott) at 1:2000.

Immunostaining using mAb3C10 was performed as described in Materials and Methods. Df(3L)Dfz2/TM6GFP flies were self-crossed to produce Df(3L)Dfz2 homozygous embryos, which were identified by their lack of staining with an anti-GFP antibody.

RESULTS

Dfz2 gene structure and identification of a P-element within the Dfz2 transcription unit

The Dfz2 gene has previously been mapped to 76A (Bhanot et al., 1996). Fig. 1 shows a restriction map of the Dfz2 locus together with the structure of the Dfz2 transcript deduced from an analysis of six cdna clones derived from a Drosophila embryo library. The Dfz2 gene consists of at least three exons, with the entire open reading frame being contained on a single exon, exon 3. We note that the full-length Dfz2 transcript may contain noncoding sequences in addition to those indicated in Fig. 1 since the size of the transcript predicted from the cdna clones isolated thus far is about 4 kb while northern blotting reveals a transcript of about 5.5 kb (Bhanot et al., 1996).

To identify a P-element integrated within or near the Dfz2 locus, we characterized five Drosophila lines in which a P-element had been reported to map in or near 76A. Genomic DNA flanking each P-element insertion site was cloned by plasmid rescue and hybridized against a panel of P1 clones carrying the Dfz2 locus. The insertion site of a single P-element line, 469, was found to reside on a subset of the P1 clones, and further mapping and sequencing showed that the 469 P-element is inserted 60 bases upstream of Dfz2 exon 1 (Fig. 1). Dfz2 transcripts from 469 embryos are indistinguishable from the wild type in size but are reduced in abundance (data not shown). Animals homozygous for this insertion show no obvious morphologic defects during embryogenesis or adulthood.

Deletion of the Dfz2 locus and construction of fz, Dfz2 double mutants

The 469 line was used in a gamma-ray mutagenesis screen and nitroblue tetrazolium (Boehringer). Embryos were cleared and mounted in methyl salicylate.

<table>
<thead>
<tr>
<th>Cross</th>
<th>UAS-Dfz2; fz, Df(3L)Dfz2</th>
<th>n</th>
<th>None</th>
<th>Weak</th>
<th>Strong</th>
<th>Naked</th>
<th>‘Ghost’</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>DaGal4, f, Df(3L)Dfz2</td>
<td>113</td>
<td>8</td>
<td>0</td>
<td>91</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>DaGal4, f, Df(3L)Dfz2</td>
<td>117</td>
<td>5</td>
<td>94</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>UAS-fz, Df(3L)Dfz2</td>
<td>190</td>
<td>2</td>
<td>59</td>
<td>0</td>
<td>0</td>
<td>39</td>
</tr>
<tr>
<td>D</td>
<td>UAS-wg; f, Df(3L)Dfz2</td>
<td>318</td>
<td>2</td>
<td>0</td>
<td>26</td>
<td>51</td>
<td>21</td>
</tr>
<tr>
<td>E</td>
<td>UAS-arm; f, Df(3L)Dfz2</td>
<td>150</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>79</td>
<td>20</td>
</tr>
<tr>
<td>F</td>
<td>+, +</td>
<td>123</td>
<td>26</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>74</td>
</tr>
</tbody>
</table>
homozygous embryos indicated that the Dfz2 locus and expression were absent (data not shown). For convenience, we will simply refer to the 469-2 deletion hereafter as Df(3L)Dfz2 or as a Dfz2 deficiency.

As described below, our initial assessment of the mutant phenotype focused on patterning in the embryonic cuticle. In wild-type larvae, the cuticle is covered by an alternating pattern of denticle belts and naked cuticle that is specified by the interaction of segment polarity genes in the embryonic epidermis. Wg action specifies both denticle diversity in the anterior half of each segment and naked cuticle in the posterior half. In wg mutants, there is loss of naked cuticle characteristic of the posterior half and reduction in the diversity of denticles covering the anterior half of each segment such that the denticles present on the cuticle are morphologically similar to those of wild-type row 5 (Fig. 2G,K; Dougan and Dinardo, 1992; Bejsovec and Wieschaus, 1993).

Dfz2-deficiency homozygotes die shortly after hatching and exhibit a subtle disorganization of denticle patterning with occasional ectopic denticles in posterior compartments. These data suggest that Dfz2 and/or other genes removed by the 469-2 deficiency play a minor or largely redundant role in cuticle patterning during embryogenesis. In light of the similarity in sequence and Wg-binding properties exhibited by Fz and Dfz2, we tested the hypothesis that Fz and Dfz2 might function redundantly by constructing double mutants in which various fz alleles (at map position 70D5) were recombined with the Dfz2 deficiency. For these experiments,
**Fig. 3.** En and Wg expression is lost in fz, Dfz2 mutant embryos. Lateral views of stage 10 embryos stained with (A-E) anti-En and (F,G) anti-Wg antibodies. (A,D,F) Wild-type embryos. (B,E,G) fz, Dfz2 mutant embryos obtained from a cross between fz^P21, Df(3L)Dfz2/fz^P21, + mothers and fz^P21, Df(3L)Dfz2/TM6 fathers. (C) wg^IN mutant embryo. (D,E) High-magnification views of single segments. Wild-type embryos (A,D) show En expression in both the CNS and epidermis (arrowhead in D) whereas fz, Dfz2 (B,E) and wg embryos (C) show a loss of En expression in the epidermis (arrowhead in E) but not in the CNS where En expression is independent of Wg signaling. In fz, Dfz2 embryos, the stripes of Wg staining (G) are both narrower and reduced in intensity compared to wild type (F).

**Fig. 4.** Defects in RP2 specification and heart morphogenesis in fz, Dfz2 mutant embryos. (A-E) Ventral views of embryos stained with anti-Eve to visualize RP2 neurons. Arrows mark RP2 neurons; arrowheads mark the positions of missing RP2 neurons. (A) Wild-type embryo. (B) fz, Dfz2 mutant embryos from a cross between fz^D21, Df(3L)Dfz2/fz^P21, + mothers and fz^P21, Df(3L)Dfz2/TM6C fathers. RP2 neurons are completely missing. (C) fz, Dfz2 embryos from a cross between fz^R52, Df(3L)Dfz2/fz^P21, + mothers and fz^R52, Df(3L)Dfz2/TM6C fathers show a variable loss of RP2 neurons. (D) wg null embryos (wg^IN/wg^IN) show a complete loss of RP2 neurons. (E) Df(3L)Dfz2/Df(3L)Dfz2 embryos show occasional loss or misplacement of RP2 neurons. See Table 2 for quantitation of the RP2 loss in the different genetic backgrounds. These embryos were derived from a Df(3L)Dfz2/GFP-TM6 stock and identified by their lack of GFP immunostaining. (F-I) Lateral views of embryos stained with anti-Eve to visualize cardiac precursors. (F) Wild-type embryos showing Eve-stained cardiac precursors. (G) fz, Dfz2 embryo from a cross between fz^P21, Df(3L)Dfz2/fz^P21, + mothers and fz^P21, Df(3L)Dfz2/TM6C fathers. (H) fz, Dfz2 mutant embryo from a cross between fz^R52, Df(3L)Dfz2/fz^P21, + mothers and fz^R52, Df(3L)Dfz2/TM6I fathers. (I) wg null embryos (wg^IN/wg^IN). (G-I) Mutant embryos show a complete loss of Eve-stained cardiac precursors. (G) The plane of focus reveals expression of Eve in the CNS. Embryos are stages 16 (A,E), 14 (B-D) and 12 (F-I).
In frizzled, Dfz2 mutants and in those described below, we have used the following fz alleles (Jones et al., 1996): (1) fz P21, a frameshift near the amino terminus that behaves as a null mutation, (2) fz R52, a premature termination codon that removes the last transmembrane domain and which behaves as a null mutation with respect to the adult tissue polarity phenotype, but which behaves as a hypomorph in the embryo experiments described below, and (3) fz D21, a deficiency that encompasses polytene bands 70D2-70E8. The fz R52 allele produces small amounts of a truncated Fz protein as determined by western blotting while fz D21 and fz P21 produce no detectable protein (Jones et al., 1996).

While embryos homozygous for the Dfz2 deficiency have essentially normal segmentation (Fig. 2E), Dfz2-deficiency embryos in which both fz alleles are also mutant display variable segmentation defects (Fig. 2B-D). The defects range from a few extra denticles in the posterior part of some segments (Fig. 2B) to a complete replacement of naked cuticle with denticles on the ventral side of the embryo (Fig. 2D; see Table 1 for quantitation of the frequencies). This “lawn of denticles” is reminiscent of phenotypes obtained with a temperature-sensitive allele of wg (Bejsovec and Wieschaus, 1993) or mutations in DTCF, the DNA-binding protein that mediates Wg signaling in the nucleus (Brunner et al., 1997; van de Wetering et al., 1997). Thus, the absence ofzygotic fz and Dfz2 produces a cuticle phenotype consistent with a partial defect in Wg signaling.

The role of maternal and zygotic fz and Dfz2 in patterning of the embryonic cuticle

While the expression of Dfz2 appears to be predominantly zygotic (Bhanot et al., 1996), the high levels of fz transcripts found in early embryos (Park et al., 1994) suggests that it has significant maternal expression. Because fz mutants are viable, maternal contribution can easily be removed by using fz, Dfz2/+ mothers (crosses H and I in Table 1). Crossing these mothers with fz, Dfz2/+ fathers results in embryos in which naked cuticle fate is lost, and the cuticle is covered with denticles (Fig. 2F) that predominantly resemble those found in row 5 in the normal cuticle (Fig. 2I). This phenotype is very similar to that of wg null mutants (Fig. 2G,K) and distinct from that of hedgehog (Hh) mutants (Fig. 2H). The difference in the phenotypes between crosses E-G and H, I in Table 1 indicate that one copy of maternal fz can partially rescue the segmentation defect.

Crosses C and D were designed to test whether either zygotic fz or Dfz2 could rescue the phenotype. In cross C, one quarter of the embryos lack maternal fz and zygotic Dfz2, but have one copy of zygotic fz. Most of the unhatched embryos from this cross showed the subtle denticle defect seen in Dfz2-deficiency homozygotes, and a few have very weak segment polarity defects. This indicates that one zygotic copy of fz can almost completely rescue the segment polarity defect. In cross D, where one half of the embryos are mutant for maternal and zygotic fz and heterozygous for Dfz2, most embryos hatch and...
the few that do not have either wild-type cuticles or only slight denticle defects. These data indicate the following order of rescuing activity: zygotic Dfz2 > zygotic fz > maternal fz.

This cuticle analysis also indicates that the fzR52 allele is not null for the segmentation defects observed. For example, fzR52, Dfz2fzR52, Dfz2 embryos (cross E in Table 1) have less severe defects (81% have cuticles similar to those in Fig. 2B or C) compared with fzD21, Dfz2fzR52, Dfz2 embryos (cross G in Table 1; 57% have cuticles similar to those in Fig. 2D or F). Also, subtle phenotypic differences can be seen in the density and type of denticle observed in cuticles lacking maternal fz as well as zygotic fz and Dfz2, with fzR21/fzD21 being more severe than fzR52/fzR52 (data not shown). The hypomorphic nature of the fzR52 allele for Wg signaling is confirmed by the immunostaining experiments described below.

In the text that follows, embryos lacking maternal fz and zygotic fz and Dfz2 will simply be referred to as fz, Dfz2 double mutants. The exact allele combinations are noted in the text, tables and figure legends. In each of the experiments described below in which fz, Dfz2 double mutants were characterized morphologically or by immunostaining, approximately 25% of the embryos showed an aberrant phenotype as expected. Since the control crosses in Table 1 indicate that the presence of one copy of fz or Dfz2 suffices to prevent severe defects in embryogenesis, we presume that in each cross the approximately one quarter of embryos that are aberrant represent the fz, Dfz2 mutant embryos. For immunostaining analyses performed late in embryonic development, the fz, Dfz2 embryos were readily distinguished by their decreased length.

**fz and Dfz2 are required during embryogenesis to maintain en and wg expression in the epidermis**

In the wild-type epidermis, wg functions in an autocrine pathway to maintain its own expression (Hooper, 1994; Yoffe et al., 1995) and in a paracrine regulatory loop to maintain expression of en in adjacent cells (DiNardo et al., 1988; Martinez-Arias et al., 1988). In the epidermis at gastrulation, when wg function is first detected, a stripe of cells in the anterior half of each parasegment expresses wg and an adjacent stripe of cells in the posterior half express en (DiNardo et al., 1988; Martinez-Arias et al., 1988). This pattern is initiated by pair-rule and gap genes, but its maintenance requires paracrine signaling by Wg to the en expressing cells and both paracrine signaling by Hh and autocrine signaling by Wg to the wg expressing cells. Thus, in wg mutant embryos the pattern of wg and en expression is initiated correctly but is not maintained.

In fz, Dfz2 double-mutant embryos, the En stripes begin to fade at stage 9/10 and are completely absent from the epidermis by mid stage 10 (Fig. 3B) similar to wg mutants (Fig. 3C). By contrast, en expression within the CNS is maintained (Fig. 3E) as it is in wg mutants (data not shown). Consistent with a defect in Wg signaling, Wg expression is greatly reduced in fz, Dfz2 mutants (Fig. 3G). The effect on En and Wg expression in the epidermis is less severe in fz, Dfz2 double-mutant embryos heterozygous for fzR52/fzD21 compared to embryos carrying fzD21/fzD21 (data not shown).

**fz and Dfz2 are required for the specification of RP2 neurons and heart precursors**

At the end of gastrulation, wg participates in the morphogenesis of various embryonic structures. In the embryonic central nervous system, wg is expressed by row 5 neuroblasts (NBs) and its function is required to specify NBs in rows 4 and 6 (Chu-Lagraff and Doe, 1993). Null mutants of wg show a loss or duplication of several NBs, the most extensively studied being NB-4. The NB-4 lineage gives rise to two RP2 motoneurons per segment that innervate the dorsal musculature and are missing in wg mutant embryos (Chu-Lagraff and Doe, 1993).

RP2 neurons are marked by their expression of even-skipped (eve; Fig. 4A,B). Mutant embryos missing maternal fz and zygotic fz and Dfz2 or missing only zygotic Dfz2 were examined using an antibody against Eve. fz, Dfz2 double-mutant embryos carrying the fzD21/fzD21 alleles show a complete loss of RP2 neurons in all hemisegments. As observed in the epidermis, the fzR52 allele shows residual activity: in fz, Dfz2 double mutants carrying the fzR52 allele, approximately 26% of the double-mutant embryos showed Eve-positive RP2 staining in 1-3 hemisegments. Interestingly, 469-2 homozygous embryos also show a weakly penetrant RP2 phenotype. In approximately 21% of the 469-2 homozygous embryos, an RP2 neuron is either missing or misplaced in 1-3 hemisegments (see Table 2 for details). We conclude that fz and Dfz2 are largely but not entirely redundant in specifying RP2 identity. These observations are in good agreement with those reported by Bhat (1998).

Wg signaling is required to specify cardiac precursor cells by directly or indirectly maintaining the expression of the homeobox gene tinman (tin) in the cardiac mesoderm (Park et al., 1996), eve is expressed in a subset of heart precursor cells at stage 10/11, and these cells are missing in wg mutants (Lawrence et al., 1995; Fig. 4I). In fz, Dfz2 double-mutant embryos, eve-expressing cardiac precursors are similarly missing (Fig. 4G,H), a phenotype that is completely penetrant even in fz, Dfz2 double mutants carrying fzR52 (Fig. 4H).

**fz and Dfz2 are required for midgut development**

In the wild-type embryo, Wg signaling is required independently in both germ layers that constitute the midgut. It is required in the visceral mesoderm for the formation of the second midgut constriction (Immergluck et al., 1990) and in the midgut endoderm (Hoppler and Bienz, 1995). At the molecular level, Wg activates the expression of the zinc-finger gene Teashirt (tsh) in the visceral mesoderm (Mathies et al., 1994; Fig. 5D) and the homeotic gene Labial (lab) in the endoderm (Immergluck et al., 1990; Reuter et al., 1990; Yu et al., 1996; Fig. 5G). In wg embryos, Tsh expression is lost from the central midgut while its anterior expression is maintained (Fig. 5F). In the endoderm of wg embryos, the stripe of lab expression is not induced to the wild-type level (Fig. 5I).

We have characterized midgut development in fz, Dfz2 double-mutant embryos using three markers. Titin, a protein expressed in somatic and visceral muscles (Bilder and Scott, 1998) was used to visualize midgut morphology (Fig. 5A-C), and Tsh (Fig. 5D-F) and Lab (Fig. 5G-I) were used as markers for Wg signaling in the visceral mesoderm and endoderm, respectively. fz, Dfz2 embryos lack the normal number of midgut constrictions (Fig. 5B). These embryos are also missing tsh expression in the central domain although its anterior expression persists (Fig. 5E). Finally, lab expression is
Ectopic expression of fz or Dfz2 rescues defects in segment polarity observed in double-mutant embryos

Since the 469-2 deficiency removes a number of genes in the 76A interval, it is possible that the defects described above are due to the loss of genes other than Dfz2. To address this possibility, we asked whether a Dfz2 transgene (UAS-Dfz2) under the control of a Daughterless GAL4 (Da-GAL4; Georgias et al., 1997) driver could rescue the fz, Dfz2 double-mutant cuticle phenotype. In this experiment, we observe that ectopic expression of Dfz2 produces nearly complete rescue (Fig. 6B), strongly suggesting that among the genes deleted in the 469-2 interval, Dfz2 is the only one that is relevant to the segment polarity and cuticle patterning defects observed in fz, Dfz2 double mutants. We note that this experiment does not eliminate the formal possibility that a third as-yet undiscovered frizzled-like gene might reside in the 469-2 interval and play a redundant role in Wg signaling. Near complete rescue of the fz, Dfz2 cuticle phenotype is also achieved by ectopic expression of a fz transgene (Fig. 6C), the expected result given that the fzP21 and fzR52 point mutations within the fz coding region. The ability of either ectopic fz or Dfz2 to rescue the segment polarity defects in the fz, Dfz2 double mutants further confirms the overlap in function of these two proteins in the embryonic epidermis.

Effect of ectopic wg and activated arm on the fz, Dfz2 double-mutant phenotype

Although the data presented thus far are most readily explained by a model in which Fz and Dfz2 act as signal transducing receptors for Wg, it is formally possible they are not bone fide Wg receptors but rather act to concentrate, transport or stabilize Wg, or present Wg to its real receptor. Proteoglycans are presumed to act in this manner and mutations affecting proteoglycan synthesis produce wg-like phenotypes in the embryo (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997) that can be rescued by high level ectopic expression of wg (Hacker et al., 1997). Moreover, in light of the diverse actions of Wg during embryonic development, it is also possible that Wg utilizes additional and as yet uncharacterized receptors and/or coreceptors that act independently or in conjunction with Fz and Dfz2. Under any of the preceding scenarios, high level ectopic expression of wg might either modify or correct the patterning defects seen in fz, Dfz2 double-mutant embryos, as was observed for the proteoglycan biosynthetic mutants. We therefore tested this possibility by overexpressing Wg in the fz, Dfz2 double-mutant embryos using a Da-GAL4 driver. In this experiment, no alterations in the fz, Dfz2 cuticle phenotype were observed, arguing against the existence of additional Wg receptors in the segment polarity pathway and against models in which Fz or Dfz2 concentrate, stabilize or transport Wg or present it to a coreceptor.

If Fz and Dfz2 act as Wg receptors, then constitutive activation of any of the intracellular components of the Wg signaling cascade should bypass the receptor defect in fz, Dfz2 double-mutant embryos. To test this hypothesis, we determined the phenotypic effect of ectopic expression of a constitutively activated arm transgene (Pai et al., 1997) in the fz, Dfz2 double-mutant background. As seen in Fig. 7C and D, the naked cuticle phenotype is produced in both wild-type and fz, Dfz2 double-mutant embryos expressing the activated arm. Taken together, the wg and arm ectopic expression experiments indicate that Fz and Dfz2 function downstream of Wg and upstream of Arm, consistent with their proposed roles as Wg receptors.

DISCUSSION

Dfz2 and Fz are redundant receptors for Wg in multiple embryonic tissues

The results presented here strongly suggest that Fz and Dfz2 are the principal Wg receptors in the embryo. The evidence for this claim is two fold. First, mutants lacking maternal fz and zygotic fz and Dfz2 exhibit defects in the embryonic epidermis, CNS, heart and midgut that are indistinguishable from those observed in wg mutants. Second, overexpression of wg does not detectably alter the patterning defects of the double-mutant embryos. The redundancy of Fz and Dfz2 explains why Wg receptors were not identified in earlier genetic screens for mutants defective in embryonic patterning (Nüsslein-Volhard and Wieschaus, 1980; Perrimon et al., 1996).

In the fz, Dfz2 mutant analysis, defects identical to wg were...
When characteristic lack of denticles is observed (A) or fz, Dfz2, "ghost" cuticles caused by the SM5a-TM6B cuticle phenotype shown (except for the from unhatched embryos had the naked cuticle phenotype shown (except for the "ghost" cuticles caused by the SM5a-TM6B balancer). Note that in D, we cannot distinguish individuals that are heterozygotes from fz, Dfz2 mutants, since all embryos in this cross have the naked cuticle phenotype and are indistinguishable from one another. See Table 3 for additional details.

found when the fzP21 or fzD21 alleles were recombined with Df(3L)Dfz2 but partial phenotypes were often observed in the presence of the fzR52 allele. The retention of partial function inferred for the fzR52 allele was probably responsible for the incomplete wg-like phenotypes that were interpreted to imply the existence of additional Wg receptors (Bhat, 1998; Muller et al., 1999).

With respect to the apparent redundancy of Fz and Dfz2 function inferred during normal embryogenesis, we note that the experiments presented here do not rule out the possibility that in a Dfz2 mutant Fz function or fz expression is altered to compensate for the absence of Dfz2, and that in a fz mutant Dfz2 function or Dfz2 expression is altered to compensate for the absence of Fz.

In the embryonic epidermis, where Wg signaling has been most thoroughly studied, Wg controls the production of naked cuticle and denticle diversity. These two functions can be independently altered by different wg alleles, leading to the hypotheses that different cuticle fates arise either from the activation of different receptors or from different levels or spatial distributions of Wg (Hays et al., 1997). The normal or nearly normal cuticular pattern seen in fz or Dfz2 single mutants and the wg-like cuticular phenotype seen in fz, Dfz2 double mutants support the latter hypothesis.

Despite the near identity of fz and Dfz2 action in the early embryo, it is possible that there are subtle differences between them that bear upon the different readouts of Wg activity. For example, Fz and Dfz2 might have different affinities for Wg, they might interact differentially with cofactors or proteoglycans that affect Wg signaling, or they might be used differentially in various tissues. If various developmental events were sensitive to different levels of Wg signaling, then small differences in receptor-ligand affinity or receptor density might be relevant in some tissues and not in others. For example, in the CNS, the residual quantities of functional or partially functional protein produced from the fzR52 allele are sufficient for specification of some RP2 neurons, whereas it is not sufficient for specifying Eve-expressing heart precursors. Conversely, in 469-2 homozygotes, there is occasional loss of RP-2 neurons but no effect on eve-expressing heart precursors. It will be interesting to extend these observations in the future by studying the effects of point mutations in Dfz2.

Combinatorial interactions between Frizzled and Wnt family members

Earlier misexpression studies have indicated that in imaginal discs Wg can signal through Dfz2 (Zhang and Carthew, 1998; Cadigan et al., 1998) but not through Fz (Zhang and Carthew, 1998; Axelrod et al., 1998; K. M. C. et al., unpublished results). The mechanism by which the binding specificity, availability or transduction capacity of Fz is altered between embryo and imaginal disc represents an interesting question for future investigation.

The question of whether endogenous Dfz2 is required for Wg signaling in imaginal discs cannot be addressed with the Dfz2 deficiency described in this report, since we have not been able to obtain somatic homozygous clones in discs (H. Lin and K. M. C., unpublished results). Once more surgical mutations in the Dfz2 gene are obtained, it will be interesting to determine whether Dfz2 acts alone or in tandem with other Frizzled family members in these tissues.

In summary, the experiments reported here indicate that in vivo there are overlapping interactions between Frizzled and Wnt family members such that a single Wnt can interact with more than one Frizzled receptor. We infer that the converse is also true based on the available data regarding Fz function in the embryo and imaginal disc. Presumably, the promiscuous binding observed between various Wnt and Frizzled family members in cell culture experiments represents the in vitro correlate of the broad in vivo specificities inferred from genetic experiments. By extension, we predict that the 16 Wnt and 9 Frizzled proteins identified thus far in mammals are likely to exhibit complex patterns of pairwise interactions in producing the full repertoire of Wnt signaling events during mammalian development.

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