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PRINCIPAL INVESTIGATOR(S):  Roel Nusse, Ph.D.

CONTRACTING ORGANIZATION:  Stanford University
                           Stanford, California  94305

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Isolation of a Receptor for WNT/Wingless Growth Factors

Roel Nusse, Ph.D.

Stanford University
Stanford, California 94305

U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

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The work done in this grant aims at elucidating the function of the Wnt signaling molecules in cancer. Wnt proteins are secreted and play important roles in the control over growth, in particular in the mammary gland. Wnt genes can also act as oncogenes in mouse mammary tumors. The work is specifically aimed at identifying a receptor for Wnt proteins. The isolation of a receptor is critical to our understanding of the mechanism of action of Wnt proteins. Using genetic and biochemical approaches we wish to identify and to clone the receptor for a Wnt gene product in Drosophila, called wingless. The receptor will be characterized and using the receptor gene, we will subsequently clone mammalian Wnt receptors by homology. During the past year, we have accomplished this goal, as a receptor for wingless has been identified.
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Introduction

The goal of this grant is to elucidate the function of the Wnt signaling molecules in cancer and in early embryogenesis, with emphasis on finding a receptor for Wnt proteins. Wnt genes encode secreted proteins involved in cell-to-cell signaling. The Wnt gene family includes a Drosophila gene wingless, which genetically has been very well characterized (3, 4). Wnt genes are important in growth control, in particular in the mammary gland and, importantly, can act as oncogenes in mouse mammary tumors (4). Problems inherent to the nature of Wnt proteins had precluded the isolation and characterization of Wnt receptors, which is central to our understanding of their mechanism of action.

Our approach to identify a Wnt receptor is two-fold:

1. We use an assay for soluble extracellular wingless protein and an in vitro cell culture assay to identify wingless receptors.

2. In Drosophila, we perform genetic screens to identify suppressors and enhancers of an wingless phenotype. These modifying genes will be cloned and their properties will be examined by sequencing and transfection.

During the past year, we have succeeded in identifying a protein, Dfrizzled-2, that fulfills the criteria to act as a receptor for wingless, and have thereby accomplished the major goal of this grant.
Body

1. *wingless* signaling *in vitro*; identification of a receptor.

Previously, we had developed an *in vitro* assay for *wingless* signaling in our laboratory, using a cell line (clone 8 or cl-8) derived from *Drosophila* imaginal discs. To measure a response to *wingless*, we examined the expression of the *armadillo* protein, the most ubiquitous downstream genetic target of *wingless* during the development of the fly. Using co-cultured *wingless*-producing S2 cells or the medium from these S2 cells, we found a large increase in *armadillo* levels in the cl-8 target cells. The soluble *wingless* protein has a rapid, concentration-dependent effect and can be depleted by an antibody to *wingless*, providing a quantitative and early effect of an extracellular *Wnt* protein (5). We also found that, in contrast to clone-8 cells, *Drosophila* S2 cells do not respond to the wg protein, indicating that they lack one or more components of Wg signaling (6). This finding suggested a complementation strategy to identify such missing components and we therefore tested whether transfection of receptor candidates would make S2 cells responsive to the *wingless* protein.

One interesting receptor candidate was identified during the characterization of a large family of putative cell surface receptors with extensive homology to the *Drosophila* tissue polarity gene *frizzled* (*fz*). Mutations in *fz* result in aberrant orientations of adult cuticular structures, and the *fz* sequence predicts an encoded protein with a cysteine-rich extracellular domain followed by seven transmembrane segments. Although wg does not appear to be involved in the tissue polarity pathway and *fz* does not appear to be involved in the segment polarity pathway, a possible link between the two pathways is suggested by the requirement in each for the function of dishevelled, another *Drosophila* segment polarity gene.

Based on the sequences of Dfz1 and of three mammalian *fz* genes, degenerate PCR primers were designed for the purpose of amplifying additional *frizzled*-like sequences. PCR amplification using *Drosophila* genomic DNA as a target revealed a novel *frizzled* family member, *Drosophila frizzled* 2 (*Dfz2*). The predicted *Dfz2* protein resembles all other members of the *frizzled* family seven putative transmembrane domains, a cysteine-rich aminoterminal extension and a long intracellular domain.

We examined the time and place of *Dfz2* expression by Northern blot analysis, in situ hybridization, and immunostaining. A 5.5 kb *Dfz2* transcript is found throughout the *Drosophila* life-cycle, most prominently during embryogenesis and late larval and pupal life. A segmental pattern of *Dfz2* expression in early embryogenesis is
reminiscent of the expression patterns of many genes in the segment polarity pathway, including wg. We found a transcript of similar size in Drosophila clone-8 cells, a cell line from imaginal discs previously shown to be responsive to Wg activity in vitro. Drosophila Schneider 2 (S2) cells, which do not respond to Wg, did not contain detectable Dfz-2 transcripts.

The absence of Dfz-2 expression in S2 cells was of interest in view of the lack of response of these cells to Wg (6). We tested a possible function of Dfz-2 expression in Wg signaling by transfecting an expression construct into S2 cells. In this construct, Dfz-2 is driven by the metal-inducible metallothionein promoter. In stable cell lines derived after selection in hygromycin, there was a baseline level of expression in cells grown in the absence of inducers, as detected with an antiserum to Dfz-2.

We tested a possible function of Dfz-2 expression in Wg signaling by following the levels of the Arm protein in response to added extracellular Wg protein. In transfected cells, the levels of the Arm protein were similar to those in non transfected cells, irrespective of whether Dfz-2 expression was elevated by copper induction. When the Dfz-2 transfected cells were incubated in the presence of soluble Wg protein, the level of the faster migrating (non-phosphorylated) form of Arm protein were increased. The elevation was similar to the response in clone-8 cells elicited by Wg. These results showed that Dfz-2 acts as a signal transducing molecule for Wg, suggesting that it is a receptor for Wg. To examine binding of Wg to the Dfz-2 transfected cells, we incubated the Dfz-2 expressing S2 cells in Wg containing conditioned medium at 4°C, and subsequently stained the cells with an antiserum to Wg. Cells expressing Dfz-2 stained brightly when incubated with Wg and the antiserum, whereas the controls (non transfected S2 cells or transfected cells without adding Wg protein) showed some spots of background staining. We conclude that the Wg protein can specifically bind to cells expressing Dfz-2.

As a test for binding of the Wg protein to Dfz-2 itself, we constructed a fusion protein containing the cysteine-rich amino-terminal domain of Dfz-2, linked to the constant domain of human IgG. We added this fusion protein to the supernatant of metabolically labeled S2 cells producing Wg. The fusion proteins and possible complexes were then retrieved by adding sepharose-ProteinA beads. The Dfz-2 fusion protein, but not a control Ig, selectively bound to labeled proteins of 52 kD, the size of the mature Wg protein. Normal S2 cells did not produce Dfz-2 binding proteins.

Hence, we have shown that the Dfz-2 gene fulfills two criteria to be a receptor for the Wg protein: Wg binds to the Dfz-2 and binding leads to a biological response; an increase in intracellular Arm concentration. In most vertebrates, more than 10 Wnt
genes have been identified. As expected, there exists indeed a large family of fz-like genes in vertebrates, likely candidates for receptors for the other Wnt proteins. At this moment, there is no genetic evidence that D fz-2 is required for Wg signaling, as no mutants at the gene are available. Possible candidates for D fz2 mutant may have arisen from the genetic screen described in 6.2.

This work was done in collaboration with the lab of Dr. Jeremy Nathans, Johns Hopkins University in Baltimore and has been published with acknowledgment of the USAMRMC support (ref 1 in part 8, see appendix).

2. A genetic screen for suppressors of a wingless phenotype in Drosophila

A second route to the identification of components of wingless signal transduction in Drosophila is to take advantage of the genetic tools developed in this organism. By performing genetic screens for suppressors of a wingless-caused phenotype in the fly, one can uncover mutations in genes that are essential to generate this phenotype. Those genes could encode components of the wingless signaling pathway, including the receptor.

We have made several P-element based constructs to obtain ectopic expression of wingless in larval imaginal discs, the progenitors of adult tissues. These include a construct in which wingless expression is driven by the sevenless promoter, pSEW-wingless, which is known to be active only in the eye imaginal disc. The transgenic flies that were obtained have a very specific phenotype in the eye: an almost complete absence of interommatidial bristles. This phenotype is 100% penetrant and easy to score with a dissecting microscope. This phenotype is also generated by a wingless temperature sensitive allele, but in a temperature dependent manner.

This penetrant adult viable wingless phenotype has been used to perform a screen for dominant suppressors or enhancers of wingless. The principle behind this screen is to search for mutations that will give a phenotype when one allele has undergone a loss-of-function mutation. Normally, complete absence of one allele will not give a phenotype. But in a genetic background where the phenotype of one gene (in this case wingless) is dosage-sensitive, absence of one copy of an interacting gene may modify this phenotype. This screen can be done in the F1 generation. Especially since the phenotype is semi-quantitative (i.e. the number of bristles on the eye can be approximated) this screen is very sensitive to dosage of gene products interacting with
*wingless* and can identify not only suppressors but also enhancers of the pSEW-
*wingless*(ts) phenotype.

We have now isolated approximately 20 suppressors and enhancers of the *wingless*
phenotype in the eye. These genes have been mapped and have been assembled into
complementation groups. We have also performed clonal analysis of these genes,
indicating that some of them also have a phenotype in the homozygous state. Five
interesting complementation groups have been found, two of them consisting of known
genes.

One is *daughterless* (*da*), a helix-loop-helix protein heterodimerizing with other such
proteins and required for neurogenesis. We found that *wg* expression in the eye
reduces the level of *da* expression, the first demonstration of regulation of *da* expression
by an extracellular signal.

A second known suppressor is a *Drosophila* tumor suppressor gene, called *warts*.
This gene encodes a protein kinase but its biochemical function is not clear. We are
currently addressing this by producing antibodies to *warts*.

Among the unknown suppressors is one that maps very close to *Dfz2* (map
position 76A). We will directly sequence the *Dfz2* gene in this mutant stock to see if the
gene is indeed affected.

Part of this work has been published with acknowledgment of the USAMRMC
support (ref 2 in part 8, see appendix)
Conclusions

Since the work started, approximately two years ago, we have made very significant progress. The main goal of the project, the identification of a *wingless* receptor, has been accomplished. We have no *Drosophila* mutants in the receptor gene, *Dfz2*, but we have found a number of suppressor mutations in *Drosophila*, one of which may correspond to the receptor gene. Further work will address the biochemical mechanism of signal transduction by the *Dfz2* receptor, and the interactions between other members of the *frizzled* receptor gene family and the various *Wnt* proteins.
References


APPENDIX
A new member of the frizzled family from Drosophila functions as a Wingless receptor

Purnima Bhanot*, Marcel Brink†, Cindy Harryman Samos†, Jen-Chih Hsieh‡, Yanshu Wang†, Jennifer P. Macke†, Deborah Andrew§, Jeremy Nathans*†‡§ & Roel Nusse†

* Department of Molecular Biology and Genetics, § Department of Cell Biology and Anatomy, ‡ Department of Neuroscience, and † Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, 725 North Wolfe Street, Room 805 PCTB, Baltimore, Maryland 21205, USA
† Howard Hughes Medical Institute and Department of Developmental Biology, Stanford University School of Medicine, Stanford, California 94305, USA

Receptors for Wingless and other signalling molecules of the Wnt gene family have yet to be identified. We show here that cultured Drosophila cells transfected with a novel member of the frizzled gene family in Drosophila, Dfz2, respond to added Wingless protein by elevating the level of the Armadillo protein. Moreover, Wingless binds to Drosophila or human cells expressing Dfz2. These data demonstrate that Dfz2 functions as a Wingless receptor, and they imply, in general, that Frizzled proteins are receptors for the Wnt signalling molecules.

There is abundant evidence that secreted Wnt proteins have important signalling functions during animal development. For example, Wnt proteins have been implicated in cell-lineage decisions in Caenorhabditis elegans, in embryonic and adult pattern formation in Drosophila, in axis formation and dorsal–ventral polarity determination in Xenopus embryos, and in central nervous system (CNS) development and oncogenesis in mice. However, the Wnt proteins have been difficult to obtain in a soluble form, a problem that has hampered the development of biochemical and cell biological assays. Most information about the mechanism of Wnt signalling has come instead from the genetic analysis of Drosophila segment polarity and the role of the Wnt gene wingless (wg; refs 2–4). Within each embryonic segment, production of the wingless protein (Wg) by a narrow stripe of cells maintains engrafted expression in an adjacent stripe of cells.

In the embryonic epidermis the wg signalling pathway is defined by several genes: dishevelled (dsh)30, zeste white 3 (zw3 or slagy); and armadillo (arm), a member of the beta-catenin gene family31, which is thought to be inactivated by zw3. The wg signal seems to counteract the inhibitory effect of zw3, leading to activation of arm31–34. In Drosophila embryos the cytoplasmic levels of the arm protein (Arm) are increased as a consequence of wg signalling5. As judged by sequence data, none of the proteins identified thus far in the signalling pathway is a Wg receptor.

On the basis of genetic interactions between wg and other genes in the wg pathway, we have established a tissue-culture system for wg signalling5. In this assay, Wg produced by Drosophila S2 cells is added in soluble form to a cell line (clone 8) derived from Drosophila imaginal discs. Like Drosophila embryos, clone 8 cells respond to Wg by specifically increasing the levels of hypophosphorylated Arm5, suggesting that these cells express a receptor specific for Wg.

Here we report the identification of a novel Drosophila gene, frizzled2 (Dfz2), and demonstrate that it functions as a Wg receptor in cultured cells. Dfz2 was identified in the course of characterizing a large family of vertebrate and invertebrate homologues of the Drosophila gene frizzled (fz)52. Mutations in fz result in aberrant orientations of adult cuticular structures, a tissue polarity phenotype53–55. The fz sequence predicts an encoded protein with an amino-terminal cysteine-rich extracellular domain followed by seven transmembrane segments56,57. These characteristics have led to the suggestion that fz is a receptor for an unidentified ligand that transmits tissue-polarity information58. Although wg does not seem to be involved in the tissue-polarity pathway and fz does not seem to be involved in the segment-polarity pathway, a possible link between the two pathways is suggested by the requirement in each for dsh function59,60.

Molecular cloning of Dfz2

Using the sequences of fz and three mammalian fz homologues, degenerate polymerase chain reaction (PCR) primers were designed for the purpose of amplifying additional fz-like sequences61. PCR amplification using Drosophila genomic DNA as a target revealed a novel frizzled family member, Drosophila frizzled2 (Dfz2). Isolation and sequence analysis of genomic and complementary DNA clones corresponding to a single coding exon containing an open reading frame of 694 amino acids (Fig. 1a). The predicted Dfz2 protein (Dfz2) resembles all other members of the frizzled family in having the following structural motifs (beginning at the N terminus): a putative signal sequence, a domain of 120 amino acids with an invariant pattern of ten cysteine residues, a highly divergent region of 40–100 largely hydrophobic amino acids that is predicted to be flexible, and seven putative transmembrane segments (Fig. 1b). The C terminus of Dfz2 resembles that of most mammalian frizzled protein in ending with the sequence S/T-X-V. A comparison with all known frizzled sequences shows that Dfz2 most closely resembles human fz5 and mouse fz8 with which it shares 49% and 45% amino acid identity, respectively. Fz and Dfz2 share 33% amino-acid identity. The Dfz2 gene resides at 76A on the polytene chromosome map as determined by in situ hybridization (data not shown).

Developmental expression of Dfz2

As a first step in elucidating the function of Dfz2 we examined temporal and spatial expression patterns by northern blot analysis, in situ hybridization, and immunostaining. A 5.5-kilobase (kb) Dfz2 transcript is found throughout the Drosophila life cycle, most prominently during embryogenesis and in late larval and pupal life (Fig. 1c). At 2 hours post-fertilization, embryos have low levels of Dfz2 RNA, which is presumably of maternal origin. Dfz2 expres-
b

Df2

fz

c

embryos

larvae

pupae

adults

0-2 2-4 4-8 8-12 12-24 24-48 48-72 72-96 96-120

Df2

fz

Consensus

RP49

Transection of Df2 in S2 cells

We also measured Df2 RNA and protein levels in clone 8 cells, which respond to added Wg as determined by an increase in Arm protein, and in Schneider (S2) cells, which are unresponsive. A Df2 transcript that matches the size of the in vivo transcript was observed in clone 8 cells, but no Df2 transcript was detected in S2 cells (Fig. 1c). Similarly, in western blots probed with affinity-purified anti-Df2 antibodies, a protein band with an apparent mobility of 65K (and comigrating with Df2 produced in transfected S2 cells; see below) was observed in samples derived from clone 8 cells but not S2 cells (data not shown). Transcripts derived from fz were not detectable in either S2 or clone-8 cells (data not shown).

It is possible that S2 cells fail to respond to added Wg because they do not express Df2. We tested this directly by transecting S2 cells with a Df2 expression construct. These cells were then

transcrip

sion is first clearly detected at stage 6, where it is found in all cells between roughly 15% and 70% of egg length, including the invaginating cells of the ventral furrow (Fig. 2a). An emerging stripe pattern is evident by early stage 8, and by stage 10 Df2 expression is clearly seen in 15 stripes in the presumptive head and

trunk regions, in the posterior midgut primordium, in a subset of cells at the site of anterior midgut invagination, and in the proctodeal lobe (Figs 2b, c). Beginning at stage 12, during germ-band shortening, Df2 expression declines in the epidermis and increases in the midgut and visceral mesoderm (Fig. 2f). Beginning at stage 9 and continuing throughout embryogenesis, Df2 expression is also seen in the developing CNS (Fig. 2d–f). By stage 17 Df2 expression becomes limited to the CNS, hindgut and dorsal vessel (Fig. 2f).

Fig. 1 isolation and characterization of Df2—a novel frizzled family member in Drosophila. a, Alignment of the predicted amino-acid sequences of Df2 and Fz. Identical residues are indicated in the consensus and conserved cysteines in the cystein-rich domain are in bold face. b, Hydropathy profiles of Df2 and Fz proteins were calculated according to ref. 37 with a window size of 15 amino acids; increasing hydrophobicity is downward. The seven putative transmembrane domains and the signal peptide are indicated by a solid line. c, Expression of Df2 RNA during Drosophila development and in clone 8 and S2 cells. Upper panel, a 950-bp segment from the Df2 coding region was used to probe a blot containing 40 μg RNA per track from the indicated stages of Drosophila development or 30 μg total RNA from S2 cells and clone 8 cells. For the embryo samples, times during development are indicated in hours. A 5.5-kb transcript was detected at all stages, including 0–2 h post-fertilization. Lower panel, a ribosomal protein 49 (RP49) probe was used to probe the same blot to control for sample loading and transfer efficiency. METHODS. D. melanogaster genomic DNA was used as a template for PCR amplification using fully degenerate primers corresponding to conserved sequences YPERPI (sense) and WFLAA (antisense) as described. A single PCR product corresponding to codons 348–425 of Df2 was obtained and used to screen a D. melanogaster genomic DNA library in bacteriophage λ. Sequence analysis of cloned genomic DNA revealed an open reading frame of 694 codons with 33% amino-acid identity to Fz. Partial sequences from 6 independent cDNA clones obtained from a 0–9 h embryo cDNA library (gift from K. Zinn) indicate that the open reading frame is contained within a single exon, that there is at least one 5' non-coding exon, and that the putative initiator methionine is the 5' most-proximal ATG codon and is located 5 codons 3' of an in-frame termination codon. The Df2 and Fz sequences were aligned using GeneWorks software, and hydropathy profiles were calculated using MacVector 3.5 software. RNA from various stages of Drosophila development was isolated from the guanidinium–phenol method or from tissue culture cells by LiCl–urea precipitation. RNA was transferred and hybridized as described.

NOTE: In this context, Wg stands for Wingless, a protein that is involved in signaling pathways regulating cell growth and development. The figure illustrates the expression patterns of Df2 and Fz across different stages of Drosophila development, showing how they are expressed in various parts of the embryo, particularly in the midgut and visceral mesoderm, and how their expression patterns differ between S2 cells and clone 8 cells.
assayed for the ability to stabilize Arm in response to added Wg. In the absence of Wg, transfected and untransfected S2 cells show similar low levels of Arm irrespective of whether Dfz2 expression was elevated by copper induction. However, when Dfz2-transfected cells were incubated in the presence of Wg, the level of the faster migrating (hypophosphorylated) form of Arm was increased (Fig. 3). This elevation was similar to the response elicited by Wg in clone-8 cells. Increasing Dfz2 above basal level by copper induction of the metallothionein promoter led to a decrease in Wg responsiveness (Fig. 3), suggesting that at high levels Dfz2 may bind non-productively to second messenger components. Four independent Dfz2-transfected cell lines derived from two separate transfections were tested, and all four lines showed Wg-dependent Arm stabilization.

**Transfection of Dfz2 confers Wg binding**

The results described above show that Dfz2 expression confers responsiveness to Wg, consistent with the idea that Dfz2 is a receptor for Wg. To examine Wg binding directly, we incubated Dfz2-expressing S2 cells with Wg at 4 °C, and subsequently stained the cells with affinity-purified polyclonal antibodies to Wg. S2 cells expressing Dfz2 show strong surface staining when incubated with
FIG. 3 Addition of soluble Wg leads to an increase in the level of Armadillo protein in Dfz2-transfected S2 cells. Clone 8 cells (left), untransfected S2 cells (centre), or Dfz2-transfected S2 cells (right) were incubated with concentrated conditioned medium either from S2 cells producing Wg (+Wg) or from control S2 cells (−Wg). Untransfected and Dfz2-transfected S2 cells were tested following growth with or without copper sulphate (+Cu or −Cu) to modulate expression of transfected Dfz2 from the metallothionein promoter. Cellular proteins were analysed on blots with antibodies against Arm (upper panel). Incubation with Wg-containing medium produces an increase in the level of Arm in clone 8 cells and in Dfz2-expressing S2 cells, but not in untransfected S2 cells. Further induction of Dfz2 expression in transfected cells by preincubation with copper sulphate leads to a lower response to Wg. As a control for loading, blots were stripped and incubated with antisera against α-catenin (lower panel).

METHODS. To produce control conditioned medium or conditioned medium containing soluble Wg protein, untransfected S2 cells or S2 cells stably transfected with a construct in which the Wg coding region is under the control of a heat-shock promoter were used as described. S2 cells stably transfected with Dfz2 under the control of the metallothionein promoter were generated by hygromycin selection following transfection with a plasmid carrying the Dfz2 coding region inserted into pMK3. Clone 8, S2, and Dfz2 transfected S2 cells were incubated with concentrated medium for 2 h. After the incubation, cells were lysed and protein extracts analysed using a monoclonal anti-Armadillo antibody 7A1 (ref. 7) or rat monoclonal anti-α-catenin antibody DCAT-1 (ref. 46). Bound antibody was visualized using the ECL system (Amer sham).

FIG. 4 Wg protein binds to cells transfected with Dfz2. Untransfected and transfected cells were incubated with concentrated conditioned medium from untransfected S2 cells (a) or from S2 cells producing Wg (b–h; see Fig. 3 legend). Following incubation with conditioned medium, the cells were washed, fixed in paraformaldehyde/PBS, and incubated with an anti-Wg antibody directed against an 85-amino-acid domain that is found in Wg but absent from all other Wnt proteins. This domain is dispensable for Wg activity (Chi-hwa Wu, C.H.S. and R.J., unpublished observations). Untransfected S2 cells (b), S2 cells transfected with a Notch expression plasmid (c), and untransfected 293T cells (human embryonic kidney-cell line 293 stably expressing SV40 Tag) (d) show a low level of fluorescent antibody binding. Roughly 80% of S2 cells stably transfected with Dfz2 and incubated with Wg show anti-Wg antibody binding to the cell surface. 10–20% of 293T cells transiently transfected with a Dfz2 expression plasmid and incubated with Wg show anti-Wg antibody binding to the cell surface. g, 293T cells cotransfected with a mixture of Dfz2 expression plasmid and a GFP expression plasmid, and incubated with Wg and anti-Wg antibodies show colocalization of green cytoplasmic fluorescence (GFP) and red surface fluorescence (Wg and anti-Wg antibody) and confirm that 10–20% of cells were transfected, and that only this subset of cells bind Wg. h, 293 cells transfected with a T-antigen expression plasmid and a truncated Dfz2 construct in which the signal sequence, the CRD, and the first half of the linker region are anchored to the cell surface by GPI, show cell-surface binding to Wg and anti-Wg antibody.

METHODS. Untransfected S2 cells and S2 cells expressing Dfz2 were washed twice in PBS and incubated with 1.5 ml of 10× concentrated conditioned medium at 4 °C for 3 h. After three 10-min washes with cold PBS the cells were fixed in 2% paraformaldehyde (Polysciences, Inc.) for 15 min at room temperature. After three more 10-min washes with PBS, affinity purified anti-Wg antibody diluted 1:25 in 5% donkey serum/PBS was added to the cells and incubated overnight at 4 °C. After additional washes in PBS, the cells were incubated with fluorescent Cy3 secondary antibody (Jackson ImmunoResearch) and mounted. For transient expression in 293T or 293 cells, the Dfz2 coding region was inserted into the pCIS expression vector under the control of the cytomegalovirus immediate early promoter/enhancer and with an optimized translation-initiation context, and transfected into 293T or into 293 cells with a T-antigen expression plasmid using the calcium phosphate method. Eight hours after transfection, 10 mM chlorate was added. Twenty-four hours later, the cells were treated with 2 μg/ml diepoxybutane (Seikagaku) for 3 h before adding Wg protein. S2 cells expressing Notch were obtained from S. Arvanitis-Tsikalas. From N to C-termini, the GPI-anchored construct consists of the first 270 amino acids of Dfz2, a myc epitope and the C-terminal 40 amino acids of decay activating factor, a GPI-anchored protein. Confocal images were collected with a Bio-Rad MRC 1000 confocal laser attached to a Zeiss Axio scope microscope. The same number of scans for each sample. Images were processed in Adobe Photoshop 3.0.
Wg and anti-Wg antibodies (Fig. 4d), whereas Notch transfected or non-transfected cells incubated either with or without Wg show a background of randomly distributed spots of low fluorescence intensity (Fig. 4b, c). Dfz2-transfected cells incubated in the absence of Wg show a similar low-intensity, spotty background (Fig. 4e). We conclude that Wg specifically binds to S2 cells expressing Dfz2.

Although this binding experiment indicates that Wg and Dfz2 probably interact directly, it is possible that expression of Dfz2 could act indirectly by inducing or masking a Wg receptor. We therefore performed a series of binding experiments using heterologous cells, in case human embryonic kidney cells (293 or 293T; both will be referred to as 293) and a variety of wild-type and mutant fz constructs. Pretreatment of the 293 cells with chlorate and heparinase lowered the overall background of Wg binding (presumably binding of Wg to extracellular matrix molecules; Fig. 4e), and revealed specific binding of Wg to the surface of 293 cells that had been transiently transfected with Dfz2 (Fig. 4f) but not to untransfected cells or cells that had been transfected with a bovine rhodopsin expression construct (Fig. 4e, and data not shown). In a second experiment in which 293 cells were cotransfected with a green fluorescent protein (GFP) expression plasmid and the Dfz2 expression plasmid, we observed that cells with green cytosolic fluorescence (caused by GFP) also had red surface fluorescence (Wg and anti-Wg antibody; Fig. 4g).

Each frizzled protein has an extracellular cysteine-rich domain (CRD) that is joined to the transmembrane domain by a variable linker. The CRD has been proposed to constitute part or all of the ligand-binding domain, which suggests that cell-surface expression of the isolated CRD segment might confer Wg binding. This possibility was tested by expressing a truncated form of Dfz2 in which the CRD and part of the linker region was displayed on the cell surface as a glycosylphosphatidylinositol (GPI)-anchored protein. This protein was detected at the surface of transfected cells by immunostaining either with antibodies directed against the Dfz2 extracellular domain or with antibodies to a myc epitope tag that was engineered near the C terminus of the GPI-anchored protein (data not shown). When 293 cells transfected with the GPI-anchored Dfz2 CRD were incubated with Wg and anti-Wg antibodies, strong surface staining was observed (Fig. 4h). We conclude from this experiment that the Dfz2 CRD constitutes either all, or a significant part of, the ligand-binding domain.

Transfection of a subset of frizzled members

In many ligand–receptor systems a single ligand can bind to more than one species of receptor, or a single receptor can bind to more than one species of ligand, or both. Among G-protein-coupled receptors there are many examples of receptor subtypes that recognize the same ligand but differ in effector coupling, tissue distribution and pharmacology. In the fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and transforming growth factor (TGF)–β/activin/inhibin systems, tissue-culture experiments show that different receptors can bind to a single ligand and that different ligands can bind to a single receptor.

As a first step in examining the question of ligand–receptor specificity in the Wnt frizzled system, we tested the ability of Wg to bind to 293 cells transfected with Drosophila fz and with six mammalian frizzled sequences. 293 cells transfected either with fz, human fz (Hfz2), or mouse fz, fz7 or fz8 (Mfz4, Mfz7 and Mfz8) bind added Wg (Fig. 5a, c), whereas transfection with Mfz3 and Mfz6 did not confer Wg binding (Fig. 5b, d). As a complement to the Dfz2 CRD GPI-anchor experiment, a derivative of Mfz4 was constructed in which the CRD was replaced with a myc epitope. In transfected 293 cells, immunostaining with an anti-myc antibody, and western blotting with an antibody specific for the Mfz4 C terminus showed, respectively, that the CRD-deleted Mfz4 protein accumulated at the cell surface and to the same percentage of membrane protein as full-length Mfz4 (Fig. 5c and data not shown). However, CRD-deleted Mfz4 did not confer Wg binding (Fig. 5d). This experiment further implicates the CRD as an essential determinant of Wg binding. Figure 6 summarizes all of the Wg–frizzled binding experiments described above.

Discussion

The experiments reported here identify a second member of the frizzled family in Drosophila, Dfz2, and show by the following two criteria that it can function as a receptor for Wg. First, transfection of S2 cells with Dfz2 confers Wg responsiveness as determined by an increase in cytoplasmic Arm concentration, and second, transfection with Dfz2 confers cell-surface binding of Wg in both homologous (S2) and heterologous (293) cells. It is important to note that these data do not rule out the possibility that additional molecules in the conditioned medium might associate with Wg and participate in its binding to the receptor. They also do not rule out the possibility that Dfz2 is part of a larger complex at the cell surface; in such a complex Dfz2 would be necessary but may not be sufficient for binding and/or signal transduction. We note that these experiments do not support the proposal that Notch is the Wg receptor, because Notch...
Drosophila fz and some members of the mammalian frizzled family also confer Wg binding supports this inference but also suggests that there may be overlapping specificities in Wnt-Frizzled interactions. From the general conclusion that frizzled family members encode Wnt receptors, we infer that in vivo the Drosophila fz protein recognizes at least one Wnt other than Wg (three of which are known)\(^{35}\), and, by extension, that the initial biochemical steps in fz-mediated tissue polarity signalling resemble the initial steps of wg-mediated segment polarity signalling.

The experiments reported here provide a new point of entry for examining the biochemistry of Wnt signalling. It should now be possible to determine which cytoplasmic proteins interact directly with the frizzled receptors, whether these interactions are modified by Wnt binding, and whether Wnt signalling is regulated by covalent or non-covalent receptor modification. It is interesting to note that many Fz proteins, including Dfz2, contain a S/T-X-V motif at their C-terminal end; this motif has been shown to interact with PDZ (or DHR) domains in a variety of proteins\(^{36}\). Dsh, one of the cytoplasmic components of Wg signalling, contains a PDZ domain\(^{37}\).

A question remaining is how frizzled and Wnt proteins might interact to initiate signal transduction. One attractive hypothesis is suggested by the relative immobility of Wnt proteins because of their affinity for the extracellular matrix, and the predicted mobility of the CRD, which we show here constitutes part or all of the ligand-binding site. The prediction that the CRD is mobile follows from the predicted lack of a stable structure in the highly divergent sequence that links it to the membrane-embedded domain. For example, in Dfz2 this linker region includes a stretch of 42 amino acids that includes 21 glycines and 15 serines. Therefore the CRD may be able to bind to an extracellular matrix-associated Wnt protein at a distance of several tens of nanometres from the plasma membrane of the cell on which the frizzled receptor resides. It is tempting to speculate that binding of a Wnt ligand to the CRD disrupts or modifies an interaction between the CRD and the extracellular face of the transmembrane domain, and that this results in a rearrangement of transmembrane a-helices. Although the frizzled proteins have no primary sequence homology to G-protein-coupled receptors\(^{38}\), this allosteric model suggests a mode of receptor activation that is reminiscent of that proposed for G-protein-coupled receptors\(^{39}\).
wingless signaling in the Drosophila eye and embryonic epidermis

Kenneth M. Cadigan and Roel Nusse

Howard Hughes Medical Institute and the Department of Developmental Biology, Stanford University School of Medicine, Stanford, California 94305, USA

SUMMARY

After the onset of pupation, sensory organ precursors, the progenitors of the interommatidial bristles, are selected in the developing Drosophila eye. We have found that wingless, when expressed ectopically in the eye via the sevenless promoter, blocks this process. Transgenic eyes have reduced expression of aceaete, suggesting that wingless acts at the level of the proneural genes to block bristle development. This is in contrast to the wing, where wingless positively regulates aceaete to promote bristle formation. The sevenless promoter is not active in the aceaete-positive cells, indicating that the wingless is acting in a paracrine manner. Clonal analysis revealed a requirement for the genes porcupine, dishevelled and armadillo in mediating the wingless effect. Overexpression of\textit{ zeste white-3} partially blocks the ability of wingless to inhibit bristle formation, consistent with the notion that wingless acts in opposition to\textit{ zeste white-3}. Thus the wingless signaling pathway in the eye appears to be very similar to that described in the embryo and wing. The\textit{ Notch} gene product has also been suggested to play a role in wingless signaling (J. P. Cousu and A. M. Martinez Arias (1994) \textit{Cell} 79, 259-272). Because\textit{ Notch} has many functions during eye development, including its role in inhibiting bristle formation through the neurogenic pathway, it is difficult to assess the relationship of\textit{ Notch} to wingless in the eye. However, we present evidence that wingless signaling still occurs normally in the complete absence of Notch protein in the embryonic epidermis. Thus, in the simplest model for wingless signaling, a direct role for Notch is unlikely.

Key words: wingless, signal transduction, Notch, Drosophila, neurogenesis, segment polarity

INTRODUCTION

The wingless (\textit{wg}) gene is the best characterized member of the\textit{ Wnt} family, which contains over fifty genes in organisms ranging from nematodes to humans (Nusse and Varmus, 1992).\textit{ Wnt} genes encode cysteine-rich proteins containing signal sequences and several members, including \textit{wg}, have rigorously been shown to be secreted (Bradley and Brown, 1990; Fradkin et al., 1995; Gonzalez et al., 1991; Papkoff and Schryver, 1990; Van den Heuvel et al., 1989; Van Leeuwen et al., 1994).

In \textit{Drosophila melanogaster}, \textit{wg} is required throughout embryogenesis and larval development for a wide range of patterning events (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994). Some of these include specifying cell fate in the embryonic epidermis (Baker, 1988; Bejsjovec and Martinez-Arias, 1991; Dougan and Dinardo, 1992), CNS (Chu-Lagriff and Doe, 1993), mesoderm (Beyles et al., 1995; Wu et al., 1995) and endoderm (Hoppler and Bienz, 1995). In larval development, \textit{wg} is required for patterning in leg (Cousu et al., 1993; Dz-Benjumea and Cohen, 1994; Struhl and Basler, 1993; Wilder and Perrimon, 1995) and wing (Cousu et al., 1994; Dz-Benjumea and Cohen, 1995; Phillips and Whittle, 1993) imaginal discs. In the eye, \textit{wg} has recently been shown to be necessary for proper spacing of morphogenetic furrow initiation (Ma and Moses, 1995; Treisman and Rubin, 1995). How one signal can produce so many responses remains an important unanswered question in developmental biology.

Consistent with being a secreted molecule, \textit{wg} is thought to execute most of its functions in a paracrine manner. In the best documented cases, the range of \textit{wg} action can vary from one (Vincent and Lawrence, 1994) to several (Hoppler and Bienz, 1995) cell diameters, though the exact limits of \textit{wg} diffusion remain unclear (Axelrod et al., 1996; Peifer et al., 1991; Theisen et al., 1994). In a few cases, \textit{wg} regulates gene expression in the same cells in which it is expressed, e.g. the activation of cut expression at the wing margin (Cousu et al., 1994) and the regulation of its own expression in the embryo (Bejsjovec and Wieschaus, 1993; Hooper, 1994; Yoffe et al., 1995). This embryonic autoregulation has been referred to as 'autocrine \textit{wg} signaling' but it is not clear whether \textit{wg} works in a truly autocrine manner. However, recent evidence indicates that \textit{wg} autoregulation may have different genetic requirements than the paracrine signaling pathway of \textit{wg} (Hooper, 1994; Manoukian et al., 1995; see discussion).

Three genes with embryonic phenotypes very similar to that of \textit{wg} have been described (Klingensmith et al., 1989; Peifer and Wieschaus, 1990; Perrimon et al., 1989; Perrimon and Mahowald, 1987), \textit{porcupine (poc)}, \textit{dishevelled (dsh)} and \textit{armadillo (arm)}). Another gene, \textit{zeste white-3} (\textit{zwo}; also known as \textit{shaggy}) has a mutant phenotype (Perrimon and Smouse, 1989; Siegfried et al., 1992) very similar to that of embryos...
where wg has been expressed ubiquitously (Noordermeer et al., 1992). Genetic epistasis (Noordermeer et al., 1994; Peifer et al., 1994b; Siegfried et al., 1994) have ordered these genes in the following genetic pathway:

\[ \text{porc} \rightarrow \text{wg} \rightarrow \text{dsh} \rightarrow \text{ze3} \rightarrow \text{arm} \]

\[ \text{K}-\text{porc} \]

This gene has been shown to be involved in either secretion or subsequent diffusion of the wg protein (Siegfried et al., 1994; van den Heuvel et al., 1993a) and the other three genes are thought to be required for receiving the wg signal (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994).

Recent work has revealed that many aspects of this embryonic wg signaling pathway are conserved in larval Drosophila tissues as well as in other organisms. Analysis of \( \text{dsh}, \text{ze3} \) and \( \text{arm} \) mutations in leg and wing imaginal discs indicates that these genes are required for wg signaling (Couso et al., 1994; Diaz-Benjumea and Cohen, 1994; Klingensmith et al., 1994; Peifer et al., 1991; Thiebaux et al., 1994). This has been best shown in the developing wing margin, where these genes mediate wg regulation of the \( \text{acheate} \) (\( \text{ac} \)) gene (Couso et al., 1994; Blair, 1994). The vertebrate homologs of these three genes have been shown to play a role in inducing dorsal mesoderm in Xenopus in a manner consistent with functioning in a Wnt signaling pathway (Domínguez et al., 1995; Helf et al., 1995; Heasman et al., 1994; Pierce and Kimmel, 1995; Rothbacher et al., 1995; Sokol et al., 1995).

The wg signaling pathway described above was first postulated based on extensive genetic analysis, but recent work indicates that some of the gene products may function directly with wg in a biochemical pathway. The \( \text{arm} \) gene encodes the Drosophila homolog of \( \beta \)-catenin (Peifer and Wieschaus, 1990), a component of vertebrate adherens junctions (Kemler, 1993). A similar junctional complex is found in flies (Peifer, 1993) but a substantial pool of cytoplasmic arm protein also exists (Peifer et al., 1994b; Van Leeuwen et al., 1994). wg signaling causes an accumulation of cytoplasmic arm protein (Peifer et al., 1994b; Van Leeuwen et al., 1994) caused by a dramatic decrease in arm protein turnover (Van Leeuwen et al., 1994). This accumulation is correlated with a reduction in phosphorylation of arm (Peifer et al., 1994a). This increase in arm protein is thought to somehow transduce the wg signal to the nucleus (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994).

Consistent with the proposed genetic pathway, mutations in the other components of the wg pathway affect arm protein levels. The normal segmentally repeated accumulation of arm protein is absent in \( \text{wg} \), \( \text{porc} \), and \( \text{dsh} \) mutants (Peifer et al., 1994b; Riggleman et al., 1990), while \( \text{ze3} \) mutants have uniformly high levels of arm protein (Peifer et al., 1994b; Siegfried et al., 1994). The \( \text{dsh} \) gene encodes a novel protein (Klingensmith et al., 1994; Thiebaux et al., 1994) containing a PDZ domain (Kennedy, 1995) that is phosphorylated in response to wg in embryos and cultured cells, and this phosphorylation is correlated with the ability of \( \text{dsh} \) to stabilize the arm protein (Yanagawa et al., 1995). \( \text{ze3} \) encodes a serine-threonine protein kinase that is homologous with mammalian glycogen synthase kinase-3 (Ruel et al., 1993a; Siegfried et al., 1994). At the present time, it is not clear whether any of the regulatory steps in the pathway are direct or how many missing components remain to be identified.

One new candidate for functioning in the wg pathway is the product of the \( \text{Notch} \) (\( \text{N} \)) gene, which encodes a transmembrane protein found on the surface of cells. N protein is thought to act as the receptor for the \( \text{Delta} \) (\( \text{D} \)) gene product in a signaling pathway involved in many aspects of development (Muskavitch, 1994; Artavanis-Tsakonas et al., 1995). Its potential role in the wg pathway is based on strong genetic interactions between \( \text{N} \) and \( \text{wg} \) mutations in several tissues, but primarily in the wing (Couso and Martinez Arias, 1994; Hong et al., 1994). It is possible that the role of \( \text{N} \) in the separate but oft-used pathway with \( \text{D} \) could mask a requirement for \( \text{N} \) in wg signaling when \( \text{N} \) mutant embryos or clones are examined. Because \( \text{N} \) is expressed at the cell surface and appears to act as a receptor, it has been postulated that \( \text{wg} \) encodes a ligand for the \( \text{N} \) protein (Couso and Martinez Arias, 1994).

This report describes a phenotype created by ectopic expression of \( \text{wg} \) during eye development. These transgenic animals lack the mechanosensory bristles normally surrounding each facet of the compound eye. This is the exact opposite effect seen in the wing, where \( \text{wg} \) is required for bristle formation (Couso et al., 1994; Phillips and Whittle, 1993). Despite this difference in regulation, the wg signal transduction machinery found in the embryo and wing also functions in the eye. Finally, the role of \( \text{N} \) in wg signaling was examined in the eye and in the embryonic epidermis, where, in the complete absence of \( \text{N} \) protein, wg signaling appears to occur normally. These data argue against a direct role for \( \text{N} \) in wg signaling.

**Materials and Methods**

**Fly Stocks**

The mutant alleles in components of the wg signaling pathway used in this study were: \( \text{wg}^{\text{ru}} \), \( \text{wg}^{	ext{D}} \), \( \text{wgo}^{	ext{C5}} \), \( \text{porc}^{	ext{ru}} \), \( \text{porc}^{	ext{D}} \), \( \text{dsh}^{	ext{U90}} \), \( \text{dsh}^{	ext{D}} \), \( \text{arm}^{	ext{DNI}} \), \( \text{arm}^{	ext{D}} \), \( \text{ze3}^{	ext{D}} \), and \( \text{ze3}^{	ext{DNI}} \). \( \text{wg}^{	ext{C5}} \) (van den Heuvel et al., 1993a,b), \( \text{dsh}^{	ext{U90}} \) (Yanagawa et al., 1995) and \( \text{ze3}^{	ext{D}} \) (Ruel et al., 1993) are null alleles. \( \text{wg}^{	ext{D}} \) encodes a non-secreted wg protein (van den Heuvel et al., 1993a,b). \( \text{wg}^{	ext{U90}} \) is a temperature-sensitive allele (Baker, 1988) and the rest are characterized phenotypically as strong alleles (Klingensmith, 1993; Siegfried et al., 1992), except for the \( \text{arm} \) alleles, which are hypomorphs but are the strongest alleles that arc cell viable when homozygous (Peifer et al., 1991). Two null alleles of \( \text{N}^{	ext{ru}} \) and \( \text{N}^{	ext{D}} \) were used. For further information, see Lindsay and Zinn (1992).

A P-element placing the wg ORF under the control of the sevenless (\( \text{sev} \)) promoter (\( \text{P}^{	ext{sev-wg}} \)) was made by inserting the \( \text{XhoI} \text{Cl} 1 \text{at blunt ended fragment of the wg} \text{cDNA, pCV}\) (Rijswick et al., 1987) into the \( \text{XhoI} \) and \( \text{BglII} \) (blunt ended) sites of \( \text{pSE} \text{WA} \) (Fortini et al., 1992, between the \( \text{sev} \) proximal promoter and 3' processing elements, \( \text{pSE} \text{WA} \) also contains three tandem repeats of the \( \text{sev} \) enhancer 5' of the promoter, \( \text{ye}^{	ext{C1}} \) embryos were co-injected with \( \text{P}^{	ext{sev-wg}} \) and \( \text{pZ25.7} \) as described previously (Rubin and Spradling, 1982) and several independent lines were established using standard balancer stocks. A stock containing the \( \text{cas} \text{Z} \) coding sequences under the control of the \( \text{sev} \) enhancer (three tandem repeats) and \( \text{kas} \) proximal promoter (\( \text{P}^{	ext{sev-lacZ}} \)). R. Conklin, personal communication) was obtained from Todd Laverty (UC Berkeley, CA).

The following heat-shock strains were used: \( \text{P}^{	ext{hsp}-\text{wg}} \) (Noordermeer et al., 1992), \( \text{P}^{	ext{hs-cw3}} \) (Siegfried et al., 1992) and \( \text{P}^{	ext{hsp-dsh}} \) (Axelrod et al., 1996). \( \text{P}^{	ext{hsp-wg}} \) is on the third chromosome, the other two on the second. The following chromosomes were created by recombination. \( \text{P}^{	ext{sev-wg}} \), \( \text{P}^{	ext{hs-cw3}} \), \( \text{P}^{	ext{hsp-wg}} \) (the white \( \text{w} \) gene in
wingless signaling in the fly eye and embryo

A P[neo-wg] transgene was inactivated by EMS mutagenesis. The P[neo-wg] insert on chromosome 3L was recombined with a Df(3R) mutation to make P[neo-wg], Df[3R]. Two different P[hs-dsh; w+] wg'd recombinants were created, one using a vg+ cn bw sp chromosome and the other a vg+dhg by rp, since both chromosomes contain a different lethal mutation unrelated to wg (Couso et al., 1994). Both P[hs-dsh; wg'd] recombinants were placed over a Sb53a-TM6B compound chromosome, so that homozygotes could be identified by the absence of the Tubby pupal marker.

Whole-mount stainings of pupal eyes and embryos

Pupal eyes were dissected and then immunostained as described (Buchholzer et al., 1993). Embryo stainings were performed essentially as previously described (Frisch et al., 1987; Grosniklaus et al., 1992). Affinity-purified rat α-cort insulin was generously provided by K. Buchholzer (Fred Hutchinson Institute, WA). Mouse α-ac monoclonal antibody was a gift of Sean Carroll (University of Wisconsin at Madison). Rabbit α-lacZ antisera was from Cappel and affinity purified rabbit α-wg antisera was kindly provided by C. Harryman-Samos (Stanford University, CA). Mouse α-N monoclonal antibody was provided by S. Artavanis-Tsakonas (Yale Univ, CT) and mouse α-en antisera by T. Kornberg (UCSF, CA). The primary antibodies were used at the following dilutions: α-c, 1:3 to 5:5, wg, 1:20, N, 1:100, cut and en, 1:300, lacZ, 1:500. For histochemistry, secondary antibodies were either biotinylated (goat α-mouse, horse α-rabbit and rabbit α-rat; all from the Ellie ABC kit, Vectorstain, used at a 1:500 dilution) or goat α-rabbit conjugated to alkaline phosphatase (from Vector, used at 1:500). For fluorescence microscopy either donkey FITC α-mouse (1:100) or donkey Cy3 α-rabbit (1:2000) were used (Jackson Immunoochemicals). Confocal images were collected with a Bio-Rad MRC 1000 confocal laser scanning module attached to a Zeiss Axioimage microscope. Images were imported into Adobe Photoshop for presentation.

In situ hybridization to whole-mount embryos using digoxigenin-labeled probes (Tautz and Pfeiffle, 1989) and antibody in situ double stainings (Manoukian and Krause, 1992) were performed as described (detailed protocol available upon request).

All whole-mount stainings were photographed with a Nikon Microphot-FXA microscope and slides were scanned into Adobe Photoshop for presentation.

Production of mosaic animals

Mutant alleles of dsh, wg, and en were recombined onto a P[hs-neo; FRT]18A chromosome, parc onto P[hs-neo; FRT]19A, wg onto P[hs-neo; FRT]40A, and a P[neo-wg; w+] mapping to 3L onto P[hs-neo; FRT]39A, all in a w background. w clones were induced in animals heterozygous with the appropriate P[mini-w+; P[FRT]] chromosome: P[mini-w+; hs-M5A], 10D, P[hs-neo; FRT]18A; P[mini-w+; FRT]19A; P[mini-w+; hs-M721C], 36F, P[hs-neo; FRT]. All FRT derivatives are as described (Xu and Rubin, 1993) except for P[mini-w+; FRT]18A, which is from the Jan lab enhancer detection collection (Bir et al., 1989). FLP recombinase was provided from the FLP-99 chromosome (Chou and Perrimon, 1992). Clones were induced by a one hour heat shock (37°C) 24-48 hours at 25°C after egg laying and scored for the absence of pigmentation in the adult eye.

For production of N germ-line clones, the N null alleles were recombined onto a P[mini-w+; FRT]19h chromosome (Chou and Perrimon, 1992). N, P[mini-w+; FRT]19h females were crossed to a w ovo D1, P[mini-w+; FRT]19h/Y; P[hs-FLP]19h stock (Chou and Perrimon, 1992) and progeny were heat shocked late 3rd instar early pupation for 2 hours at 37°C (earlier heat shocks resulted in high lethality due to somatic clone). Mosaic mothers were crossed to P[hs-lacZ]C; P[hs-lacZ]C; P[hs-wg]TM3 males. Embryos with no β-gal staining lacked both maternal and zygotic expression of N.

Heat shocks and other temperature shifts

The P[hs-neo] phenotype was induced by multiple heat shocks as previously described (Nordehoefer et al., 1992). Late larvalearly pupal temperature shifts were performed by submerging glass vials in a water bath of the appropriate temperature (37°C for heat shocks). At all other times, larvae and pupae were kept at 25°C. Formation of white pupae was used as the reference point (0 hours AEF).

Histology

Flies were prepared for scanning electron microscopy by serial dehydration in ethanol and Freon 113 (EM Sciences) as described (Kimmel et al., 1990). Dried samples were mounted with colloidal graphite, and a 10 nm gold-platinum coat was applied with a Hummer spotter coater. The samples were viewed with an AMR1000 SEM and photographed using Polapan 400 film (Kodak). Pupal eyes were surface stained with Co(NO3)2·6H2O and (NH4)2S as described (Kimmel et al., 1990).

RESULTS

wg blocks SOP formation in the eye

During the course of our attempts to create a dominant adult wg mutant through limited misexpression of wg during larval development, we found a highly penetrant phenotype when wg was placed under control of the eye-specific promoter svpr. As shown in Fig. 1, the eyes of P[svpr-wg] flies appear normal, except that the interommatidial bristles, normally found at alternating vertices in the compound eye’s hexagonal array, are almost completely missing. Sections through adult eyes (data not shown) and surface staining of pupal eyes with cobalt sulfide (Fig. 1E,F) revealed no other detectable abnormality in
adult eye. The bristles are replaced in the repeated structure of the eye with tertiary pigment cells. Thus, at the level of ectopic wg expressed from the P[sev-wg] transgene, the effect of wg on eye development is very specific.

Interommatidial bristles are mechanosensory organs composed of four cells that are derived from a single sensory organ precursor (SOP; Cagan and Ready, 1989a). Larval SOP determination has been best described in the wing imaginal disc (Campuzano and Modolell, 1992; Jan and Jan, 1993). The process begins with small groups of cells expressing basic helix-loop-helix proteins such as achaete (ac) and scute (Cubas et al., 1991; Skeath and Carroll, 1991). All the cells in these proneural clusters have the ability to become the SOP, however, in a wild-type background, only one does. This cell is thought to become the SOP by reaching a threshold level of ac and/or scute after which it inhibits these genes' expression in its neighbors (Ghysen et al., 1993; Simpson, 1990). This lateral inhibition is mediated by the neurogenic pathway, in which the products of the DI and N genes are thought to act as ligand and receptor, respectively (Aravanis-Tagkous et al., 1995; Muskavitch, 1994). The initiation of SOP development is correlated with the expression of a new set of genes, such as neutralized (Huang and Dambly-Chaudière, 1991) and for some SOPs, cut (Buchholinger et al., 1993). The SOP undergoes to two divisions to generate the four cells that will give rise to the mature bristle organ (Bodmer et al., 1989; Hartenstein and Posakony, 1989).

The events leading to SOP formation in the eye have many similarities to those occurring in other tissues. ac protein becomes detectable shortly after white prepupa formation (data not shown). At 3 hours after the white prepupa stage (3 hours APF), the ac gene is expressed in small clusters of cells throughout the eye (Fig. 2C). Unlike the photoreceptors and cone cells, the appearance of the ac-positive cells is not related to the distance from the morphogenetic furrow, although the cells anterior of the furrow do not express ac (see arrows in Fig. 2C). By 6 hours APF, only one cell per cluster still expresses ac, again with the anterior-most portion of the eye showing a less mature pattern (data not shown). At 15 hours APF, after the eye disc events, ac protein is gone, but the daughters of the SOPs can be observed by staining with α-cut antisera (Fig. 2A). Because of the complicated morphogenetic movements associated with the eye/brain disc eversion, we have been unable to stain tissue between 6 and 15 hours APF.

In the P[SEV-wg] eyes, ac expression is greatly reduced compared to controls though not completely absent (Fig. 2D,F). After disc eversion, no SOPs are found, as judged by cut staining (Fig. 2B) and an enhancer detector line for the neutralized gene (data not shown). Thus, wg appears to act at the level of the proneural genes, i.e., ac, to inhibit SOP formation.

**wg-dependent SOP inhibition is a paracrine effect**

The activity of the sev promoter has been well studied in third instar larva, by monitoring endogenous sev expression (Tomlinson et al., 1987) and with chimeric constructs (Boettiger et al., 1989) using sev enhancer and promoter elements similar to the ones in P[sev-wg]. The enhancer is active in the cone cells and in a subset of the underlying photoreceptor precursors. No description of sev expression has been reported after pupation, so the possibility existed that wg was expressed in the proneural cells of P[sev-wg] eyes, suggesting a possible autocrine effect.

This question was addressed by examining the distribution of wg protein in P[sev-wg] eyes. Though wg is a secreted protein, it is found at the highest levels on the surface of the same cells that synthesize it (Bejojev and Wieschaus, 1995; Cousu et al., 1994; van den Heuvel et al., 1993). In P[sev-wg] eyes, the highest levels of wg protein were found around the four cone cells (Fig. 3A) and accumulated on their apical surface (Fig. 3B). In more basal sections of the eye, wg protein was associated with the photoreceptors, which extend basally to the same plane as the ac-positive cells (Fig. 3C). There was no significant overlap between wg protein and the remaining cells expressing ac.

To confirm that the sev enhancer was not active in the proneural clusters, we stained eyes of flies that contained a P[sev-laZ] transgene (see Materials and Methods) for products of laZ and ac. As found for wg in P[sev-wg] eyes, most of the β-gal was found in the cone cells (data not shown). In the same focal plane as the ac-expressing cells, there is no overlap (Fig. 3D). Thus, the inhibitory effect of wg on ac expression is paracrine in nature.
The wg signal transduction pathway in the eye

Extensive genetic analysis, confirmed by recent biochemical experiments, has identified four genes that encode probable components of the wg signaling pathway, *porc*, *dsh*, *zw3* and *arm* (Klingensmith and Nasse, 1994; Siegfried and Perrimon, 1994; see introduction). Mosaic analysis (using the w gene as a marker) was performed to determine if these genes were required for the P[sev-wg]-dependent bristle inhibition. Control clones still lack bristles (Fig. 4A), as do clones mutant for the endogenous wg gene (Fig. 4C). In clones that lack the P[sev-wg] transgene, bristles are found almost to the clonal boundary (Fig. 4B). Likewise, 89% of the mutant clones for *porc*, *dsh* and *arm* had the full array of bristles within the clone (Fig. 4D-F and Table 1) and an additional 9% had a partial rescue of the bristleless phenotype. The remaining 2% that still lacked bristles were small in size and probably not completely mutant since the absence of the w gene cannot be detected on the surface of the eye at the cellular level. These experiments indicate that *porc*, *dsh* and *arm* are required for wg-dependent bristle inhibition.

*zw3* is unique among the known genes required for wg signaling because it must be inhibited for the wg signal to be transduced (Klingensmith and Nasse, 1994; Siegfried and Perrimon, 1994). Thus, loss of zw3 should be equivalent to activation of wg signaling. Therefore, a zw3 mutant clone in the eye might be expected to lack bristles. This straightforward analysis cannot be employed because the cells in zw3 clones in the eye imaginal disc do not differentiate into eye tissue (Treisman and Rubin, 1995; data not shown). This is probably due to the fact that high levels of wg signaling activity prevent the morphogenetic furrow from progressing, blocking any subsequent differentiation (Treisman and Rubin, 1995).

If zw3 must be inhibited for the wg signal to be transduced, then flooding cells with zw3 protein might silence the signal. This has been shown to be the case in *Xenopus* where overexpression of the homologue of zw3, glycogen-synthase kinase 3, blocks Wnt gene induction of dorsal mesoderm (Dominguez et al., 1995; He et al., 1995). We attempted a similar experiment by creating flies with one copy of P[sev-wg] (we chose one of the weaker P[sev-wg] lines, which at one copy has approximately 20 bristles/eye) and one or two copies of a heat-shock construct expressing the zw3 gene, P[hs-zw3] (Siegfried et al., 1992). zw3 was induced by heat shock shortly before and twice after the onset of pupation (see Fig. 5 legend for details). Though the results were not entirely conclusive (Fig. 5), many pupal eyes showed a significant response especially when the ratio of P[hs-zw3]/P[sev-wg] is two (Fig. 5C). Other heat-shock regimes were not as effective at suppressing the P[sev-wg] phenotype. These results are consistent with the current model for zw3 function in wg signaling.

Overexpression of dsh has previously been found to mimic wg signaling in cultured cells (Yanagawa et al., 1995), frog embryos (Sokol et al., 1995; Rothbacher et al., 1995) and in the wing imaginal disc (Axelrod et al., 1996). The same P[hs-dsh] transgenic stock used in the wing can also duplicate the effect of wg in the eye. Induction of dsh at 3 hours (data not shown) or 6 hours APF (Fig. 6B) could block bristle formation, but heat shock at 9 hours APF (Fig. 6C) failed to inhibit bristles in the interior of the eye, though inhibition still occurred toward the periphery. This can be explained by previous work...
Table 1. Summary of the clonal analysis in a P[sev-wg] background (see Materials and Methods for details)

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Bare</th>
<th>Partial</th>
<th>Full</th>
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<tr>
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<td>0</td>
<td>1</td>
<td>17</td>
</tr>
<tr>
<td>w</td>
<td>25</td>
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<tr>
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<td>yw* [B]</td>
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<td>yw* [D]</td>
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<td>w* [A]</td>
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The P[sev-wg; w+] clones are w+/+ clones surrounded by P[sev-wg; w+] tissue. The rest are clones of the homozygous genotype indicated and the entire eye, including the cells in the clone, are P[sev-wg; w+]. Bare means no bristles found within the clone and full means the normal wild-type bristle density.

(Cagan and Ready, 1989a,b), which showed that SOP determination occurs first in the center of the eye and radiates outward concentrically. The same time requirements were seen when the bristles were inhibited using P[hs-wg] (data not shown).

Genetic and biochemical evidence places dsh downstream of wg in the signal transduction pathway (Klugerman et al., 1994; Noordermeer et al., 1994; Theisen et al., 1994; Yanagawa et al., 1995), suggesting that the overexpression of dsh can bypass wg function. However, in the wing, where dsh causes an expansion of the wing margin, it appears that wg gene activity is needed to see the dsh effect (Axelrod et al., 1996). In the eye, the opposite appears to be true. In pupa homozygous for a wg temperature-sensitive mutation, induction of dsh after 6 hours at the restrictive temperature still inhibited SOP formation (Fig. 6E). Thus it appears that dsh in the eye can act independently of wg, though caveats remain (see discussion).

The role of N in wg signaling in the eye

A strong interaction between mutations in the N and wg genes has been described (Cousin and Martinez Arias, 1994; Heng et al., 1994), which suggests that the two genes have common developmental targets in some tissues. One report suggested that wg encodes a ligand for N, based on these genetic interactions and the fact that N encodes a transmembrane receptor-like protein (Cousin and Martinez Arias, 1994). In the eye, N activity is required for almost every differentiated cell type (Cagan and Ready, 1989b), so examining N clones in a P[sev-wg] background was not possible. Therefore, we utilized N[+], a temperature-sensitive allele (Cagan and Ready, 1989b). When these flies were reared at the restrictive temperature for 3-11 hours APF in a P[sev-wg] background, a strong suppression of the wg bristleless phenotype was seen (Fig. 7B). This is consistent with a proposed role for N in transducing the wg signal. However, removal of DI activity for the same time period also suppresses the P[sev-wg] phenotype (Fig. 7C).

N and DI are key components in the lateral inhibition pathway (functioning as receptor and ligand respectively) that insures the proper number of bristles in the eye (Cagan and Ready, 1989b; Parody and Muskavitch, 1993; note the abnormally high bristle density in Fig. 7B and C). This pathway is independent of wg, since mutant clones of wg, porc, dsh and arm in an otherwise wild-type background have the normal number of bristles (data not shown; see also Fig. 4D-F). Thus, the observation that loss of DI activity can suppress the P[sev-wg] phenotype as well if not better than loss of N raises the possibility that the interaction between N and wg in the eye is due to the role of N in the lateral inhibition pathway.

If a higher level of wg expression is used (via a heat-shock promoter) all the bristles in the N[+]/N[+] background can be inhibited (data not shown; pupa were placed at the restrictive temperature for 6 hours before a 30 minute heat-shock pulse was given at 6 hours APF). However, it is known that the N[1] allele does not completely remove N activity (Cousin and Arias, 1994; Hartenstein et al., 1992) so this result is inconclusive. In the eye, it is not possible to determine whether wg works...
Fig. 6. Overexpression of dsh can inhibit bristle formation independently of wg. (A-C), SEM micrographs of P[hs-dsh] eyes given no heat shock (A) or a 30 minute heat shock (37°C) at 6 hours APF (B) or 9 hours APF (C). When dsh was induced at 6 hours APF, more than half the eyes had no or only a few bristles in the center of the eye (n=11) and the rest had a small patch of bristles in the center (n=8). At 9 hours APF, bristles were found over the interior two thirds of the eye but bristles were still missing toward the periphery (n=17). (D,E), cut stainers of P[hs-dsh], wg<sup>act</sup> homozygotes that were raised at 29°C (the permissive temperature) and then incubated at 39°C for 0-12 hours APF; without (D) or with (E) a 30 minute heat shock at 6 hours APF. Antibody stainings were done at ~30h APF. The cut-positive SOPs (now at the 4-cell stage) are completely absent in the heat shocked eyes (n=8). wg<sup>act</sup> homozygotes were identified as described in Materials and Methods.

through N or in a parallel pathway converging at proneural gene expression.

Role of N in wg signaling in the embryo

In order to more rigorously test the requirement of N for wg signaling, a tissue is needed where a putative N-wg connection can be separated from the wg-independent functions of N. One suitable place is the embryonic epidermis. Embryos mutant for N undergo a dramatic neural hyperplasia; almost all of the cells of the epidermis delaminate and become neuroblasts (Campos-Ortega, 1993). However, the epidermis remains relatively intact until full germ-band extension, after significant wg signaling has already occurred. Null N embryos were generated by making germ-line clones (Chou and Perrimon, 1992; see Materials and Methods). Antibody staining revealed no detectable N protein in N germ-line clones that have received a paternal Y chromosome (Fig. 8F). Thus we can examine wg signaling in a tissue that has never contained N protein.

Two well-characterized targets of wg signaling in the embryo are the engrailed (en) gene (DiNardo et al., 1988; Martinez-Arias et al., 1986) and the wg gene itself (Belyaev and Wieschaus, 1993; Hooper, 1994; Yoffe et al., 1995). Careful analysis of expression of both genes has revealed that, in wg mutants, wg transcript begin to fade before the embryo reaches full germ-band extension (stage 9; all stages according to (Campos-Ortega and Hartenstein, 1995), and is gone by the beginning of stage 10 (Manoukian et al., 1993). en protein in the adjacent posterior cells fades shortly thereafter. By mid-stage 10, both en protein and wg transcripts are completely gone from wg<sup>act</sup> homozygous embryos (Fig. 8B). In N null embryos at early stage 10, wg and en patterns are indistinguishable from wild type (data not shown). At mid-stage 10, both sets of stripes are still clearly present (Fig. 8C,D). The stripes do appear a little ragged, and we believe this is a consequence of the beginning of the disintegration of the epidermis, which is well underway by late stage 10 (about 15-20 minutes later than the embryos shown in Fig. 8).

Despite the results in Fig. 8, it might be argued that in N mutants, perhaps wg and en expression no longer depended on wg activity. To address this, we examined the effect of global wg expression on en transcript distribution in a N mutant background. As previously reported (Noordermeer et al., 1992, 1994), overexpression of wg via a heat-shock promoter in an otherwise wild-type background causes a dramatic posterior expansion of the en stripes so that they are about twice as wide as normal (compare Fig. 9A and B). This expansion in still seen in embryos lacking N protein (Fig. 9D) and is dependent on the presence of the P[hs-wg] transgene (Fig. 9C). In the complete absence of N protein, wg signaling appears normal as late as we can reliably assay for it.

Fig. 7. Removal of N or DI activity can suppress the P[svw-wg] bristleless phenotype. SEM micrographs of P[svw-wg]<sup>+/+</sup> (A), N<sup>101</sup>Y; P[svw-wg]<sup>+/+</sup> (B) and P[svw-wg], Df<sup>PRODIEE</sup> (C) flies that were reared at 17°C and incubated at 32°C for 3 to 11 hours APF (7 hours APF at 17°C corresponds to 3 hours APF at 25°C) and then kept at 17°C until eclosion or dissection of pharate pupa. Control and N<sup>101</sup> Y hemizygotes were made by crossing P[svw-wg]<sup>+</sup> males to either w<sup>1118</sup> or N<sup>101</sup> Y females. All males then had the desired genotype. P[svw-wg], Df<sup>PRODIEE</sup> and Df<sup>PRODIEE</sup> individuals were crossed and appropriate animals identified by the absence of the dominant Tubby marker (found on TM3). All N<sup>101</sup> Y hemizygotes (n=20) and Df<sup>PRODIEE</sup> transheterozygotes (n=9) showed the dramatic increase in bristle number. Note the higher than normal bristle density, indicative of the role these genes play in lateral inhibition. The DI mutant combination consistently gave a more severe bristle hyperplasia than N<sup>101</sup>Y in both a P[svw-wg] and non-transgenic background.
K. M. Cadigan and R. Nusse

DISCUSSION

wg inhibits SOP formation at the level of the proenulean genes

The interommatidial bristle is a 4-cell sensory organ that arises from a single SOP which is selected from a group of cells expressing pronuclear basic helix-loop-helix proteins (Campuzano and Modolell, 1992; Jan and Jan, 1993a). Our data strongly suggests that P[seg-wg] derived wg protein blocks SOP formation in the eye by inhibiting pronuclear gene expression. Levels of ac protein are much lower in P[seg-wg] expression in the eye compared to controls (Fig. 2C-F). 12 hours later, after the expression has been evoked, no SOP daughters cells are seen in the transgenic eye (Fig. 2A,B). Though disc evisceration prevents us from directly showing that no SOPs ever form in P[seg-wg] eyes, the time window when P[hs-sog] or P[hs-dpp] can inhibit bristle formation (no later than 6 hours APF for the central portion of the eye; Fig. 6 and results) is consistent with the model that, once an SOP is determined, wg signaling activity can no longer influence its fate.

The ac protein is not the only proenulean gene product monitored in this study and we are by no means suggesting that the wg signaling pathway acts directly on the ac promoter. In fact, loss of the ac gene alone does not result in the complete elimination of interommatidial bristles; a related gene, scute (sc) must also be removed (Brown et al., 1991). The expression patterns of ac and sc are nearly identical (Cubas et al., 1991; Skeath and Carroll, 1991). This is most likely achieved by a combination of shared enhancer elements (Gómez-Skarmeta et al., 1995) and auto- and transactivation between the two genes (Martínez and Modolell, 1991; Skeath and Carroll, 1991; Van Doren et al., 1992). In addition, there are important negative inputs from other BHLH proteins such as extramacrochaete (Cubas and Modolell, 1992; Van Doren et al., 1992) and hairy (Brown et al., 1991; Van Doren et al., 1994). wg could be acting to inhibit ac (and presumably sc) expression at any of these regulatory levels. Further studies are needed to address this issue.

The P[seg-wg] bristleless phenotype was unexpected, because in the wing imaginal disc, wg has been shown to have the opposite effect, i.e. it promotes bristle development. In the absence of wg activity, the pronuclear ac-positive clusters fail to form (Cubos et al., 1994; Phillips and Whittle, 1993). It is not clear why wg activates ac in one tissue and inhibits it in another, but this is a simple example of how one signal can generate different responses in different tissues.

wg is not normally expressed in the interior of the eye, but it is present at the periphery, forming a ring around the pupal eye (Cadigan and Nusse, unpublished data). Interestingly, the edge of the eye lacks bristles (Cagan and Ready, 1989b; Fig. 1A). Clones of arm at the periphery contain ectopic bristles (Cadigan and Nusse, unpublished data), suggesting that wg normally inhibits bristles there. However, large wg clones do not show this effect. We are currently examining this in more detail.

The wg signal transduction pathway in the eye

A genetic pathway for wg signal transduction has been elucidated in which the gene products work in the following order: nars → wg → dsh → T w2 → arm (Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994). Studies in the wing imaginal disc have indicated that dsh, w2 and arm are also required for wg signaling (Cousso et al., 1994; Díaz-Benjumea and Cohen, 1994; Klingensmith et al., 1994; Peifer et al., 1991; Theisen et al., 1994; Wilder and Perrimon, 1995). This study extends these findings; porc, dsh and arm are clearly required for the ability of wg to inhibit eye bristles (Fig. 4; Table 1). The overexpression experiments with w2, while not as conclusive (Fig. 5), are entirely consistent with the favored model, where wg acts by antagonizing w2 gene activity. While there may be exceptions to this (below), it seems that most tissues use the same wg signaling components to achieve a variety of results.

The mammalian counterpart of w2, glycoen synthase kinase-3, has been shown to function in ras-dependent wg signaling (Stambolic and Woodgett, 1994). This raises the possibility that members of the ras and wg pathways share components in flies. In the eye, differentiation of photoreceptor cells is absolutely dependent on ras-dependent signaling (Simon et al., 1991). However, in clones of dsh and arm, all photoreceptors are present (S. Kac, K.M. Cadigan and R. Nusse, unpublished observations). In the wing, clonal analysis with members of the ras pathway demonstrated that, unlike wg, they were not required for wing margin development (Díaz-Benjumea and Hafen, 1994). Thus, no interaction between these two pathways has yet been observed in Drosophila.

Fig. 8. wg signaling appears to be normal in N null mutant embryos. (A-D) Whole-mount staining for wg transcripts (blue) and for en protein (brown) in wild-type (A), wg;en (B) or N;en (C,D) mutant embryos. All embryos are at mid-stage 10 (Campos-Ortega and Hartenstein, 1985). Both wg and en are absent at this stage from the epidermis of the wg mutants, but remain robust in the N mutant background (these embryos were also stained for β-gal protein, to unambiguously identify maternal and zygotic N mutants (see Material and Methods). (E,F) Confocal images of N antibody staining with a monoclonal antibody directed against the intracellular domain of N (Fehon et al., 1990) in N;seg germline clones receiving a paternal P[seg-lacZ] (E) or Y (F) chromosome. N signal is completely lacking in the embryos that are negative for β-gal protein. Similar results in wg, en and N expression were obtained with a second N null allele, N;seg;en (data not shown).
Fig. 9. The effect of ubiquitous expression of wgl on en transcript distribution is still seen in a N null mutant background. All embryos are whole-mount stainings of en transcripts. (A) P[hs-wg] embryo with no heat shock. The en stripes are normal in appearance. (B) P[hs-wg] embryo after three 20 minute heat shocks (37°C) during early gastrulation. The en stripes have expanded posteriorly, to about twice the width (N=20). (C) N\textsuperscript{R434} null mutant after the three heat shocks. The stripes are somewhat ragged, but still present at the normal level. (D) P[hs-wg] embryo after heat shock treatment. The stripes have broadened as they do in a N\textsuperscript{R434} background. All embryos were mid-stage 10 and the same results were obtained using the N\textsuperscript{R434} allele. N null embryos were created and identified as described in Materials and Methods.

wgl expression is subject to positive autoregulation in the embryo (Bajovicek and Wieschaus, 1993; Hooper, 1994; Yofie et al., 1995) and recent evidence suggests that this occurs through a distinct signaling mechanism (Hooper, 1994; Manoukian et al., 1995). Some discrepancies exist between the two reports, but Manoukian et al. (1995) provide strong evidence that wgl autoregulation requires porc but not dsh, zv3 and arm. They suggest a model where porc functions only in wgl autoregulation and the other three genes in wgl paracrine functions.

Our results in the eye indicate that, at least in the eye, porc is required for wgl paracrine signaling. While we could clearly see sev enhancer-driven wgl expression in cone cells and photoreceptors, we found no expression in the proneural clusters, the targets of our experiments (Fig. 2). The endogenous wgl gene was not required for the P[sev-wg]-dependent bristle inhibition (Fig. 4C), ruling out a paracrine-autocrine circuit. Our results indicating a role for porc in paracrine wgl signaling are consistent with the observation that sevcretion or diffusion of wgl protein is blocked in borc mutant embryos (Siegfried et al., 1994; van den Heuvel et al., 1993a).

Overexpression of dsh can mimic the action of wgl in the eye (Fig. 6A) as has been shown previously in the wing (Axelrod et al., 1996) and in cultured cells (Yanagawa et al., 1995). In the wing, this effect of dsh requires wgl. This does not appear to be the case in the eye (Fig. 6E). This is an important point because it speaks as to whether dsh can completely bypass the requirement for wgl or whether overexpression of dsh simply potentiates wgl signaling. It may be that there is residual wgl activity left in our experiments (we could only rear the animals for 6 hours at the restrictive temperature before induction of dsh; longer times killed the organism before disc evisceration). Another possibility is that a much higher threshold of wgl activity is needed to transform wing blade to wing margin than is needed to inhibit eye bristles. The data of Axelrod et al. (1996) show that the transformation of identity is more penetrant closest to the normal wing margin, where wgl is expressed. Thus, overexpression of dsh in the wing blade may not easily reach the necessary level of signaling to trigger the change in cell fate. In the eye, dsh is active (at 3 hours APF) to inhibit bristles in the middle of the eye (far from endogenous wgl expression) just as efficiently as bristles closer to the periphery. That dsh can bypass the need for wgl is also supported by the cell culture experiments (Yanagawa et al., 1995) where no detectable wgl protein was observed under conditions where dsh could stabilize arm protein. In addition, Park et al. (1996) have recently shown that overexpression of dsh in the embryo can induce wgl targets in a N null background.

Is N required for wgl signaling?
On the basis of genetic interactions between mutations in the two genes, the N protein was proposed to be a receptor (or part of a receptor complex) for wgl (Couso and Martinez Arias, 1994). In the eye, we also observed strong genetic interactions between N and wgl (Fig. 7). However, the interpretation of these experiments is complicated, for N is known to affect bristle development independently of wgl, and because, for technical reasons, we could not completely remove N activity to determine whether wgl signaling could still occur. Likewise, the previously published genetic interactions involve animals where wgl and N activities are only partially removed (many of the experiments were done with double heterozygotes of various wgl and N alleles), and are therefore subject to the same limits of interpretation.

Unlike the eye, wgl signaling in the complete absence of N activity can be assayed in the embryonic epidermis until just after germ-band extension is complete (mid-stage 10), right before the absence of N causes most of the epidermis to delaminate and become neuroblasts. We found no significant change in the expression of wgl and en in N null mutants at this time (Fig. 8), even though their expression fades at early stage 10 in wgl mutants and mutants in dsh or arm (Manoukian et al., 1995; Van den Heuvel et al., 1993b). Indeed, the effect of overexpression of wgl on the en stripes is still seen in a N null mutant background (Fig. 9). Couso and Martinez Arias (1994) reported that the en stripes were affected in about half the N mutants they examined, but they used hyperplasia of the nervous system as their method for determining which embryos were N mutants. This happens after mid-stage 10, thus any effect on the stripes may be a secondary consequence of the epidermis falling apart. Therefore, we conclude that in N mutant embryos, wgl signaling occurs normally, at least with regard to the two markers we assayed.

A similar conclusion with regards to N-wgl interactions has been reached in the wing (Rulifson and Blair, 1995). They showed that wgl could still regulate an expression in homozygous clones for a N null allele. These mutant clones should completely lack N, barring prolonged perdurance of the N protein. Of equal importance is their finding that N activity is required for wgl expression at the wing margin (see also Diaz- Benjumea and Cohen, 1995; Doherty et al., 1996). This means that all of the genetic interactions between wgl and N in the wing can potentially be explained by a reduction in N activity causing a reduction in the amount of wgl signal, not the ability of wgl to signal.
Another link between wg and N has been proposed by Axelrod et al. (1996), who have presented evidence that dsh protein can bind to and inhibit N activity in the wing imaginal disc. They suggest that part of the ability of wg to induce bristles in the wing is achieved by inhibition of N through dsh.

Such an antagonistic relationship does not appear to be occurring in the eye since wg, dsh and N all inhibit bristle formation, although we can not rule out a mechanism where wg and dsh activate N to inhibit ac expression.

A subtle role for N in transducing the wg signal cannot be entirely ruled out. However, our results and those of Russell and Blair (1995) suggest that the signal in tissues where the direct test can be done, i.e., can wg signaling occur in cells that lack N protein, N is not required. A better candidate for a wg receptor is the product of the Drosophila frizzled2 gene, which can bind to and transduce the wg signal in cultured cells (Bianet et al. 1996). N showed no activity in this wg-binding assay. In the absence of any biochemical data suggesting that the proteins interact, the simplest models for wg signal transduction should exclude a direct role for N.

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