RESEARCH COMMUNICATION

Wingful, an extracellular feedback inhibitor of Wingless

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Secreted peptide signals control many fundamental processes during animal development. Proper responses to these signals require cognate inducible feedback antagonists. Here we report the identification of a novel *Drosophila* Wingless (Wg) target gene, *wingful* (*wf*), and show that it encodes a potent extracellular feedback inhibitor of Wg. In contrast to the cytoplasmic protein Naked cuticle (Nkd), the only known Wg feedback antagonist, Wf functions during larval stages, when Nkd function is dispensable. We propose that Wf may provide feedback control for the long-range morphogen activities of Wg.

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Pattern formation during animal development is largely controlled by intercellular signaling events. Peptide signals secreted by some cells regulate a variety of cellular processes in other cells by activating signal transduction pathways, which induce cell fate changes and morphogenetic responses. Over the past decade it has been recognized that the precision of developmental signaling depends largely on the employment of positive- and negative-feedback control (Perrimon and McMahon 1999; Freeman 2000). Negative-feedback inhibitors are widely used to spatially and temporally limit responses to developmental signals, enabling an intricate accuracy of pattern formation (Freeman 2000).

Wnt proteins are secreted signaling molecules that direct growth and cell fates in processes as diverse as embryonic segmentation, CNS patterning, and limb development (for review, see Wodarz and Nusse 1998). The protein encoded by the *Drosophila* segment-polarity gene *wingless* (*wg*) is a defining member of the Wnt family (for review, see Nusse and Varmus 1992). In embryos, Wg acts as a short-range inducer: it is secreted by subpopulations of cells within each segment and received by their immediate neighbors (van den Heuvel et al. 1989). Later in development, Wg plays an essential role in the primordia of the adult appendages, where it acts directly on cells, over a range of many cell diameters, to organize their patterns of differentiation (Zecca et al. 1996; Neumann and Cohen 1997).

A negative-feedback inhibitor has recently been iden-

tified that limits the effects of the Wg signal (Zeng et al. 2000). The *naked cuticle* (*nkd*) gene encodes an EF hand protein that regulates embryonic Wg activity by acting as an inducible antagonist of the Wg transduction component Dishevelled (Rousset et al. 2001). Unexpectedly, the *nkd* product plays no discernible role at later stages of development, such as during the patterning of imaginal discs (Zeng et al. 2000). This is particularly surprising because the spatial patterning activities of the Wg morphogen are especially demanding during these processes (Zecca et al. 1996; Neumann and Cohen 1997; Strigini and Cohen 2000). It is tempting to suggest, therefore, that another feedback inhibitor is used to antagonize Wg activity in larval tissues.

Most, if not all, genes encoding negative-feedback inhibitors are transcriptionally induced in all cells transducing the respective signal, with no apparent stage or tissue specificity. This behavior contrasts that of the ef-

Figure 1. Expression pattern of *wf*, a target of Wg signaling. (A–C) Wing imaginal discs (anterior to the left, dorsal up), showing the activity of the Gal4 enhancer trap insertion *P[S145]* revealed by GFP fluorescence (A), or in situ hybridizations of a wild-type (B) and a *dpp-Gal4 UAS-wg* disc (C) with an RNA probe derived from *CG13076*. Note the ectopic expression of *wf* in a stripe along the anteroposterior compartment boundary in C. (D–F) Leg imaginal discs, showing the expression of *P[S145]* (D) or of *CG13076* transcripts in wild-type (E) or *dpp-Gal4 UAS-wg* animals (F). Note the ectopic expression of *wf* in dorsal regions of the leg disc. In D, a haltere disc is visible to the upper right of the leg disc. (G,H) Dorsal view (anterior to the right) of embryos showing the *wf* expression pattern visualized by *P[S145]* activity (G) or in situ hybridization (H).

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Figure 2. *wf* encodes a secreted protein with homology to pectin acetylsterases. (A) Structural organization of the *wf* locus, indicating the position of the Gal4 *P*-element insertions. Boxes represent exons, coding sequences are colored. (B) Alignment of the Wf protein sequence with the putative pectin acetylsterase family member F3L24.31 from *Arabidopsis thaliana* (on chromosome III BAC F3L24, GenBank accession no. AC011436). The position of the stop codon present in allele *wf*^{l41} is indicated by an asterisk. The complete *wf* mRNA and protein sequence has been deposited to GenBank (accession no. AY078993). (C) Western blot of an immunoprecipitation experiment using supernatants (S) and cell lysates (L) from S2 cells transfected with HA-tagged *wf* (HA-*wf*), *dispatched* (HA-*disp*), and *wg* (HA-*wg*). Proteins were precipitated with a monoclonal anti-HA antibody. HA-Wf and HA-Wg are readily secreted as they are detected in lysates and supernatants. (D) Supernatants of HA-*wg*-expressing cells (D) and HA-*wf*-expressing cells (E) have been incubated with nontransfected S2 cells. Binding to cell surfaces was monitored by an anti-HA antibody, followed by an Alexa 594 goat anti-mouse secondary antibody, and is shown to the right of the bright field images.

factor targets of these signals, the induction of which generally depends on the developmental state of the cell receiving it (Freeman 2000). For example, although the expression of the Hedgehog (Hh) feedback antagonist Patched is up-regulated in all cells known to transduce the Hh signal (Forbes et al. 1993; Goodrich et al. 1996), one of the key effector targets of the Hh signal in discs, *decapentaplegic* (*dpp*), is only induced in larval but not embryonic cells (Basler and Struhl 1994; Tabata and Kornberg 1994). Similarly, the induction of the Smad feedback inhibitor Dad is a universal response to Dpp signaling (Tsuneizumi et al. 1997), whereas nonfeedback targets of Dpp, such as *pannier*, *tinman*, *labial*, and *op-*

tomoter-blind, are expressed in different subsets of Dpp-induced cells (for review, see Affolter et al. 2001).

Here we set out to identify a larval Wg feedback antagonist on the basis of its putative expression pattern. By screening a large collection of enhancer trap lines, we isolated a pan-Wg-target, *wingful* (*wf*); we show that it encodes a secreted protein with sequence homology to hydrolytic enzymes. A loss-of-function mutation in *wf* results in phenotypes reminiscent of Wg gain-of-function situations, whereas overexpression of Wf severely inhibits Wg signaling activity. Thus Wf functions, indeed, as a potent feedback inhibitor of Wg and complements the embryonic *nkd* system both temporally and mechanistically by shaping the larval response gradient to Wg morphogen signaling.

Results and Discussion

Identification of *wingful* as a Wg target gene

A library of 2000 Gal4 enhancer trap *P*-element insertions was established, each of which reports a gene expression pattern in the wing imaginal disc (Gerlitz et al. 2002). This collection was screened with a *UAS-GFP* reporter for lines that show a *wg*-like expression pattern. There were 11 insertions identified that reported *wg*-like gene expression in the embryonic epidermis and all imaginal discs (Fig. 1A,D,G). Four of these lines (S180, ND382, S476, S554) contained an insertion in the *wg* gene itself (data not shown); the other seven lines (S141, S145, S163, S330, ND337, ND339, ND634) all carried a *P*-element insertion at cytological position 72D, only a few base pairs upstream of gene *CG13076*, referred to as *wingful* (*wf*). These enhancer trap insertions indeed report the expression of *wf*, as revealed by RNA in situ hybridization (Fig. 1B,E,H). *wf* is ectopically expressed upon *wg* misexpression (Fig. 1C,F), indicating that *wf* is a Wg target throughout larval development.

wf encodes a secreted protein with structural homology to pectin acetylsterases

RACE analysis revealed that the first exon of *wf* is incorrectly predicted in the *Drosophila* genome annotations (<http://flybase.bio.indiana.edu>) and instead is located 5.3 kb further upstream (Fig. 2A). The resultant open reading frame of *wf* codes for a presumptive protein of 671 amino acids, with an N-terminally situated signal

sequence. The *wf* product is readily secreted from transfected *Drosophila* cells (Fig. 2C) and has a noticeable propensity to adhere to the surfaces of intact cells (Fig. 2D,E). The analysis of the Wf protein sequence reveals a significant structural homology to a subfamily of poorly characterized hydrolases related to plant pectin acetyl-esterase (Fig. 2B; Breton et al. 1996). Together, these results suggest that the product of the *wf* gene may catalyze the hydrolytic cleavage of an extracellular substrate.

Loss of Wf function causes a gain of Wg activity

To test our hypothesis that the pan-Wg-target *wf* encodes an inhibitor of Wg activity, we sought to abolish *wf* function by genetic means. From a collection of six EMS-induced lethal mutations, located between the distal breakpoint of *Df(3L)st-f13* and the proximal breakpoint of *Df(3L)brm11* (Meléndez et al. 1995), we identified a putative null allele of *wf* with a stop codon at amino acid position 141, encoding a severely truncated protein. Animals homozygous for *wf¹⁴¹* or animals of the genotype *wf¹⁴¹/Df(3L)st-f13* die during pupal stages, and show various phenotypes. The most prominent of these are patterning defects in the wing imaginal disc.

Wg signaling plays at least two distinct roles during wing development. Early reduction of *wg* activity results in a wing-to-notum transformation, indicating a requirement for Wg in defining the wing blade primordium (Morata and Lawrence 1977; Ng et al. 1996), but later reductions cause the loss of wing margin and adjacent tissue, indicating its subsequent role in specifying the wing margin and organizing wing blade development (Couso et al. 1994; Diaz-Benjumea and Cohen 1995; Rulifson and Blair 1995). As shown in Figure 3, *wf* animals show phenotypes opposite to both classes. Wing discs mutant for *wf* are enlarged with an extended wing blade region (hence the name *wingful*). Often these discs contain two wing pouches at the expense of notal structures (Fig. 3B). Although we did not detect an apparent expansion of *Distalless-lacZ* (*Dll-lacZ*) expression along the dorso-ventral axis, there was a significant increase in the number of cells expressing *neuralized* (Fig. 3C), a high-threshold Wg target expressed in neural wing margin cells (Zecca et al. 1996). Consistent with this observation, rare adult escapers mutant for *wf* show a dramatically increased number of mechanosensory bristles in the wing (Fig. 3J). Wg signaling also distinguishes between sternite and ventral pleura development in the adult abdomen (Shirras and Couso 1996). *wf* adult escapers show extra sternite bristles (Fig. 3F), an effect that was also observed with ectopic expression of *wg* (Shirras and Couso 1996). Finally, *wf* adults show extra dorsocentral bristles (Fig. 3H), sensory organs on the notum whose specification has been shown to depend on *wg* activity (Phillips and

Figure 3. *wf* loss-of-function phenotypes. (A–D) Wing imaginal discs stained for the expression of *Dll-lacZ* (A,B) or *neur-lacZ* (C,D). The discs in panels A and C are derived from wild-type larvae, the discs in B and D from *wf¹⁴¹/Df(3L)st-f13* mutant animals. (E,F) Abdominal sternites of a wild-type adult (E) and a *wf¹⁴¹/Df(3L)st-f13* escaper. The number of sternite bristles per wild-type segment is 19 ± 2 and in *wf* mutants 28 ± 2 ($n = 20$). (G,H) Dorsal views of wild-type (G) and *wf¹⁴¹/Df(3L)st-f13* mutant (H) thorax. Instead of two dorsocentral bristles, *wf* mutants show four (arrows in H). (I,J) Anterior wing margins of wild-type adults (I) and *wf¹⁴¹/Df(3L)st-f13* mutant escapers (J). (K) A *wf* mutant clone is marked by the absence of *yellow⁺* function; the *wf⁻* genotype leads to a reduction of Wf levels and a corresponding formation of extra sensory bristles within and adjacent to the clone. Small *wf⁻* clones show no mutant phenotype, as they are rescued by adjacent *wf⁺* tissue.

Whittle 1993; Calleja et al. 1996). Taken together, these results show that the absence of *wf* function causes a gain of Wg activity in developing adult tissues. Therefore, the function of the wild-type *wf* product is to limit Wg signaling activity.

Gain of Wf function causes a loss of Wg signaling

A further prediction of our assumption that Wf functions as a Wg feedback inhibitor is that *wf* overexpression should lead to *wg* loss-of-function phenotypes. We present three lines of evidence to show that this is, indeed, the case. First, we replaced one of the *wf* enhancer trap *P*-element insertions with an EP element positioning 10 *UAS* sites upstream of the *wf* gene, rendering it transcriptionally responsive to Gal4 expression. Alterations of *wf* expression had unusually potent effects, as all commonly used *Gal4* drivers caused lethality in combination with *UAS-wf*. The only exceptions were *S168-Gal4* and *scalloped-Gal4*, which are expressed in the wing pouch and represent Wg targets (Fig. 4A,C), providing a self-regulating circuit in combination with *UAS-wf* (see below). Adult animals carrying the *S168-Gal4* and *UAS-wf* transgenes have severely reduced wings that lack all wing margin structures (Fig. 4E,F). Second, we analyzed the expression of two target genes in this context. *S168-Gal4* expression was virtually abolished by *UAS-wf* expression (Fig. 4A,B), whereas the expression domain of *wg* itself is expanded. It had previously been reported that Wg narrows its own domain of expression, because

Figure 4. *wf* gain-of-function phenotypes. (A,B) Expression of *S168-Gal4* visualized with *UAS-GFP* (in green) and Wg protein (red) in the absence (A) and presence (B) of the *UAS-wf* transgene. Note the reduction of *S168* expression and widening of the *wg* expression domain along the dorso-ventral boundary in the presence of higher Wf levels (see arrows). (C,D) Expression of *scalloped-Gal4* visualized with *UAS-GFP* (in green) and Wg protein (red) in the absence (C) and presence (D) of a *UAS-wf* transgene. Note the absence of *scalloped* and *wg* pouch expression owing to the wing-to-notum phenotype induced by overexpression of Wf. Insets in A–C show low-magnification images of the Wg staining. (E,F) Adult wings of wild-type (E) and *S168-Gal4 UAS-wf* adults (F). (G) Wing-to-notum transformation of a *scalloped-Gal4 UAS-wf* adult. A higher magnification of the extra notum is shown in the inset. (H) Wing imaginal disc expressing *UAS-wf* under the control of *dpp-Gal4* (green), resulting in a wing-to-notum transformation and a concomitant symmetrical expression of Wg (red). (I,J) *Dll-lacZ* expression (green) in wing discs expressing *UAS-wg* under *dpp-Gal4* control in the absence (I) or presence (J) of the *UAS-wf* transgene. Wg expression is visualized in red. (K,L) *Dll-lacZ* expression (green) in wing discs expressing *UAS-NRT-wg* under *dpp-Gal4* control in the absence (K) or presence (L) of the *UAS-wf* transgene. Wg expression is visualized in red. Note the elimination of *NRT-wg*-induced *Dll-lacZ* expression (green) by ectopic Wf.

a reduction in Wg signal transduction causes ectopic *wg* transcription (Rulifson et al. 1996). Finally, and perhaps most strikingly, driving expression of *wf* with *scalloped-Gal4* results in a wing-to-notum transformation (Fig. 4D,G), the founding loss-of-function phenotype of the *wg* gene (Sharma and Chopra 1976).

Wf blocks the signaling activities of free and tethered Wg

Based on its structural features as a secreted protein with homologies to pectin acetyl esterases, Wf could exert its function by modifying polysaccharide-based properties of cell surface proteins and thereby impeding the intercellular movement of the Wg protein. Alternatively, Wf could counteract Wg signaling by modifying the transducing properties of Wg or one of its receptors. To distinguish between these two possibilities, we tested whether Wf also antagonizes a derivative of Wg, Nrt-Wg, that is tethered to the cell surface and does not move

through tissue (Zecca et al. 1996). Expression of Wg or Nrt-Wg driven by *dpp-Gal4* results in a robust activation of ectopic *Dll-lacZ* expression (Fig. 4I,K; Zecca et al. 1996). Surprisingly, *wf* expression extinguishes *Dll-lacZ* expression induced by tethered Wg, as well as that induced by free Wg (Fig. 4J,L). From this experiment it can be ruled out that the primary function of Wf is to impede the extracellular transport of Wg. Therefore, Wf must interfere with the signaling activities of either Wg or its receptor components. Because we were unable to detect a physical interaction between Wf and Wg, Frizzled-2 (Dfz2), and its LRP-like partner Arrow in tissue culture systems (data not shown), we favor the view that Wf inhibits the activity of a coreceptor component, such as Dally or Dally-like (Dly), proteoglycans that appear to participate in Wg reception (Lin and Perrimon 1999; Tsuda et al. 1999; Baeg et al. 2001). Wf may inhibit such receptor components via its presumptive esterase activity, for example, by modifying Dally or Dly glycosaminoglycan chains. A definitive proof for this mode of action could be achieved by the genetic demonstration that in larval *dally dly* double-mutant situations, loss of *wf* function has no antagonistic effect.

Temporal and mechanistic differences between feedback inhibitors of Wg

The discovery of Wingful as an essential Wg feedback antagonist may provide an explanation of why Nkd has no apparent role in imaginal tissues of *Drosophila*. The function of Nkd may be superseded by that of Wf, which functions in a powerful negative-feedback loop in adult development. Conversely, when we removed both maternal and zygotic components of *wf*, no obvious requirement was observed for Wf in embryonic development, possibly because the *nkd* system is operative at this stage of development. Both Naked and Wf can, however, inhibit Wg signaling throughout development if they are overexpressed, but each of them is operating more effectively at only one of the two stages. It may not be coincidence that Nkd as the intracellular feedback antagonist is used during embryonic patterning, where Wg functions at short range, whereas Wf as a secreted extracellular antagonist primarily regulates patterning processes that depend on long-range Wg signaling. Wf functions nonautonomously (Fig. 3K) and, like Argos, a secreted feedback antagonist of the *Drosophila* EGF system (Golembo et al. 1996), may have a different range of action compared with the primary signal, providing an intricate means to shape the range and slope of the cellular responses to a morphogen gradient.

Materials and methods

Drosophila stocks and genetics

The establishment of the Gal4 enhancer trap collection is described in Gerlitz et al. (2002). *P-element P[S145]*, which maps 8 bp upstream of the 5' end of the *wf* transcription unit, was replaced with the *y*⁺-marked EP element *DA530inv* (D. Nellen and K. Basler, unpubl.) essentially as described by Sepp and Auld (1999). Experimental larvae for dissection were identified by the absence of the *Tubby* genotype present on the balancer chromosome *TM6b. Dll-lacZ, neur-lacZ, UAS-HA-wg*, and *UAS-HA-Nrt-wg* are described in Zecca et al. (1996).

Immunoblotting and histochemistry

S2 cells were transfected with UAS constructs in combination with a *tubulin α 1-Gal4* plasmid. Cell lysates and supernatants were incubated with anti-HA antibody; immunocomplexes were resolved by SDS-PAGE, followed by blotting with anti-HA antibodies. Cell surface staining was assayed as follows: S2 cells were incubated with supernatant of *HA-wf*- or *HA-wg*-expressing cells at room temperature for 2 h, washed with serum-free medium, and stained with mouse monoclonal α -HA and polyclonal goat Alexa 594 secondary antibodies. Imaginal discs from third instar larvae were fixed and stained by standard techniques with mouse monoclonal α -Wg (gift from S. Cohen, EMBL, Heidelberg, Germany) and polyclonal α - β Gal (Cappel); mouse monoclonal α -HA (BabCO), and Alexa 488 and 594 fluorescent secondary antibodies (Molecular Probes).

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