Wingful, an extracellular feedback inhibitor of Wingless

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Abstract
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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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 identified 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].
decapentaplegic (dpp), one of the key effector targets of the Hh signal in discs, receiv- ing the Hh signal (Forbes et al. 1993; Goodrich et al. 1996), is only induced in larval but not embryonic cells (Basler and Struhl 1994; Tabata and Kornberg 1994). Similarly, the induction of the Smad effector 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 Hh 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 the 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 acetyl esterases

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 peptide.

Figure 2. wf encodes a secreted protein with homology to pectin acetyl esterases. [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 acetyl esterase 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 wf141 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.
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 hydrodases 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)brm11* and the proximal breakpoint of *Df(3L)st-f13* (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 *wf*141 or animals of the genotype *wf*141/*Df(3L)st-f13* die during pupal stages, whereas the discs in panels A and C are derived from wild-type larvae, the discs in B and D from *wf*141/*Df(3L)st-f13* mutant animals. 

**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
a reduction in Wg signal transduction causes ectopic wg transcription [Rulifson et al. 1996]. Finally, and perhaps most strikingly, driving expression of wfl 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 acetyltransferases, 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, wfl 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 Wfl and Wg, Frizzled-2 (Dflz2), 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 wfl 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 wfl, 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, whereas Wf 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.

Figure 4. wfl 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–wfl 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–wfl transgene. Note the absence of scalloped and Wg pouch expression owing to the wing-to-notum phenotype induced by overexpression of Wf. Insects in A–C show low-magnification images of the Wg staining. [E,F] Adult wings of wild-type (E) and S168–Gal4 UAS–wfl adults (F). [G] Wing-to-notum transformation of a scalloped–Gal4 UAS–wfl adult. A higher magnification of the extra notum is shown in the inset. [H] Wing imaginal disc expressing UAS–wfl 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–wfl under dpp–Gal4 control in the absence (I) or presence (J) of the UAS–wfl transgene. Wg expression is visualized in red. [K,L] Dll–lacZ expression (green) in wing discs expressing UAS–NRT–wfl under dpp–Gal4 control in the absence (K) or presence (L) of the UAS–wfl transgene. Wg expression is visualized in red. Note the elimination of NRT–wfl–induced Dll–lacZ expression (green) by ectopic Wfl.

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Materials and methods

Drosophila stocks and genetics

The establishment of the Ga4 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 w[+] transcription unit, was replaced with the y-marked EP element DAS350tn [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. Dil-lacZ, UAS-HA–wg, and UAS–HA–Nrt–wg are described in Zeczka et al. (1996).

Immunoblotting and histochemistry

S2 cells were transfected with UAS constructs in combination with a tubulin–1-Ga4 plasmid. Cell lysates and supernatants were incubated with anti-HA antibody; immunocomplexes were resolved by SDS-PAGE, transferred to nylon membranes, and detected with anti-rabbit secondary antibodies.

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