The porcupine gene is required for wingless autoregulation in Drosophila

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INTRODUCTION

The segment polarity genes wingless (wg) and engrailed (en) are transcribed in adjacent, non-overlapping stripes of cells in each of the 14 developing segments of the Drosophila embryonic epidermis (Baker, 1987; DiNardo et al., 1988; Martinez-Arias et al., 1988). Each of these genes is required to maintain the expression of the other in neighboring stripes of cells (reviewed in Perrimon, 1994). wg encodes a secreted glycoprotein which is a member of the Wnt family (Baker, 1987; Rijsewijk et al., 1987; reviewed in McMahon, 1992; Nusse and Varmus, 1992). wg protein (Wg) is secreted and enters en-expressing cells; thus it is thought that Wg itself may be the paracrine signal leading to maintenance of en expression (van den Heuvel et al., 1989; Gonzalez et al., 1991, review by Varmus, 1992). However, wg appears to be abnormally confined to the cells where it is transcribed, it has been postulated that porc is required for normal secretion of the Wg protein (van den Heuvel et al., 1993a; Seifried et al., 1994). It is also possible that the main role for porc could be in the regulation of wg transcription.

Because wg is required for maintenance of en transcription (DiNardo et al., 1988), and en is in turn required for that of wg (Martinez-Arias et al., 1988), wg may regulate its own transcription indirectly through a paracrine feedback loop (Ingham and Hidalgo, 1993). However, wg also has a second, distinct autoregulatory role that may reflect an autocrine wg activity that is independent of signaling via en (Hooper, 1994; Yoffe et al., 1995). Since it appears that this second autoregulatory function of wg is required prior to en activity (Yoffe et al., 1995; this work), we will refer to it as ‘direct autoregulation’ for simplicity. As wg stripes fade in porc, dsh and arm mutant embryos (van den Heuvel et al., 1993b), it is possible that these genes are crucial components of direct autoregulation as well as paracrine signaling by wg. Alternatively, these genes may only be components of the latter, the loss of wg being a secondary result of the loss of en. To date the genetic components of the two...
modes of wg autoregulation have been examined only indirectly, in mutants for the patched (ptc) gene (Hooper, 1994).

We have tested the genetic basis of wg maintenance by attempting to identify components that may be distinct to direct wg autoregulation versus the paracrine wg signaling. We have used three assays: (1) monitoring the timing of disappearance of wg versus en expression in porc, dsh and arm embryos, (2) determining the requirement for these three genes in zw3 mutants, in which wg paracrine signaling is ‘constitutive’ (Siegfried et al., 1992) and (3) assaying the autoregulatory potential of exogenous Wg in the absence of the genes porc, dsh and arm. Our results suggest that dsh, zw3 and arm are required specifically in the paracrine signaling, while porc is required for direct autoregulation but not paracrine signaling. Thus these two wg pathways appear to be genetically distinct.

MATERIALS AND METHODS

Fly strains

wg-G22 is a protein null allele of wg (van den Heuvel et al., 1993a). wg-en11 is a null wg allele on the CyO balancer chromosome that expresses lacZ in the wg pattern (Kassis et al., 1992). en-CXI makes a nonfunctional En protein and behaves genetically as a null allele (Heemskerk et al., 1991). arm-XM19 and dsh-C26 have all been previously described as molecular and genetic nulls during embryogenesis (Peifer et al., 1991; Klingensmith et al., 1994). dsh75, zw3-M11 and porc-PB16 have been described as being genetic nulls (Perrimon et al., 1989). hGAL4 (also known as 1J3) is an insertion of the GAL4 construct pGawB at the hairy (h) locus on the third chromosome and has been previously described (Brand and Perrimon, 1993). UASwg was constructed to express the wgIL14 temperature-sensitive allele which is active at 16°C. The UASwg insertion is located on the third chromosome and is homozygous viable (Wilder and Perrimon, 1995).

Generation of embryos

armXM19, dsh75; porc-PB16, zw3-M11, armXM19 zw3-M11, zw3-M11 dsh75 and zw3-M11 porc-PB16 mutant embryos were generated by heterozygous females having homozygous mutant germlines (germline clone females), as previously described (Siegfried et al., 1994). Using the hGAL4 line, we can direct the expression of UASwg in a h ‘pair-rule’ stripe pattern (Brand and Perrimon, 1993). wg-en11; hGAL4/UASwg and en-CXI; hGAL4/UASwg embryos were generated as described in Yoffe et al. (1995). hGAL4-UASwg (h-wg) is a recombinant third chromosome which carries both the hGAL4 and UASwg inserts, and was introduced into mutant embryos by crossing h-wg/TM3 males to dsh, arm and porc germline clone females. Thus one half of the non zygotically rescued (e.g. dshhY) embryos bear the h-wg chromosome. dsh-C26; h-wg and armXM19; h-wg, embryos were identified by their predominantly ‘seven stripe’ expression patterns of endogenous wg. For each experiment, at least 100 progeny were examined. The percentage of rescued embryos matched the predicted number that should have been generated in the genetic cross (1 out of 4) in the dsh and arm mutant backgrounds. However, not all embryos are rescued in a perfect seven stripe pattern and the extent of rescue was often incomplete. porc-PB16

h-wg embryos were identified by the fading of wg transcription in embryos displaying seven broad (h-wg) Wg stripes. This analysis was accomplished through Wg antibody and endogenous wg mRNA double labeling experiments (Manoukian and Krause, 1992): Wg (in a h pattern) can be detected whereas endogenous wg transcription is lost. Hundreds of embryos were examined and wg transcription was never rescued in the epidermis of porc; h-wg embryos. All experiments were

Fig. 1. wg activity is required for autoregulation prior to its requirement for en maintenance. The left panels show the cuticle phenotype of wild-type (A) and wg-G22 (C) larvae. wg mutants show the characteristic lawn of denticles on the ventral surface of the cuticle. The right panels show the expression pattern of wg transcripts (blue) and En protein (brown) in the ventral epidermis of wild-type (B) and wg-G22 (D) embryos. In wild-type embryos, wg is expressed in a series of 14 single-cell-wide stripes. In the absence of functional wg protein, these 14 stripes of expression fade starting at stage 9 before En has faded. This shows that wg is required for its own expression prior to that of en and thus exhibits direct autoregulation. All figures are oriented anterior to left, dorsal up unless otherwise specified.
carried out at 16°C, the permissive temperature of UAS wg (see Wilder and Perrimon, 1995; Yoffe et al., 1995).

Cuticles preparations
Cuticles were prepared by clearing in Hoyer’s medium (Struhl, 1989) and photographed under dark-field optics.

Embryo stainings
Fixation and hybridization and/or immunostaining of embryos and detection of expression patterns were as previously described (Manoukian and Krause, 1992; Yoffe et al., 1995). A digoxigenin-labeled probe that detects endogenous wg RNA but not exogenous h-wg transcripts was generated by PCR, using a 5’ wg untranslated specific sequence (Yoffe et al., 1995). En antibodies (Patel et al., 1989) were used at a 1:2 dilution, and Wg antibodies (van den Heuvel et al., 1989) were used at a 1:100 dilution.

RESULTS
porc is required for direct wg autoregulation
The cuticle phenotypes of arm, dsh and porc embryos are virtually identical to that of wg, having a uniform ‘lawn’ of ventral denticles (Perrimon et al., 1989; Figs 1C, 2A,C). In these embryos, both wg and en expressions fade (Peifer et al., 1991; van den Heuvel et al., 1993b). Previously it has been shown that, in wg mutant embryos (which produce wg RNA but no protein), stripes of endogenous wg fade during embryonic stage 9, before the disappearance of the En stripes (Yoffe et al., 1995; Fig. 1D). This result suggested that wg has a more direct autoregulatory activity than the en-dependent positive feedback loop. We therefore simultaneously studied the timing of disappearance of wg versus En expression in porc, dsh and arm mutant embryos in order to distinguish possible differences in temporal requirements for these three genes. We note a difference in the timing of the loss of wg transcription in porc versus dsh or arm mutants, as monitored using En expression as an assay. In the absence of dsh (Fig. 2B) or arm (not shown) activity, wg transcription fades after or simultaneously with En. As dsh and arm are indispensable for the regulation of en transcription by wg (Noordermeer et al., 1994), this result suggests that dsh and arm may not be components of direct wg autoregulation. Rather, they may be required for wg transcription only indirectly via the paracrine feedback loop. In contrast, porc appears to be required for direct wg autoregulation since, just as in wg mutant embryos, wg fades before En in porc mutants (Fig. 2D).

Fig. 2. The order of fading of wg RNA versus En protein in dsh and porc mutant embryos. Shown on the left are the cuticle phenotype of dsh75 (A) and porcPB16 (C) larvae. Both dsh and porc mutants resemble wg mutants in phenotype (see Fig. 1). The right panels show expression of wg and En in dsh75 (B) and porcPB16 (D) mutant embryos, oriented to show the posterior ventral epidermis. In dsh (b) and arm (not shown) mutant embryos, En fades before or simultaneously with wg transcripts. By contrast, in porc mutant embryos, wg transcription fades before En is affected. This is not due to the increased stability of En in porc mutant embryos, since en transcripts fade at the same stage as En in the absence of porc (not shown). These events occur mid stage 9 of embryogenesis.
**porc is epistatic to zw3 in the regulation of wg**

The segment polarity mutant zw3 has a reciprocal cuticular phenotype to that of the wg class of mutants. In zw3 mutants, virtually all ventral denticles are replaced with naked cuticle (Perrimon and Smouse, 1989), similar to embryos in which Wg has been expressed uniformly from a heat-shock promotor (Noordermeer et al., 1992). In the absence of zw3 activity, en expression expands posteriorly, away from the wg-expressing cells, in each segment during gastrulation (Siegfried et al., 1992). After this expansion of en, ectopic wg stripes appear posterior to the expanded en expression domain, resulting in embryos with twice the normal number of wg stripes (Siegfried et al., 1992; Fig. 3A). The expansion of en stripes also occurs in zw3; wg double mutants. The loss of zw3 activity therefore uncouples en-expressing cells from their requirement for wg. Notably however, in zw3; wg double mutant embryos, all wg stripes fade by late stage 11, even though en stripes remain broadened (Hooper, 1994; A. S. M. and K. B. Y., unpublished observations). This indicates that, although wg activity is no longer required for its paracrine function in zw3 embryos, wg activity is still required for the maintenance of wg stripes. Thus zw3 does not appear to mediate direct wg autoregulation.

We examined the expression of wg RNA in double mutants for zw3 and either dsh, arm or porc. Unlike zw3; wg double mutants, zw3 dsh double mutants display stable wild-type and ectopic wg stripes (in addition to broadened en stripes), just as in zw3 single mutants (Fig. 3B; Siegfried et al., 1994). Thus, although wg activity is required for maintenance of wg transcription in zw3 embryos, dsh is not. In contrast to zw3 dsh, all wg stripes are lost in arm zw3 double mutants (Fig. 3C). Since this could be a secondary effect of the complete loss of en expression in these double mutants (Siegfried et al., 1994; Peifer et al., 1994), we cannot assess from this experiment alone whether arm is required for direct wg autoregulation.

In zw3 porc embryos, en expression is expanded as in zw3 mutants (Siegfried et al., 1994). Thus if direct wg autoregulation requires porc, it follows that wg transcription would fade in zw3 porc even in the presence of this ectopic en expression. Indeed, wg transcription ceases in zw3 porc double mutant embryos (Fig. 3D), just as in zw3; wg double mutant embryos. Therefore both wg and porc are indispensable for the maintenance of wg expression, even in the case of the ‘constitutive’ paracrine wg signaling observed in zw3 mutant embryos (Siegfried et al., 1992).

**porc is required for autoregulation by exogenous Wg**

In wg, porc, dsh and arm embryos, wg expression fades during stage 9 (Figs 1 and 2). In order to directly test the requirements of arm, dsh and porc in wg autoregulation, we have used the

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**Fig. 3.** The epistatic relationship of dsh, arm and porc to zw3. Transcription of wg is shown in stage 11 embryos lacking maternal and zygotic zw3 M11 (A), zw3 M11 dsh 25 (B), zw3 M11 arm XM19 (C) and zw3 M11 porc Fb16 (D) activity. In zw3 mutant embryos (A), wg is expressed in a 28-stripe pattern rather than the wild-type 14-stripe pattern. These ectopic stripes are thought to be induced by the ectopic en expression which is apparent in zw3 embryos (Siegfried et al., 1992). This phenotype is still observed in zw3 dsh double mutants (B; Siegfried et al., 1994), but in zw3 arm embryos (C) wg fades (Siegfried et al., 1994). Since in zw3 arm embryos en expression fades, the lack of wg expression in these double mutants could be secondary to this loss of en. In zw3 porc embryos (D), wg transcripts fade even though en stripes persist and are expanded (not shown; Siegfried et al., 1994). Ectopic wg stripes initially appear in these embryos with all the wg stripes fading by stage 11. This result would appear to be at odds with previous work showing that wild-type and ectopic Wg protein stripes persist in zw3 porc double mutants (Siegfried et al., 1994). However, this discrepancy can be explained by the fact that Wg protein is abnormally stable in the absence of porc activity (van den Heuvel et al., 1993; Siegfried et al., 1994) and thus is still present in zw3 porc embryos even when wg transcripts are gone.
GAL4 system (Brand and Perrimon, 1993) to misexpress Wg in the absence of each of these gene products. By utilizing a hairy (h) GAL4; UASwg recombinant chromosome (Wilder and Perrimon, 1995), which we will refer to as h-wg, we are able to drive exogenous Wg in the spatial pattern of the h pair rule gene. This exogenous Wg misexpression persists in the epidermis, overlapping alternate endogenous wg and en stripes, from stages 8-10 (Yoffe et al., 1995). In h-wg embryos, ectopic endogenous wg stripes are induced, resulting later in the transformation of alternate denticle belts to naked cuticle in the larva (Yoffe et al., 1995; Wilder and Perrimon, 1995). In wg; h-wg embryos, alternate endogenous wg stripes are often restored while the others fade, leading to ‘seven striped’ embryos (Yoffe et al., 1995). This incomplete wg expression pattern allows us to unambiguously identify wg mutant embryos with exogenous Wg. Using h-wg, we have previously shown that exogenous Wg can activate endogenous wg in en mutants, demonstrating an en-independent autoregulatory mechanism (Yoffe et al., 1995).

The dsh and arm genes are both required for paracrine wg signaling in the positive regulation of en and the specification of naked cuticle (Siegfried et al., 1994; Noordermeer et al., 1994). Consistent with this, we found that in dsh; h-wg or arm; h-wg embryos en expression fades (not shown), and the ‘lawn’ phenotypes of dsh and arm are not affected (Fig. 5A,B). However, h-wg can activate endogenous wg in the absence of either dsh or arm, based on the persistence of wg transcription in dsh; h-wg and arm; h-wg embryos (Fig. 4B,C). This rescue of alternate endogenous wg stripes is reproducible but fairly weak (see Materials and Methods). This inefficiency of wg maintenance in the absence of dsh or arm could be due to the weak activity of hGAL4 in our experiments (Yoffe et al., 1995), or to the absence of en in these embryos (see Discussion). Nonetheless, we conclude that these two components of the wg paracrine feedback loop are not absolutely required for Wg to autoregulate in this assay.

In contrast, h-wg cannot activate endogenous wg in porc mutants (Fig. 4D), indicating that porc is absolutely required for wg autoregulation. We examined Wg protein in porc; h-wg embryos and found that exogenous Wg persists at high levels in seven broad h-like stripes through stage 12 (Fig. 4E,F), long past the time when hGAL4 expression ceases in the epidermis (stage 10; Yoffe et al., 1995). Endogenous and exogenous Wg in porc; h-wg embryos appears to be restricted within the wg-transcribing cells instead of secreted as in wild type (van den Heuvel, 1989, 1993a; Siegfried et al., 1994; Fig. 4E,F). Although expressed with increased stability, this intracellular Wg in porc mutant embryos is nonetheless unable to activate the endogenous wg gene. However, the absence of porc does not completely abolish wg activity, since exogenous Wg is capable of restoring en expression and naked cuticle in porc mutants (Noordermeer et al., 1994; Fig. 5C).

DISCUSSION

Autoregulation and paracrine signaling are distinct activities of wg

It has been established that wg has a crucial positive role in the maintenance of en expression and specification of diverse cell types in the developing embryonic segment (reviewed in Peifer and Bejsovec, 1992; Siegfried and Perrimon, 1994), a role referred to as paracrine signaling. Since en is in turn required for wg maintenance, then paracrine signaling represents a positive feedback loop that could be the primary mechanism through which wg regulates its own transcription. However, wg appears to have an autoregulatory function distinct from this paracrine feedback loop, which we have referred to as direct autoregulation. Recently it has been shown that (1) direct autoregulation differs temporally from paracrine wg signaling and that (2) epidermal cells appear to require direct exposure to Wg protein in order to express the wg gene (Yoffe et al., 1995).

The initial suggestion for direct wg autoregulation, however, came from analyses of ptc mutant embryos (Bejsovec and Wieschaus, 1993). In ptc embryos, epidermal wg stripes expand from their normal width of about one cell to a width of approximately half the segment. It has been postulated that, in the absence of ptc function, wg maintenance no longer requires signaling from the en-expressing cells, this signal perhaps being encoded by the secreted hedgehog (hh) product (Ingham et al., 1991; Mohler and Vani, 1992; Lee et al., 1992; Tabata et al., 1992). Notably, although hh activity is not required for efficient wg transcription in ptc embryos (Ingham et al., 1991), wg activity is still required: in wg ptc double mutants, wg expression is weak and inconsistent (Ingham and Hidalgo, 1993; Bejsovec and Wieschaus, 1993; Hooper, 1994). Hooper (1994) has used ptc mutants to examine potential components of the direct (or autocrine) wg autoregulatory pathway. In this work, we have analyzed wg autoregulation using three additional approaches. We suggest that porc, but not arm, zw3 or dsh, is a crucial component of direct wg autoregulation.

The role of porc in wg function

We have shown a crucial role for porc in wg autoregulation. In the absence of Porc, wg expression fades prior to the disappearance of En (Fig. 2D) indicating that, like wg, porc is required prior to en for wg maintenance (Yoffe et al., 1995; Fig. 1). We find that exogenous Wg is unable to rescue the lost wg expression in porc embryos (Fig. 4F). Since exogenous Wg is capable of maintaining en expression and specifying naked cuticle in porc mutant embryos (Noordermeer et al., 1994; Fig. 5C), we suggest that porc is required for direct wg autoregulation but not paracrine wg signaling (Fig. 6). This possibility is further supported by our observation that, although zw3 is epistatic to porc in the maintenance of en and the specification of naked cuticle (Siegfried et al., 1994), porc is epistatic to zw3 in the regulation of wg (Fig. 3D).

It has been noticed that, in porc mutant embryos, Wg protein appears to be confined to the cells in which it is transcribed (van den Heuvel et al., 1993; Siegfried et al., 1994). While the significance of this confinement and increased stability of Wg in porc mutant embryos is unknown, this particular role of porc is not crucial for wg paracrine signaling (Noordermeer et al., 1994; Fig. 5C). It is possible that proper secretion or processing may be mandatory for Wg to regulate its own transcription. Molecular characterization of the porc gene product might give insights into these roles in wg function.

dsh, zw3 and arm may not be components of direct wg autoregulation

In both dsh and arm mutant embryos, wg RNA expression
fades following the disappearance of En (Fig. 2B). Since en activity is required for wg maintenance (Martinez-Arias et al., 1988; Bejsovec and Martinez-Arias, 1991), these results imply that the loss of wg in dsh and arm mutants is a secondary consequence of the loss of paracrine wg signaling, and not directly due to a block in direct wg autoregulation. Hh, which is co-expressed with en in the epidermis, is postulated to encode a secreted factor involved in the regulation of wg transcription. We have not followed the expression of Hh in these embryos. It is possible that hh is differentially regulated in porc versus dsh or arm embryos - whether this difference is detectable at the level of Hh antibody staining is unclear. It is therefore possible that the difference between porc and dsh or arm in our experiments could be due to the differential regulation of hh transcription or activity in these mutants. As Hh protein enters the wg-transcribing cells, we cannot exclude the possibility that hh functions in both paracrine and direct wg autoregulation. Hooper (1994) has suggested that autocrine (or direct) wg autoregulation is hh-independent. In any case, we have demonstrated a relevant difference in the regulation of wg transcription in porc versus dsh or arm embryos (Figs 2, 3 and 4). We suggest that dsh and arm, two crucial positive mediators of wg paracrine signaling (Noordermeer et al., 1994; Siegfried et al., 1994), do not appear to be crucial for direct wg autoregulation (Fig. 6).

zw3 acts between dsh and arm in the wg paracrine signaling
Fig. 6. The two pathways through which wg regulates its own transcription. One pathway involves porc and is required for ‘direct’ wg autoregulation. The second pathway involves the exclusion of en in neighboring cells. en is in turn required for the maintenance of wg transcription, thus completing a ‘paracrine feedback loop’. The latter pathway includes the dsh, zw3 and arm genes and is also required for the specification of naked cuticle and the generation of cell type diversity.

downstream of dsh and upstream of arm in the paracrine wg signaling pathway (Noordermeer et al., 1994; Siegfried et al., 1994; Peifer et al., 1994), the exclusion of exogenous Wg has no effect on the cuticle phenotype of en in this pathway. We can therefore only propose the inclusion of porc in the direct wg autoregulatory pathway.

As Wg is a secreted molecule, the existence of transcription factor(s) mediating wg autoregulation must be postulated. The gooseberry protein (Gsb) has been shown to be involved in an autoregulatory loop with wg, perhaps functioning as a transcription factor (Li and Noll, 1993). Therefore, it is possible that the dsh- or arm-independent autoregulation of wg in our h-wg experiments may be mediated by gsb. As gsb expression is a target of wg signaling as is wg expression (Li and Noll, 1993), this would again indicate a dsh- or arm-independent function of Wg.

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