Wingless gradient formation in the *Drosophila* wing

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Background: Secreted signaling proteins of the Wingless (Wg)/Wnt, Hedgehog and bone morphogenetic protein (BMP)/Decapentaplegic (Dpp) families function as morphogens to control growth and pattern formation during development. Although these proteins have been shown to act directly on distant cells in the developing limbs of the fruit fly *Drosophila*, little is known about how ligand gradients form *in vivo*. Wg protein is found in vesicles in Wg-responsive cells in the embryo and imaginal discs. It has been proposed that Wg may be transported by a vesicle-mediated mechanism.

Results: A novel method to visualize extracellular Wg protein was used to show that Wg forms an unstable gradient on the basolateral surface of the wing imaginal disc epithelium. Wg movement did not require internalization by dynamin-mediated endocytosis. Dynamin activity was, however, required for Wg secretion. By reversibly blocking Wg secretion, we found that Wg moves rapidly to form a long-range extracellular gradient.

Conclusions: The Wg morphogen gradient forms by rapid movement of ligand through the extracellular space, and depends on continuous secretion and rapid turnover. Endocytosis is not required for Wg movement, but contributes to shaping the gradient by removing extracellular Wg. We propose that the extracellular Wg gradient forms by diffusion.

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Background

Secreted signaling proteins of the Wingless (Wg)/Wnt, Hedgehog and bone morphogenetic protein (BMP)/Decapentaplegic (Dpp) families function as morphogens to control growth and pattern formation during development in a variety of organisms. In *Drosophila*, Wg, Dpp and Hedgehog are secreted from localized sources and have been shown to signal directly to distant cells in the developing limbs, leading to the proposal that they function as morphogens [1–5]. Cells close to the source exhibit responses attributed to high levels of signaling activity, whereas cells that are further away exhibit lower threshold responses. In vertebrates, there are many developmental contexts in which the homologous proteins are thought to function as morphogens, though in most cases the evidence is less direct [6–8]. The best characterized example is Activin, which exhibits concentration-dependent patterning effects and direct action at a distance (reviewed in [9,10]).

Morphogen activity can be modulated by locally increasing or decreasing production of the ligand (for example, see [1,3–5,11]). It has also been possible to modulate the sensitivity of cells to a given level of ligand by changing the level of receptor or the activity of downstream signaling proteins ([12–17], reviewed in [18]). Other factors may also modulate gradient formation *in vivo*. For example, a number of proteins involved in the biosynthesis of proteoglycans appear to modulate sensitivity of cells to ligand or ligand movement *in vivo* [19–24]. It is not clear at present whether the proteoglycans whose synthesis depends on these activities function in the Wg signaling pathway by acting as coreceptors or more indirectly by modulating the levels of the ligand. Attempts to visualize gradients of secreted signaling proteins by antibody staining *in situ* have met with limited success. Wg and Hedgehog proteins can be visualized by antibody staining [4,25]. Antigen levels are higher in cells close to the source of synthesis than in cells at a distance. Antibody staining has, however, not been sensitive enough to detect levels of ligand known to be biologically active in distant cells.

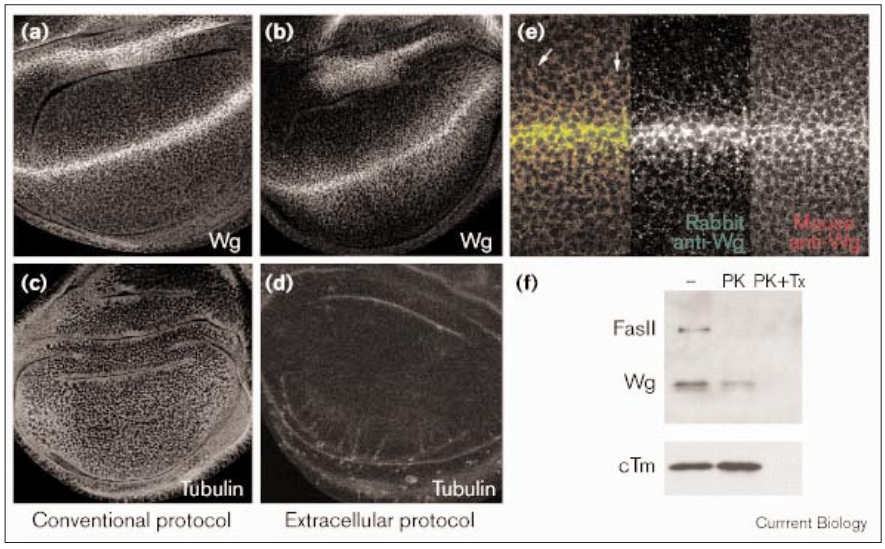
Here, we report a new method for visualizing the extracellular Wg protein gradient. Using this method, we have analyzed the properties of gradient formation *in vivo*. The Wg gradient forms by rapid movement and rapid turnover of extracellular ligand and depends on a continuous high-throughput of Wg protein. Endocytosis is not required for Wg movement, but contributes to shaping the gradient by removing extracellular Wg. We propose that the extracellular Wg gradient forms by diffusion.

Results

An extracellular Wg gradient

Using conventional antibody labeling methods, Wg protein has been detected in Wg-expressing cells at the dorsoventral (DV) boundary of the wing disc and in an irregular pattern of spots in nearby cells (Figure 1a; [4,13]). The

Figure 1



An extracellular Wg protein gradient. **(a,b)** Wing imaginal discs labeled with mouse anti-Wg antibody using (a) conventional and (b) extracellular labeling protocols. The extracellular labeling method involves antibody binding in tissue held at about 0°C until fixation. Under these conditions, it is unlikely that secretion or endocytosis would continue; molecules that happen to be free in solution would continue to diffuse, however. To the extent that free diffusion is responsible for Wg movement, it would cause us to underestimate the steepness and extent of the gradient. **(c,d)** Wing discs labeled with anti-tubulin antibody using (c) conventional and (d) extracellular protocols. **(e)** Detail of the wing pouch of a disc labeled with mouse anti-Wg antibody using the extracellular protocol (red), followed by rabbit anti-Wg antibody using the conventional protocol (green). The individual channels are shown separately in white. Most of the spots of Wg detected by conventional labeling were not labeled with the extracellular protocol and so appear green (arrows indicate some examples). Spots of Wg that also labeled with the extracellular protocol appear yellow-orange. **(f)** Immunoblots of disc extracts labeled for Wg, Fasciclin II (FasII) and cytoplasmic tropomyosin (cTm). A dash indicates that no proteinase K was added; PK, discs were incubated with proteinase K; PK + Tx, discs were incubated with proteinase K plus Triton X-100 to permeabilize cells. The upper panel was probed with mouse anti-FasII antibody (1:200) and mouse anti-Wg antibody (1:100). The lower panel is the same sample reprobed with rabbit anti-cTm antibody (1:2,000). Quantitation indicated that the Wg level is reduced to 40% when normalized for loading to the cTm control in the PK digest. Comparable levels of Wg and cTm digestion were obtained in eight independent experiments.

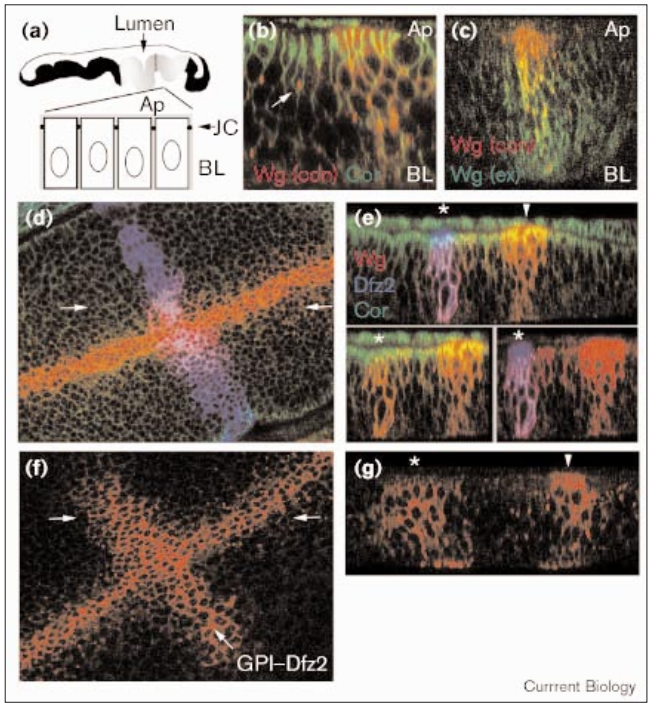
intensity and number of spots decreases with distance from the source of Wg, providing indirect evidence that Wg protein forms a gradient across the disc. The conventional antibody labeling protocol involves incubating anti-Wg antibody with fixed and permeabilized wing discs. When discs were incubated with anti-Wg antibody before fixation, we observed a gradient of Wg protein that appeared broader, shallower and less punctate than that observed with the conventional protocol (Figure 1b). Control experiments showed that tubulin, an abundant intracellular protein, is readily visualized using the conventional protocol, but was not detected using the extracellular staining protocol (Figure 1c,d). Thus, Wg visualized in this way reflects the distribution of the secreted extracellular protein.

The distribution of extracellular and conventionally labeled Wg protein was compared by sequentially labeling discs using anti-Wg antibodies produced in different species. Most of the spots of Wg visualized at a distance from the DV boundary by the conventional protocol did not colocalize with extracellular Wg, suggesting that these spots are vesicles containing Wg protein that has been internalized by responding cells (Figure 1e). To determine what proportion of Wg protein is extracellular, intact wing discs were treated with proteinase K. Digestion of Wg was compared with digestion of Fasciclin II, a glycosyl phosphatidyl inositol (GPI)-linked membrane protein, and with cytoplasmic tropomyosin in immunoblots of total disc extracts (Figure 1f). In the absence of detergent, Fasciclin II was completely digested, and cytoplasmic tropomyosin was not digested. Wg levels were reduced considerably. Wg and cytoplasmic tropomyosin were completely digested when disc cells were permeabilized with detergent during the protease treatment. These observations indicate that much of the Wg protein in the discs is extracellular and accessible to protease digestion or to antibody binding. The remaining Wg protein is presumably intracellular.

The Wg gradient forms on the basolateral surface of the disc epithelium

Imaginal discs consist of a single-layered sac of polarized epithelial cells, with the apical surface of the cells oriented towards the lumen of the disc (illustrated in Figure 2a). The polarity of the epithelial cells can be visualized using antibody to Coracle, which labels the junctional complex that separates apical from basolateral surfaces (Figure 2b). Using the conventional labeling protocol, most of the Wg appeared to be concentrated above the nuclei, near the junctional complex, in Wg-expressing cells (Figure 2b). In contrast, extracellular Wg was mainly associated with the basolateral surface of cells (Figure 2c). The pattern of extracellular Wg labeling was the same in discs that were deliberately cut open to disrupt the peripodial membrane

Figure 2



Comparison of extracellular and intracellular Wg distribution. **(a)** Schematic representation of a cross section of the wing imaginal disc. The disc is a polarized epithelial sac. The apical surface of the epithelium (ap) is directed towards the lumen. Junctional complexes (jc) located near the apical surface separate the apical from basolateral (bl) plasma membrane. The region of the epithelium that makes the wing and body wall structures is pseudostratified. The tops and bottoms of all cells are located at the same level, but nuclei are located at different levels (see next panel). The gray shading represents the wing pouch. **(b)** Optical cross section of a wing disc including the stripe of Wg-expressing cells. Junctional complexes were visualized with antibody to Coracle (Cor; green). Wg labeling with the conventional protocol (con) is shown in red. Apical and basolateral are indicated. Nuclei appear as unlabeled circles surrounded by thin rims of cytoplasm. The nuclei appear to be stacked in layers because of the close packing of the cells. Most of the Wg is apical to the nuclei in the Wg-expressing cells. The arrow indicates a vesicle of internalised Wg protein. **(c)** Optical cross section of a wing disc including the stripe of Wg-expressing cells. The disc was labeled for intracellular Wg (con; red) and extracellular Wg (ex; green). **(d)** Wg protein (red) visualized with the conventional protocol in a disc overexpressing full-length Dfz2 (blue) in a stripe perpendicular to the endogenous Wg stripe using the *decapentaplegic* (*dpp*) promoter to drive expression of the *GAL4*-encoded transcriptional activator (*dpp–GAL4*). **(e)** Optical cross section at the position indicated by the arrows in (d). The Coracle and Wg channels are shown in the lower left panel, and the Dfz2 and Wg channels in the lower right panel. Note that Dfz2 is expressed on the apical and basolateral surfaces (above and below the junctional complex). Wg accumulates below the junctional complex on Dfz2-expressing cells. The arrowhead indicates the endogenous Wg stripe where Wg is mostly above the nuclei. **(f,g)** Wg labeling in a disc overexpressing GPI–Dfz2 under *dpp–GAL4* control (lower arrow in (f)). (g) Optical cross section at the position indicated by the upper arrows in (f). Wg distribution appears comparable to that in cells expressing the full-length receptor in both domains. In imaginal disc tissue, GPI-anchored proteins localize mainly to the basolateral surface of epithelium. This is the opposite to what is seen for internal epithelia in *Drosophila*, for example, in the salivary gland and in the commonly studied polarized epithelial cell lines of vertebrate origin where GPI-anchored proteins localize mainly to the apical surface [26,27]. Consequently, GPI–Dfz2 can accumulate Wg only on the basolateral cell surface. In (e,g), the asterisks indicate the ectopic Wg stripe and the arrowheads indicate endogenous Wg expression.

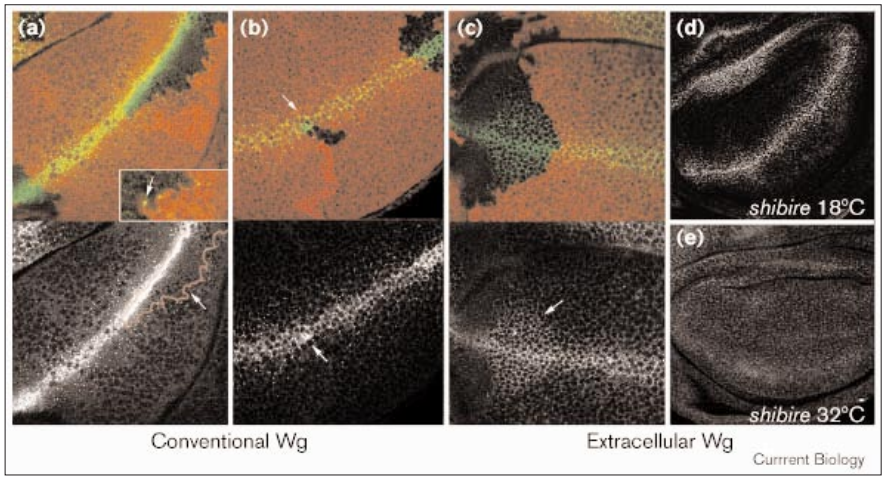
and ensure free access of the antibody to the lumenal surface of the wing pouch (data not shown). The spots of Wg visualized by conventional labeling in cells away from the source appear to reflect vesicles of internalized Wg protein (Figure 2b, arrow).

The observation that extracellular Wg appears to be concentrated on the basolateral surface of Wg-expressing and nearby cells prompted us to investigate whether Wg moves across the apical (that is, lumenal) or basolateral surface of the epithelium. To distinguish between these possibilities, we made use of the observation that overexpression of the *Drosophila* Wg receptor Frizzled 2 (Dfz2) causes accumulation of Wg on cells at a distance from the DV boundary [13]. Wg accumulation was compared in cells expressing full-length Dfz2 and cells expressing the Wg-binding domain of Dfz2 as a GPI-anchored protein (GPI–Dfz2). Full-length Dfz2 is expressed uniformly on the apical and basolateral surfaces of the epithelium (Figure 2e), whereas GPI-anchored proteins localize to the basolateral surface of the imaginal disc cells ([26,27]; S. Eaton, personal communication) and so can only accumulate Wg on the basolateral surface (Figure 2f,g). In both cases, Wg accumulated to high levels on the basolateral surface of the epithelial cells but was absent from the region above the nuclei (Figure 2e,g, asterisks), where most Wg protein was found in Wg-expressing cells (arrowheads). These observations suggest that the extracellular Wg gradient forms on the basolateral surface of the wing disc.

Dynamin-mediated endocytosis is not required for Wg movement

The extracellular Wg gradient correlates with a graded distribution of intracellular Wg vesicles. Does Wg internalization play a role in gradient formation? Previous studies have suggested a role for *shibire*-dependent endocytosis in Wg transport in the embryo [28,29]; *shibire* encodes the *Drosophila* homolog of the GTPase dynamin [30,31]. Dynamins have been implicated in the internalization of clathrin-coated endocytic vesicles and in the internalization of caveolae [32–35]. To determine whether *shibire*-dependent vesicle traffic is required for Wg gradient formation, we examined Wg distribution in wing discs carrying clones of *shibire*^{ts1} mutant cells. Clones were allowed to grow under conditions in which the temperature-sensitive Shibire protein is active (18°C). The larvae were then shifted to 32°C for 3 hours to inactivate Shibire. Under these conditions, punctate Wg was not observed in the mutant cells using the conventional labeling protocol (Figure 3a), indicating that dynamin activity is required

Figure 3



Wg distribution in *shibire* mutant clones. **(a,b)** Wg (green) visualized by conventional labeling in discs carrying *shibire* mutant clones. Cells mutant for *shibire* are marked by the absence of β-galactosidase protein (red). In (a), the arrows point to Wg internalized in wild-type cells on the far side of the *shibire* clone. The inset is a higher magnification view to show the Wg-containing vesicles in wild-type cells immediately adjacent to the clone. Wg expression is shown alone in the lower panel and the clone is outlined. In control experiments, larvae were treated for 3 h at 32°C, then shifted back to 18°C and allowed to continue development. After 2 days of recovery, no difference was observed in the relative size of mutant clones and wild-type twin spots compared with discs kept at 18°C (56 clones in 10 discs at 18°C and 55 clones in six discs at 32°C). This indicates that the effects of transient shifts to the restrictive temperature are fully reversible in wing tissue. (b) Small clones that touch the margin also accumulate Wg (arrows), even when mutant cells were mostly surrounded by wild-type cells that internalize Wg normally. **(c)** Wg expression visualized by extracellular labeling in a disc carrying a large *shibire* mutant clone. Cells mutant for *shibire* are marked by the absence of β-galactosidase (red). Wg expression is shown separately below. Note the accumulation of extracellular Wg on the mutant cells (arrow). The level of Wg also appeared to be somewhat higher in cells at the DV boundary, perhaps reflecting an incomplete impairment of Wg secretion in this sample. **(d,e)** Wg expression in *shibire* mutant discs incubated at 18°C or 32°C before labeling with the extracellular protocol. Extracellular Wg was reduced in *shibire* mutant discs at 32°C. In this example, Wg was not detectable. In approximately 50% of mutant discs, a weak stripe of Wg could be seen (as in (c)), perhaps reflecting incomplete impairment of Wg secretion when Shibire is inactivated.

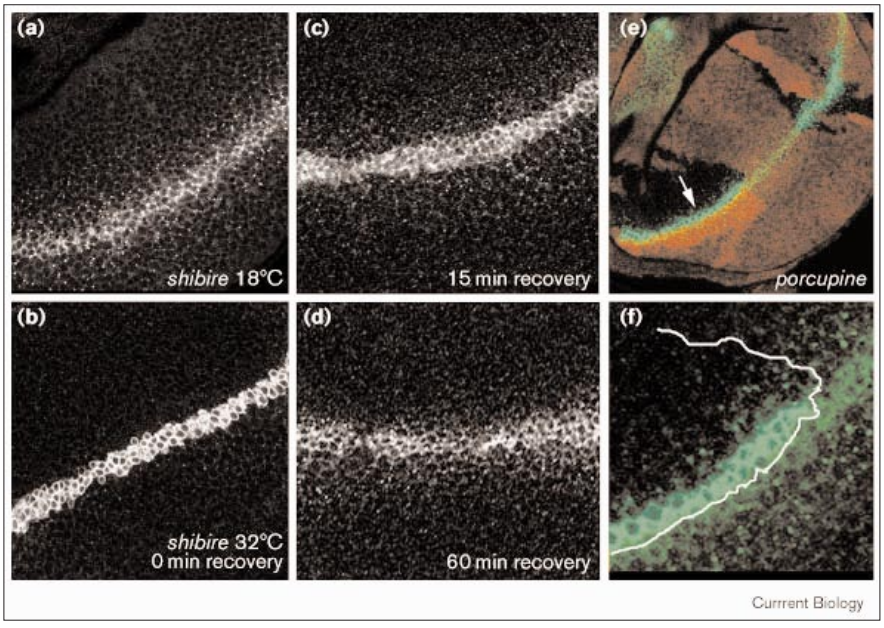
for Wg internalization. As an independent test of the ability of the *shibire* mutation to block endocytosis under these conditions, we monitored uptake of fluorescently labeled DTAF–Transferrin in discs expressing the human transferrin receptor in the posterior compartment. Internalized DTAF–transferrin formed spots in posterior cells in heterozygous *shibire*/+ larvae at 32°C. In *shibire* mutant discs at 32°C, DTAF–transferrin accumulated on the cell surface and the number of spots of internalized DTAF–transferrin was strongly reduced, indicating that the block to internalization is effective in *shibire* mutant discs (see Supplementary material).

Although spots of internalized Wg were not seen in *shibire* mutant cells, Wg was internalized by wild-type cells adjacent to the clone (Figure 3a, inset). The presence of Wg in these cells may reflect movement of Wg across the mutant tissue to reach wild-type cells. In support of this view, we observed extracellular Wg on the surface of *shibire* mutant cells (Figure 3c). The level of extracellular Wg was higher than on nearby wild-type cells. The interpretation that Wg can move across the *shibire* mutant tissue depends on the assumption that extracellular Wg is not simply stabilized in the *shibire* mutant tissue. To determine whether removing dynamin function stabilizes extracellular Wg, we examined Wg distribution in *shibire* mutant discs. At 18°C, extracellular Wg distribution in *shibire* mutant discs was comparable to that in the wild type (Figure 3d). When Shibire was inactivated at 32°C for 3 hours, little or no extracellular Wg was detected (Figure 3e). This indicates that extracellular Wg turns over rapidly when the entire disc is *shibire* mutant. We conclude that the Wg on the *shibire* mutant clones reflects Wg secreted by nearby wild-type tissue that has moved across the clone and that the local accumulation reflects impaired endocytosis. Thus, dynamin-mediated internalization does not appear to play a role in Wg transport in the wing disc. In contrast, dynamin-mediated endocytosis appears to play a role in removing secreted Wg from the extracellular space and, therefore, may help to maintain a steep gradient.

Dynamin activity is required for Wg secretion

The loss of extracellular Wg from homozygous mutant *shibire* discs suggested that Wg secretion might be impaired by removing dynamin activity. Conventional staining showed an intense band of intracellular Wg accumulation at the DV boundary under these conditions (Figure 4a,b), which resembled the accumulation of Wg in clones of *porcupine* mutant cells (Figure 4e,f). The *porcupine* gene encodes a protein that resides in the endoplasmic reticulum and is required for post-translational processing and secretion of Wg in the embryo [36,37]. Comparable Wg accumulation was observed in *shibire* mutant clones that include Wg-expressing cells (for example, Figure 3a). Wg accumulated only in Wg-expressing mutant cells, even when the clone was small and most of the surrounding cells

Figure 4



Reversible block of Wg secretion in *shibire* mutant discs. **(a,b)** Wg expression in *shibire* mutant discs incubated at 18°C or 32°C before labeling with the conventional protocol. At 18°C, the Wg distribution was equivalent to wild type (see Figure 1). (b) Wg accumulates in Wg-expressing cells. Punctate spots of internalized Wg were not seen in nearby cells in discs fixed after 3 h at 32°C. **(c)** Wg expression in a disc shifted back to 18°C after 3 h at 32°C and allowed to recover for 15 min before fixation. **(d)** Wg expression in a disc allowed to recover for 60 min at 18°C before fixation. **(e,f)** Wing disc with large *porcupine* mutant clones marked by the absence of β-galactosidase protein (red). Wg was visualized using the conventional labeling protocol (green). (f) Detail of the large clone in (e). The clone border is outlined. Wg accumulates to high levels in Wg-expressing cells within the clone (arrow in (e)). Spots of internalized Wg are seen in the clone.

were wild-type (Figure 3b). No intracellular Wg accumulation was seen in *shibire* mutant clones that abut but do not include Wg-expressing cells (data not shown). As *shibire* encodes the only *Drosophila* dynamin identified to date [38], our results suggest that Shibire protein may have a role in the formation of transport vesicles at the trans-Golgi network, comparable to that reported for dynamin-2, one of its vertebrate homologs [39]. If so, the traffic of vesicles from the trans-Golgi to the plasma membrane might be blocked in *shibire* mutant cells. Alternatively, blocking Shibire-dependent endocytosis might impair membrane traffic, and indirectly reduce Wg secretion.

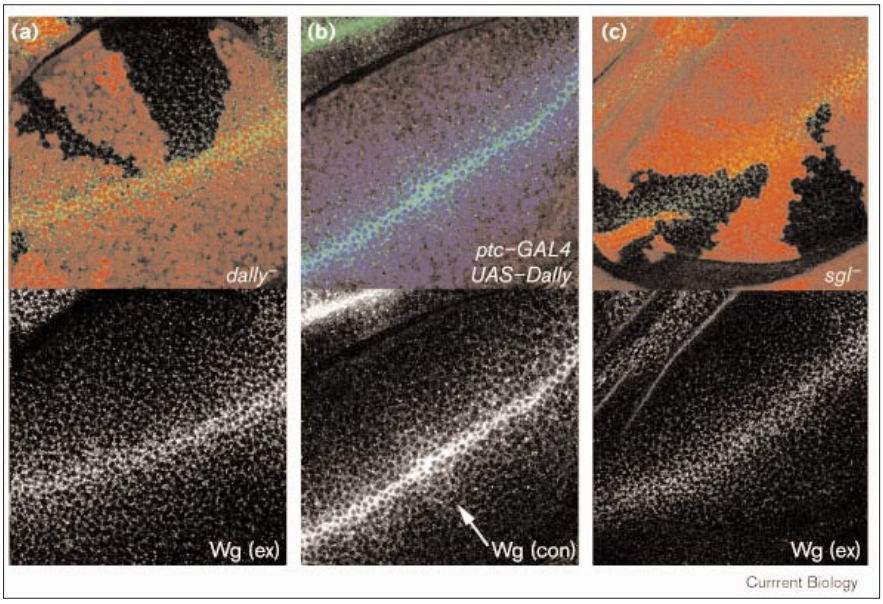
Rapid gradient formation following a reversible block to Wg secretion

The temperature-sensitive defect in dynamin function caused by the *shibire^{ts}* mutation is rapidly reversed by shifting flies back to the permissive temperature (reviewed in [38]). This provides a means to reversibly block Wg secretion. The extracellular Wg gradient was depleted within 3 hours in *shibire^{ts}* mutant discs at 32°C (Figure 3), indicating that extracellular Wg is rapidly lost or degraded even in the absence of endocytosis. Furthermore, internalized Wg was almost entirely cleared from the disc by 3 hours at 32°C (Figure 4a,b). Shifting *shibire* mutant larvae back to the permissive temperature provides the opportunity to monitor the time course of gradient formation when Wg secretion is reinitiated. With 15 minutes of recovery at 18°C, spots of internalized Wg protein could again be detected across the disc (Figure 4c). By 30–60 minutes, the Wg distribution resembled that in control discs kept at 18°C (Figure 4d), indicating that Wg protein had traveled across the disc and been internalized by distant cells. These observations indicate that Wg moves rapidly through the tissue to form a gradient covering at least 50 μm in approximately 30 minutes. For comparison, in *Xenopus* explants, the signaling protein Activin can form a gradient over more than 250 μm by diffusion in a few hours [40]. Thus, the rate of Wg movement is compatible with diffusion through the extracellular space.

Proteoglycans and extracellular Wg

Wg binds avidly to glycosaminoglycans [41]. Mutations that affect glycosaminoglycan biosynthesis phenocopy weak *wg* mutations and genetically interact with *wg* mutant alleles [19–23]. Recent reports have implicated the GPI-anchored proteoglycan Dally as a cofactor in Wg signaling [22,23]. Dally might facilitate Wg signaling by improving retention and movement of Wg along the basolateral surface of the epithelium. We were unable to detect a significant alteration in the distribution of extracellular Wg in *dally^{P2}* mutant discs (data not shown) or in *dally^{P2}* mutant clones (Figure 5a). Overexpression of Dally using the *patched* (*ptc*) promoter to drive expression of the *GAL4*-encoded transcriptional activator (*ptc–GAL4*) caused little or no additional accumulation of Wg in cells near the DV boundary (Figure 5b). This contrasts with the effects of overexpressing Dfz2 using *dpp–GAL4* (a weaker *GAL4* driver; Figure 2d). These observations suggest that Dally may have a relatively low capacity to bind Wg *in vivo* or that it is present in excess, and that Dfz2 is the limiting factor in Wg binding. Our observations strengthen the proposal that Dally might serve as a coreceptor with Dfz2 [22,23], and suggest that Dally does not play a significant

Figure 5



Dally and Sugarless (Sgl) do not affect the extracellular Wg gradient. **(a)** Extracellular (ex) Wg expression in *dally*⁻ (*dally*^{P2}) mutant clones. Wg is shown in green. Clones were marked by the absence of the green fluorescent protein (GFP, red). Wg distribution was not affected in *dally*⁻ mutant cells (shown separately below). **(b)** Wg expression (green) visualized by conventional labeling (con) in discs overexpressing Dally under *ptc–GAL4* control. Very slight accumulation of Wg was visible outlining cells close to the DV boundary (arrow). There is no effect on Distalless (Dll) or Dfz2 expression (shown in blue and red, respectively). **(c)** Extracellular Wg expression in *sgl*⁻ (*sgl*^{08310}) mutant clones. Wg is shown in green. Clones were marked by the absence of GFP (red). Wg distribution was not affected.

role in shaping the Wg gradient. We also examined clones of cells mutant for *sugarless*^{08310} and saw no effect on Wg distribution (Figure 5c). These observations leave open the question of whether other proteoglycans might contribute to Wg gradient formation.

Discussion

How does Wg protein move through the epithelium to form a gradient? Comparison of the embryonic ectoderm and the imaginal discs suggests that different mechanisms may be used. In both tissues, Wg is thought to form a gradient, and vesicles of Wg protein are found in cells near the source of Wg production [4,42,43] (Figure 1). In the wing imaginal disc, the Wg protein gradient is symmetric about its source, but this is not the case in the embryonic ectoderm where Wg is asymmetrically distributed [43] and evidence has been presented that posterior-ward movement of Wg is actively limited by a Hedgehog-dependent process [44]. It has been proposed that dynamin-mediated internalization plays a role in Wg transport in the embryo, where blocking Shibire activity causes accumulation of Wg at its site of production and decreases its apparent range of action [28,29]. Dynamins are required for internalization of clathrin-coated endocytic vesicles as well as for internalization of caveolae and so should disrupt both receptor mediated endocytosis and internalization of some GPI-anchored proteins (reviewed in [38]). Our results indicate that dynamin-mediated endocytosis is not required for movement of extracellular Wg. Rather, dynamin is required for Wg secretion. This may reflect a function related to that reported for vertebrate dynamin-2 [39], or may be a secondary consequence of reduced endocytosis. It is possible that reduced secretion of Wg in the embryo might decrease the apparent range of Wg movement. Recent studies on Wg movement in the embryo also cast doubt on the proposed role of dynamin in transport [45].

In the wing imaginal disc, Wg moves rapidly through the tissue to form a long-range extracellular gradient. We favor the model that gradient formation depends on diffusion of Wg in the plane of the disc epithelium. Although our results do not directly address this issue, we suggest that it is important to constrain ligand movement to the surface of the epithelial cell layer because of the folding of the imaginal discs. The wing imaginal disc is relatively flat in the wing pouch, so the effects of disc folding are less apparent than in the leg disc. Data presented in the Supplementary material illustrate how folding of the leg disc epithelium poses a problem if gradient formation were to occur out of the plane of the epithelial cell layer. Cells that are at very different positions along the proximodistal axis of the leg are brought into close proximity by the folding of the epithelial sheet. Wg and Dpp have been shown to act directly at a distance along the proximodistal axis, and their activities are high in the center of the disc and decrease toward the periphery defining a series of concentrically arranged domains of gene expression [46,47]. It is difficult to imagine how graded responses to Wg and Dpp in the leg disc would be possible unless the ligands are constrained to form their gradients in the plane of the epithelium. Extracellular Wg was also basolateral in the leg disc (data not shown).

We have presented evidence that formation of the extracellular Wg gradient depends on continuous secretion, rapid turnover and rapid movement of extracellular Wg on

the basolateral surface of the disc epithelium. The Wg and Dpp gradients form in a growing field of cells. Use of the temperature-sensitive dynamin mutation to reversibly block Wg secretion has allowed us to monitor the rate of movement of Wg through the tissue. Wg can spread over 30–50 μm in approximately 30 minutes. This suggests that formation of the long-range gradient can occur by diffusion, independent of the rate of growth of the tissue. Evidence has been presented that the Dpp morphogen gradient forms more slowly. Although it has not been possible to directly monitor the rate of Dpp movement through tissue, the rate can be inferred from the time required to activate target genes as a function of distance around a point source of Dpp. This has led to the proposal that Dpp spreads slowly through the tissue, independent of the rate at which it is synthesized, and raises the possibility that Dpp might be spread in part by growth of the tissue [14]. In the case of Dpp, cytonemes might provide another means to regulate ligand transport [48]. The properties of these gradients have intriguing implications for the relationship between pattern formation and size regulation by diffusible morphogens in growing tissues.

Materials and methods

Antibodies

The antibodies used were mouse anti-Wg [49], rabbit anti-Wg [13], anti-Fasciclin II [50], guinea pig anti-Coracle [51], rabbit anti-Dfz2 (unpublished; provided by Susan Cumberledge), rabbit anti-cytoplasmic tropomyosin (unpublished; provided by Anne-Marie Michon and Anne Ephrussi), rabbit anti-β-galactosidase (Cappel), and mouse anti-tubulin (Sigma).

Fly strains

Fly strains expressing Engrailed–Gal4 [52], dpp–Gal4 [53] and UAS–Dfz2–GPI [13] were used. UAS–Dfz2 (encoding full-length Dfz2) was provided by M. Boutros and M. Mlodzik. UAS–hTf-R was constructed by cloning a cDNA encoding the human Transferrin receptor into pUAST [54]. UAS–Dally was provided by Scott Selleck.

Antibody labeling

For labeling extracellular proteins, larvae were dissected in ice-cold Schneider's M3 medium (Sigma) and incubated with antibody for 30–60 min on ice. Larvae were rinsed three times with ice-cold PBS and fixed for 20 min in ice-cold PBS containing 4% formaldehyde. Subsequent processing was the same as in the conventional antibody labeling protocol (described in [4]). For extracellular labeling, antibodies were used at threefold higher concentration than for conventional labeling. Thus far, we have only had success using this protocol with monoclonal antibodies. Fluorescent and HRP-conjugated secondary antibodies were from Jackson labs. Westerns were developed with Bioluminescence reagents (Amersham).

Protease digestion of discs

For each sample, 20–24 discs were removed from larvae in ice-cold PBS. Discs were incubated in 50 μl PBS for 12 min at 32°C. Where indicated, proteinase K was added to 0.1 mg/ml and Triton X-100 to 0.5%. Reactions were stopped by adding three aliquots of 0.5 μl each of 100 mM PMSF in isopropanol, before homogenization of tissue in SDS sample buffer.

DTAF–transferrin labeling

Tb^+ larvae from a cross between *shibire^{ts1}* females and *engrailed–Gal4/CyO; UAS–hTf-R/TM6B* were incubated for 30 min at 32°C, dissected in Schneider's medium at 32°C and incubated for 20 min at 32°C with DTAF–transferrin (5 μg/ml final concentration). Samples were rinsed twice in PBS and fixed as for antibody labeling. The results are shown in the Supplementary material.

Genotypes of larvae for clonal analysis

The *porcupine* mutant clones were *w f porc^{PB16} FRT18A/w P[w$^+$, arm–lacZ] FRT18A; HSFlp2/+*. The *dally* mutant clones were *y w HSFlp1/+; dally^{P2} FRT80B/ P{w$^+$, UbiGFP] FRT80B*. The *sugarless* mutant clones were *y w HSFlp1/+; sugarless^{08310} FRT80B/ P{w$^+$, UbiGFP] FRT80B*. The *shibire* mutant clones were *w shibire^{ts1} FRT18A/w P[w$^+$, arm–acZ] FRT18A; HSFlp2/+*. Clones were induced by heat shock treatment of first or second instar larvae for 45–60 min at 38°C. For *shibire* mutant clones, larvae were allowed to grow at 18°C and were shifted to 32°C for 3 hours before fixation and antibody labeling.

Supplementary material

Supplementary material including a figure showing DTAF–Transferrin labeling of *shibire* mutant wing discs, and a figure showing an optical cross section of a leg disc to illustrate the folded arrangement of the epithelium is available at http://current-biology.com/supmat/supmatin.htm.

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