The *Drosophila rhomboid* gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signaling pathway

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The *rhomboid* (*rho*) gene, which encodes a transmembrane protein, is a member of a small group of genes (ventrolateral genes) required for the differentiation of ventral epidermis in the *Drosophila* embryo. The ventrolateral genes include *spitz*, which encodes an EGF-like ligand, and *Star*. The receptor for *spitz* may be the gene encoding the *(Drosophila)* epidermal growth factor-receptor (*Egf-r*) because the phenotype resulting from partial loss of function of *Egf-r* is similar to that of ventrolateral group mutants. Among ventrolateral genes encoding cell-surface or secreted proteins, *rho* is the only member expressed in a localized pattern corresponding to cells requiring the activity of the ventrolateral pathway. In this paper we provide evidence that spatial localization of *rho* plays an analogous role in establishing vein pattern in the adult wing. *rho* is expressed in early wing disc cells likely to be wing vein primordia and later is sharply restricted to developing veins. Flies homozygous for the viable *rho ve* allele have missing veins and *rho* fails to be expressed in *rho~* mutant wing discs. Ectopic expression of *rho* during wing development leads to the formation of extra veins. Gene dosage studies among ventrolateral genes suggest that the *rho* product (Rho) may facilitate Spi–EGF-R signaling, resulting in activation of RAS. We discuss models for how localized expression of Rho may amplify signaling mediated by ubiquitously distributed ligand and receptor components.

[Key Words: rhomboid; Drosophila; wing veins; imaginal disc development; tyrosine kinase signaling; EGF-R]

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The *rhomboid* (*rho*) gene is required early for differentiation of ventral epidermis in response to the dorsal morphogen gradient in the *Drosophila* embryo. In the absence of *rho* function, longitudinal strips of ventrolateral cells in the early blastoderm embryo that would ordinarily give rise to ventral epidermis of the larva fail to differentiate [Jürgens et al. 1984; Mayer and Nüsslein-Volhard 1988]. As a consequence of this early defect, *rho* mutant larvae lack portions of ventral epidermis. The *rho* gene is also required at other times during embryogenesis in a variety of capacities. For example, *rho* is necessary for the proper development of cells along the ventral midline, for the formation of two stretch receptor organs in each abdominal hemisegment, and for the formation of the first row of denticles in each segment [Mayer and Nüsslein-Volhard 1988; Bier et al. 1990]. In addition to these embryonic phenotypes, flies homozygous for the viable *rho ve* allele have missing veins and *rho* fails to be expressed in *rho~* mutant wing discs. Ectopic expression of *rho* during wing development leads to the formation of extra veins. Gene dosage studies among ventrolateral genes suggest that the *rho* product (Rho) may facilitate Spi–EGF-R signaling, resulting in activation of RAS. We discuss models for how localized expression of Rho may amplify signaling mediated by ubiquitously distributed ligand and receptor components.

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Volhard 1988; Klämbt 1993). Sequence data suggest that the spi gene encodes a growth factor-type molecule (Spi) of the epidermal growth factor/transformation growth factor-α (EGF/TGF-α) class that may be either membrane bound or secreted (Rutledge et al. 1992). The S gene has also been cloned (A. Kolodkin, U. Banerjee, and C.S. Goodman, pers. comm.) and is predicted from conceptual translation to encode a single pass (type II) transmembrane protein. Because mutations in this group of genes, known as the spitz-group or ventrolateral group, result in strikingly similar developmental defects, it is likely that they function in a common developmental pathway. This ventrolateral pathway may also involve the gene (Egf-r) encoding the Drosophila EGF-receptor (EGF-R). A role for EGF-R in ventrolateral signaling is supported by the following evidence: (1) Embryos homozygous for a weak embryonic lethal allele of Egf-r exhibit a phenotype very similar to that of ventrolateral group mutants, including a loss of two lateral stretch receptor organs (Clifford and Schüpbach 1992; Raz and Shilo 1992); (2) spi encodes an EGF/TGF-α-like growth factor (Rutledge et al. 1992); and (3) defects in tissues

Figure 1.  [See facing page for legend.]
derived from ventrolateral positions in the blastoderm resulting from rho, spi, S, and pnt mutations are primarily restricted to epidermal derivatives [Mayer and Nüsslein-Volhard 1988; Mayer et al. 1991]. These observations suggest a model in which the product of the rho gene is required for some aspect of signaling between Spi and EGF-R. Because both the putative ligand [Spi] and receptor [EGF-R] are expressed ubiquitously in the embryo and in imaginal discs [Cammermeyer and Wadsworth 1990; Zak et al. 1990; Katzen et al. 1991; Rutledge et al. 1992; Zak and Shilo 1992; M. Sturtevant, in prep.], it is likely that localized rho expression is important for restricting the spatial and temporal activity of the ventrolateral pathway.

In this paper we show that localized rho expression in wing vein primordia directs these cells to differentiate as veins. rho expression is largely eliminated in rho" wing discs correlating with the absence of veins in adult wings. Conversely, ectopic rho expression causes extra vein formation. We also report dosage-sensitive interactions between rho, spi, EGF-R, and components of the RAS system, which suggest that they operate in a common signal transduction pathway. Finally, we outline models for how local activity of the rho protein might amplify or facilitate interaction between Spi and EGF-R, which are ubiquitously expressed.

**Results**

rho is expressed in wing vein primordia during wing disc development

As a first step in analyzing rho function during wing development, we determined the pattern of rho expression by tissue in situ hybridization to wild-type wing discs with a digoxigenin-labeled rho RNA probe. rho expression in pouch-stage discs from wild-type wandering third-instar larvae and early prepupae consists of a pattern of intersecting stripes [Fig. 1A], as well as a patch of cells likely to give rise to wing hinge veins (arrowhead in Fig. 1A; see also fate map in Campuzano and Modolell 1992). The position of the strong transverse band of rho-expressing cells corresponds well with the known location of cells that will become the margin of the wing, including vein L1 along the anterior margin. These marginal cells can be resolved as two closely spaced parallel rows of rho-expressing cells separated by a small gap of nonexpressing cells. The four stripes of rho expression that cross the future margin extend into separate primordia for the dorsal wing surface (which lies proximal to the future margin) and the ventral surface (cells distal to the future margin). Although there are no known definitive markers for all wing vein primordia at this early developmental stage, a pattern of evenly spaced stripes crossing the future wing margin is what would be expected from the fate map of the uneverted disc [Campuzano and Modolell 1992]. A similar pattern of rho expression is observed slightly later when the disc has begun to evert (~5 hr after pupariation [AP] [Fig. 1B]). By this stage the longitudinal stripes of rho expression intersecting the margin have been brought into register on both the dorsal and ventral surfaces of the disc. Because we suspected that the transverse stripes in third-instar discs correspond to the primordia for the four longitudinal wing veins (L2–L5), we performed double-labeling experiments with known wing vein markers. Although rho is the earliest known gene to be expressed in all presumptive longitudinal wing veins, the A101 P-lacZ enhancer trap in the neutralized gene marks sensory mother cells along the margin and vein L3 starting in third-instar discs [Bellen et al. 1989]. In addition, an antibody to the product of the hairy gene labels the wing margin and wing vein L2 and L3 at a later stage during early pupariation [Carrol and Whyte 1981]. We visualized the ex-
expression of lacZ from the A101 enhancer trap [Fig. 1C] with antibodies followed by peroxidase reactions (brown staining) and rho RNA by whole-mount in situ hybridization followed by an alkaline phosphatase reaction (blue staining). The coincidence of A101 and rho staining along the presumptive margin and vein L3 [arrow] supports the view that rho is expressed in register with wing vein primordia. rho expression is also coincident with Hairy along presumptive veins [not shown].

Additional evidence that rho is expressed in cells forming wing veins was obtained by examining expression at later stages of wing development when the pupal cuticle separates from the underlying tissue permitting access to histological reagents. In these older developing wings (25–35 hr AP), rho transcripts are sharply restricted to veins [Fig. 1D]. rho is expressed in vein cells on both the dorsal and ventral surfaces of the wing, as can be seen in preparations in which the surfaces of the wing have separated slightly during mounting [Fig. 1D, inset]. At this stage, a strip of cells adjacent to rho-expressing cells [between two arrows, Fig. 1D] can be distinguished morphologically from the more loosely packed cells in central portions of intervein domains. As much of the extra wing vein material formed in wings of flies ubiquitously expressing rho is the result of thickening of pre-existing veins [see below and Fig. 2E, F], this strip of densely packed cells may be most susceptible to ectopic rho activity. We conclude that rho is likely to be the earliest known gene to be expressed in longitudinal wing vein primordia and that rho continues to be expressed in developing wing veins during the partitioning of the wing into vein versus intervein territories.

It should be noted that spi and Egf-r are expressed ubiquitously in wing discs [spi expression [M. Sturtevant, unpubl.], Egf-r expression [Kammermeyer and Wadsworth 1990, Katzen et al. 1991, Zak and Shilo 1992, M. Sturtevant, unpubl.]]. Thus, in wing discs, as in the embryo, rho is expressed in a strictly localized pattern, whereas other members of the signaling pathway such as the ligand [spi] and receptor [Egf-r] are more generally distributed.

rho expression in wing vein primordia is required for adult vein formation

The homozygous viable wing vein mutation ve has been determined to be allelic to rho [Diaz-Benjumea and Garcia-Bellido 1990a; E. Bier, unpubl.]. As loss-of-function rho alleles are embryonic lethal, one possibility is that the viable rho· allele is a rho promoter mutation. This possibility is consistent with genetic transfection data [E. Bier, unpubl.] and with the existence of a polymorphism in the rho promoter region in rho· genomic DNA, which from Southern blot analysis appears to be a 600-bp to 800-bp deletion [M. Roark, unpubl.]. To test this interpretation of the rho· mutation, we hybridized a rho probe to discs obtained from larvae homozygous for rho·. rho·/rho· flies lack distal segments of wing veins, especially veins L4 and L5 [see Fig. 2B, below]. At the early pouch stage [Fig. 1E] and during early disc eversion, rho is expressed in the normal pattern along the future margin but is absent from the presumptive longitudinal wing veins, except for weak expression in part of the third vein primordium. Lack of rho staining in presumptive wing vein cells is not an artifact of poor staining reactions, as expression is normal in other imaginal discs isolated from rho· larvae and in other parts of the wing disc, although a patch of staining in the presumptive wing hinge region is shifted relative to wild type [Fig. 1E, arrowhead]. By 30 hr AP, rho expression in the developing wing forecasts the defects in rho· wings [not shown]. The loss of veins can also be observed at this stage with a probe to the Delta gene, which is normally restricted to vein cells at this time [M. Sturtevant and E. Bier, in prep.].

Localized rho expression is required for restricted wing vein formation

To test our hypothesis that localized rho expression dictates cell fate choices, we placed a full-length rho cDNA clone under the control of a heat-inducible promoter in the hs-CaSpeR P-element transformation vector [see Materials and methods]. When transformant lines were heat-shocked during late larval and early pupal stages, we recovered adults with a range of abnormal wing phenotypes and roughened eyes. The most weakly affected wings had slight deltas at the junction of longitudinal veins with the margin. Moderately affected wings had more pronounced deltas and, often, the rudiments of ectopic veins. Strongly affected wings had thickened or fused longitudinal veins, extensive ectopic veins, and often had large blisters where the two wing surfaces separated in a bubble. The ectopic veins can be identified as such by a variety of criteria, including dark pigmentation, thickened and raised cuticle, and increased density of bristles [two- to threefold]. The presence of extra vein material is evident during pupal development when extra vein precursor structures known as lacunae can be observed by Nomarski optics and by the expression of several pupal-stage markers for wing veins [M. Sturtevant and E. Bier, in prep.].

Without heat induction, the transformant lines described above have either wild-type wings or exhibit only minor wing vein deltas [Fig. 2, cf. A and C]. Several other transformant lines have more severe wing vein defects in the absence of heat induction [Fig. 2D–F]. The constitutive wing vein phenotypes of different transformant lines can be ordered in a progression parallelling the phenotypic series resulting from heat-shocking transformant lines with weak constitutive phenotypes. Presumably, constitutive phenotypes are the result of various degrees of leaky or enhancer-driven expression of the heat shock promoter in wing primordia. For the sake of simplification, we have classified the various transformant lines as weak [rhoWR HS], moderate [rhoMOD HS], or strong [rhoMG HS] based on the severity of the constitutive wing vein phenotype.

As expected, rho expression was elevated throughout...
Figure 2. Wing vein phenotypes resulting from insufficient rho function and from excess rho activity. [A] A wild-type wing. The major wing veins are the marginal vein (L1); the longitudinal veins (L2–L4, numbered from anterior to posterior); and two cross veins (the anterior cross vein connects vein L3 to vein L4; the posterior cross vein connects vein L4 to vein L5). The marginal L1 vein ends where it intersects L3, although a subset of sensory bristles that line the margin extend further, ending between L3 and L4. [B] A typical rho^{ve/ve} wing. Longitudinal veins L3–L4 are shortened and do not reach the margin. Veins L4 and L5 are the most affected. L2 is often normal in our rho^{ve} stock [as shown here], but in some individuals it is drawn back a short distance from the margin. Cross veins are not affected. [C] A rho^{ve/HS+} wing. Small deltas form at the junction of L3 and L4 with the margin, and an ectopic spur perpendicular to the posterior cross vein is often observed. [D] A rho^{HS+/+} wing. Prominent deltas form at the junction of L3 and L4, and a rudiment of an extra vein is frequently observed equally spaced between L3 and L4. [E] A rho^{ve/HS+} wing. L3 and L4 deltas are greatly enlarged, becoming continuous along the margin with r3,4. Longitudinal veins are thickened especially L3, and extra longitudinal vein material lies between the margin and L2. Blisters often form centered around the anterior cross vein, causing separation of the dorsal and ventral surfaces. [F] A rho^{ve/HS+} wing. Longitudinal veins are greatly broadened and often fuse into large continuous vein surfaces. Large blisters occupying most of the wing surface are also common. [G] A rho^{ve/ve} wing. Heterozygous rho^{ve/Hs} (which has little phenotype on its own) significantly suppresses the rho^{ve/ve} loss-of-vein phenotype. Veins L3 and L4 are complete, and L5 is longer than in rho^{ve/ve}. [H] A rho^{HS+/+} wing. Heterozygous rho^{ve} (which alone has no phenotype) significantly suppresses the rho^{ve/Hs} excess vein phenotype. Deltas at the junction of L3 and L4 with the margin are reduced, and the extra vein rudiment between L3 and L4 rarely forms.

Dosage-sensitive interactions between rho^{ve} and HS–rho

The reciprocal effects of rho^{ve/ve} and HS–rho alleles on rho expression in discs and the fact that these mutations have opposite phenotypes in discs is consistent with rho function being reduced in rho^{ve/ve} and increased in HS–rho insertions. To test this idea further, we asked whether there are dosage-sensitive interactions between rho^{ve} and HS–rho insertions. Although one copy of a rho^{Wk HS} insertion causes only a very weak constitutive excess vein phenotype [Fig. 2C], heterozygous rho^{Wk HS} strongly suppresses the rho^{ve/ve} loss-of-vein phenotype [Fig. 2G]. The interaction between rho^{ve} and HS–rho works both ways as there is also considerable dominant suppression of the rho^{ve} phenotype by heterozygous rho^{ve} [Fig. 2H]. We note that rho^{ve} is a purely recessive mutation in a wild-type background. These reciprocal interactions support the view that rho^{ve} is a partial loss-of-function allele and that rho^{HS} insertions act as dominant gain-of-function alleles.

Genetic interactions between rho and other ventrolateral group mutations

To determine whether there are dosage-sensitive interactions between rho and other ventrolateral group genes, we combined the rho^{ve} allele and several HS–rho inserts of differing constitutive strengths with alleles of Egfr and other ventrolateral group genes. The results of these crosses are summarized in Table 1, and examples of enhanced or suppressed rho^{ve} or HS–rho phenotypes are shown in Figure 3. All interactions observed are consistent with models in which rho acts synergistically with EGF-R signaling components. Flies homozygous for the partial loss-of-function
torpedo allele of Egf-r (Egf-r\textsuperscript{top}) usually have wild-type wings but occasionally lack vein segments, whereas individuals transheterozygous for Egf-r\textsuperscript{top} and an Egf-r deficiency (DfEgf-r) always lack a large section of vein L4 (Fig. 3A). Mosaic analysis of stronger Egf-r alleles has shown that EGF-R function is required for the formation of all wing veins [Diaz-Benjumea and Garcia-Bellido 1990b]. The dominant gain-of-function Ellipse allele (Egf-r\textsuperscript{Elp}) alone has a very weak wing phenotype when heterozygous [Baker and Rubin 1989, 1992], but generates delta and sporadic spurs along other veins in some individuals when homozygous [Fig. 3B]. Interaction of rho and Egf-r mutations is revealed in several combinations of alleles. Homozygous Egf-r\textsuperscript{top} or heterozygous DfEgf-r enhances the rho\textsuperscript{ve}/rho\textsuperscript{ve} loss-of-function phenotype [Fig. 3C, cf. Fig. 2B], and flies of the genotype Egf-r\textsuperscript{top}/DfEgf-r, rho\textsuperscript{ve}/rho\textsuperscript{ve} lack virtually all wing veins [Fig. 3E]. This latter phenotype is much more severe than the sum of vein deletions observed in Egf-r\textsuperscript{top}/DfEgf-r and rho\textsuperscript{ve}/rho\textsuperscript{ve} separately. If the phenotypes were only additive, the double mutant combination would resemble the rho\textsuperscript{ve}/rho\textsuperscript{ve} phenotype in eye development, presumably by increasing the endogenous GTPase activity of RAS (Gaul et al. 1992). In the wing, Gap1 mutations also appear to antagonize RAS activation as semilethal alleles of the Gap1 gene act as dominant enhancers of HS-rho phenotypes [Table 1; Fig. 3H, cf. Fig. 2D]. A role for Gap1 in vein formation is also revealed by escaper Gap1/Gap1 or Gap1/DfGap1 flies, which have ectopic veins typical of rho\textsuperscript{Mod} HS\textsuperscript{+} phenotypes [Fig. 3G, see also Fig. 1M in Gaul et al. 1992]. No dominant interactions were observed between HS-rho insertions and sos [sos encodes a nucle-
Interactions between HS--rho alleles are not fully penetrant (e.g., the presence of the r3,4 extra vein spur between L3 and rho formation is also likely because vein L2 is significantly shortened in spi/+; rho++/rho++ flies and the rho++/rho++ phenotype is more enhanced by double heterozygous spi than by heterozygous S alone (Table 1). In contrast to the strong interactions between rho and Egfr alleles and between rho and S, we did not observe any interaction between HS--rho and pnt.

The interactions described above are quite specific. We have also examined interactions between HS--rho insertions and many other wing vein mutants and have found very few other interactions comparable with those described above (Table 1). For example, there are no obvious dominant interactions between HS--rho insertions and mutants in the integrin-encoding genes l(1)myospheroid gene or inflated (Brower and Jaffe 1989, Wilcox et al. 1989; Zusman et al. 1990). Combinations of mutant alleles of these genes induce blisters and might be expected to interact non-specifically with HS--rho insertions, yet they do not. Interestingly, the few strong genetic interactions that we have observed outside of those described above include genes encoding EGF-type receptor repeats such as Notch, Delta, and Serrate (Table 1; M. Sturtevant and E. Bier, in prep.). As further evidence for the specificity of the above interactions, we are currently conducting a screen for dominant enhancers of rho++/rho++ yielding phenotypes equivalent to that observed with DIegf-r/+ of S/+ and have found only a small handful of loci acting as such strong enhancers.

Discussion

In the wing disc, as in the embryo, rho is expressed in a localized pattern. This localized expression in presumptive
Figure 3. Interactions between mutant alleles of rho and other ventrolateral genes in wing vein formation. (A) A Egf-tisp/DfEgf-t wing. (B) A Egf-tisp/Egf-tisp wing. A prominent delta typically forms at the junction of L2 with the margin, and L3 and L4 frequently bifurcate near the margin. (C) A Egf-tisp/Egf-tisp, rhoV~ rhoVe wing. A similar phenotype is observed with DfEgf-t/+; rhoV~ rhoVe/+ wing. All longitudinal veins are shortened (L2 most dramatically). L5 is drawn back behind the posterior cross vein, which never occurs in our rhoV~ rhoVe stock. (D) A Egf-tisp/Egf-tisp, rhoV~ rhoVe wing. The loss of all L3, most of L2, and the proximal segments of L4 and L5 in this double mutant is striking, as neither Egf-tisp/DfEgf-t nor rhoV~ rhoVe alone disrupt these vein segments. (E) A Egf-tisp/Egf-tisp, rhoV~ rhoVe wing. The rhoV~ rhoVe extra vein phenotype is enhanced. L3 and L4 deltas are accentuated, and extra vein spurs are frequent (often posterior to L4 at the margin or posterior to L5 near the posterior cross vein). (G) A Gap1Ec2/DfGap1 wing. The Gap1Ec2 allele is the result of an insertion of the P-lacW enhancer trap vector [Bier et al. 1990] into the Gap1 locus. Extra vein material is typically found between L2 and the margin, and L2 is broadened. Wings of flies homozygous for the Gap1Ec2 allele described by Gaul et al. (1992) look virtually identical to rhoV~ rhoVe/+; rhoV~ rhoVe/+ wings. (H) A Gap1Ec2/+; rhoV~ rhoVe/+ wing. Heterozygous Gap1Ec2 (a recessive mutation) significantly enhances the rhoV~ rhoVe extra vein phenotype. Ectopic vein rudiments form in addition to exaggeration of the rhoV~ rhoVe phenotype. (I) A S/+; rhoV~ rhoVe wing. Heterozygous S enhances the rhoV~ rhoVe loss-of-vein phenotype. L2 is greatly shortened, and L5 is drawn back behind the posterior cross vein. This enhancement is yet more severe in S spi/+; rhoV~ rhoVe wings demonstrating a role for spi in vein formation. spi/+ also enhances rhoV~ rhoVe (particularly L2) and reduces the frequency of the extra vein phenotype observed in rhoV~ rhoVe flies. (J) A S/+; rhoV~ rhoVe HS/+ wing. Heterozygous S strongly suppresses the rhoV~ rhoVe phenotype. Blistering is completely eliminated, broadening of veins and deltas are greatly reduced, and the extra vein rudiment between L3 and L4 is suppressed. Wings of this genotype can be difficult to distinguish from wild type.

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tive wing vein primordia is crucial because lack of rho expression in rhoV~ rhoVe presumptive vein cells leads to loss of adult veins, whereas ectopic expression of rho during wing development induces the formation of extra veins. Dosage-sensitive interactions between rho alleles and mutations in other ventrolateral genes support models in which rho functions by hyperactivating EGF-R signaling. Because both the putative ligand (Spi) and recep-
tor (EGF-R) are ubiquitously expressed in wing discs, rho is likely to provide spatial specificity for restricting wing vein formation to appropriate locations.

**rho is expressed in wing vein primordia**

rho is the earliest known gene to be expressed in a pattern of intersecting rows of cells in wing imaginal discs likely to be the primordia of wing veins. The correspondence of rho stripes with vein primordia is supported by double-labeling experiments in which rho expression colocalizes with known wing vein markers. The rho stripes marking presumptive veins L2–L5 cross the future margin. Because the margin divides the wing disc into dorsal and ventral anlagen, vein formation is likely to be initiated independently on both surfaces of the wing. Cells at the margin also express rho in two tightly spaced rows, one on the dorsal surface and the other on the ventral surface. The expression of rho on both separated presumptive wing surfaces is consistent with genetic observations suggesting that rho expression disrupts wing vein formation during the third larval instar prior to disc eversion and subsequent apposition of the dorsal and ventral surfaces (García-Bellido 1977).

When the two wing surfaces come together as the wing events, refinement may be necessary to bring the two surfaces into strict alignment. The independent initiation of pattern in two separate regions destined to form a single structure as a result of subsequent morphogenetic events is reminiscent of the formation of midline genes in the embryo from initially separate ventrolateral rows of blastoderm cells. rho expression in tracheal pits is another example of isolated groups of cells that will later join to form a unified structure. Perhaps, the rho protein is functioning in each of these cases to facilitate the mutual recognition and merging of spatially distinct groups of cells into a single coherent structure.

**Localized rho expression is required for wing vein formation**

rho expression along presumptive veins in wing discs is required for vein formation because rho expression is greatly reduced or missing entirely from longitudinal wing vein primordia in rho<sup>ve</sup>/rho<sup>ve</sup> mutant discs and large portions of longitudinal veins are missing in rho<sup>ve</sup>/rho<sup>ve</sup> flies. On the other hand, rho expression along the presumptive margin is normal in rho<sup>ve</sup>/rho<sup>ve</sup> discs and the margin is unaffected in rho<sup>ve</sup>/rho<sup>ve</sup> flies. The loss of rho expression along longitudinal veins in rho<sup>ve</sup>/rho<sup>ve</sup> discs is more severe than the loss-of-vein phenotype. This discrepancy is not likely to be the result of low but functionally important levels of rho expressed along vein primordia in rho<sup>ve</sup> that are not detected by in situ hybridization, because in mosaics, clones homozygous for the rho<sup>delet</sup> null allele only delete vein segments in portions of the wing lacking veins in rho<sup>ve</sup> mutants (M. Sturtevant and E. Bier, in prep.). These data suggest that either rho is unimportant for formation of portions of veins that are intact in rho<sup>ve</sup> mutants or that other spatial cues act in concert with rho to determine the location of vein formation. Genetic interactions [see below] favor the latter alternative, that rho establishes the vein versus intervein cell fate choice in combination with other partially redundant cues.

Ectopic rho expression during wing development is sufficient for inducing wing vein fates in many more cells than normal. Weak and moderate HS–rho phenotypes are predominantly restricted to distal portions of wing veins. Because the loss of veins in rho<sup>ve</sup>/rho<sup>ve</sup> flies is restricted to distal portions of the veins, it appears that this region of the wing is most sensitive to the level of rho expression. Strong HS–rho phenotypes include thickening of wing veins and fusion of veins, which when extreme converts much of the wing surface into vein tissue, causing large blisters. As loss of rho expression leads to the truncation of wing veins while ectopic rho expression induces the formation of excess wing vein material, we conclude that rho activity is necessary and sufficient for inducing vein differentiation in many wing imaginal cells. Thus, rho functions during wing vein development as it does during embryogenesis to translate positional information established by patterning genes into coherent differentiation programs.

**Genetic interactions between rho and Egf-t mutations in wing development**

There are often dosage-sensitive interactions between mutations in genes functioning in a common genetic pathway. Strong dosage-sensitive interactions can indicate physical interactions between gene products or synergistic function in a particular process. We combined both loss-of-function rho<sup>ve</sup> and gain-of-function rho<sup>HS</sup> alleles of different constitutive strengths with mutations in other ventrolateral group genes to determine which genes rho might interact with most intimately. From these crosses we observed strong interactions between rho and Egf-t. Combining gain-of-function alleles of both rho<sup>HS</sup> and Egf-t (Egf-t<sup>elip</sup>) enhances formation of extra veins, whereas combining loss-of-function alleles of these genes (rho<sup>ve</sup> and Egf-t<sup>ops</sup>) greatly exacerbates the loss of vein phenotype. In addition, combining loss-of-function rho<sup>ve</sup> and gain-of-function Egf-t<sup>elip</sup> leads to nearly wild-type wing development. Although these double mutant combinations of partial loss-of-function alleles are consistent with a model in which rho acts upstream or in parallel with EGF-R, such an epistatic relationship cannot be established without performing mosaic analysis with stronger loss-of-function alleles.

The virtually complete suppression of the rho<sup>ve</sup>/rho<sup>ve</sup> phenotype by homozygous Egf-t<sup>elip</sup> suggests that rho is not the only molecule providing spatial cues for vein development because rho expression in rho<sup>ve</sup>/rho<sup>ve</sup> discs, which is undetectable in any longitudinal veins other than a short segment of vein L3, is not restored by Egf-t<sup>elip</sup> (M. Sturtevant, unpubl.). On the other hand, the enhancement of rho<sup>ve</sup>/rho<sup>ve</sup> by decreased Egf-t or S dosage suggests that rho is likely to play a role in the formation of wing veins that are not deleted in rho<sup>ve</sup>/rho<sup>ve</sup>.
These data suggest that rho plays a key role in the formation of all longitudinal wing veins in combination with other spatially localized cues. We also observed strong genetic interaction between rho and S, as well as interaction between rho and spi. S has also been observed to interact strongly with Egf-r\textsuperscript{top} in the wing (J. Price, pers. comm.). In addition, the recessive viable asteroid allele of S has been shown to be a strong enhancer of the Egf-r loss-of-vein phenotype when homozygous (Diaz-Benjumea and Garcia-Bellido 1990b). The intense genetic interaction between rho and S suggests that the S product may directly interact with rho (see Fig. 4). Alternatively, S may be the most limiting component of the ventrolateral pathway.

All of the genetic interactions that we have observed support a model in which rho is required to permit or to amplify Spi–EGF-R signaling. Because interactions with rho as strong as these are rare, they are very likely to represent meaningful functional interactions between rho and genes of the Spi–EGF-R signaling pathway. It should be emphasized, however, that no physical evidence for a direct interaction of Spi with EGF-R has yet been reported. Thus, signaling via other ligands and/or tyrosine kinase receptors may be enhanced by rho activity. Such potential components of the ventrolateral signaling pathway may be identified by searching for dominant suppressors of HS–rho phenotypes.

Relation of the ventrolateral pathway to other developmental systems

Signaling via the EGF-R tyrosine kinase and the sev tyrosine kinase receptor are important for different steps in the development of the compound eye (Hafen et al. 1987; Tomlinson and Ready 1987; Rubin 1991; Simon et al. 1991; Fortini et al. 1992). These studies suggest that the Sev tyrosine kinase receptor when activated by the Boss ligand (Reinke and Zipursky 1988; Hart et al. 1990; K\"{a}rmer et al. 1991) activates Drosophila RAS1 (Simon et al. 1991; Fortini et al. 1992). Activated RAS is presumably in the GTP bound form as mutations in the Gap1 gene suppress a sev phenotype by leading to unregulated RAS1 activity (Fortini et al. 1992, Gaul et al. 1992). Mutations in the genes involved in RAS regulation also modify the severity of the Egf-r\textsuperscript{top} allele, suggesting that both Sev and EGF-R activate a common intracellular signaling cascade (Simon et al. 1991). RAS2 may also play a role in eye development because activated RAS2 causes a roughened eye phenotype that is distinct from that induced by activated RAS1 and does not involve sev signaling (Fortini et al. 1992).

The observations that homozygous Gap1 mutants have a wing phenotype typical of moderate rho overexpression (Fig. 3J; Gaul et al. 1992) and that rho\textsuperscript{HS} and rho\textsuperscript{SS} interact strongly with Egf-r, Ras1, and Gap1 alleles support the view that wing vein formation depends on the activation of RAS by EGF-R. As expression of activated RAS2 in wing discs leads to a phenotype virtually indistinguishable from that of rho\textsuperscript{HS} + \textsuperscript{SS} (A. Brand and N. Perrimon, pers. comm.), it would be interesting to test whether EGF-R signaling also involves RAS2 in wing vein formation.

A developmental pathway strongly resembling the Drosophila ventrolateral pathway has recently been described for the development of vulval cells in the nematode Caenorhabditis elegans. In vulval development a signal secreted from the anchor cell directs cell fate choices. The putative secreted signal encoded by the lin-3 gene, which like the spi gene product shares significant sequence similarity with EGF and TGF-\alpha (Hill and Sternberg 1992), activates the C. elegans EGF-R encoded by let-23 (Aroian et al. 1990), which functions through a RAS protein encoded by let-60 (Han and Sternberg 1990). Thus, the EGF-R signaling pathway is not only used for several unrelated developmental decisions in Drosophila but appears to be conserved in detail throughout evolution.

Localized amplification of ubiquitously distributed ligand and receptor

Receptor tyrosine kinases have been implicated in several other important developmental decisions. For exam-
ple, activation of the Torso receptor at the poles of the Drosophila embryo is required for formation of the terminal portions of the larva [Klinger et al. 1988], and, as mentioned above, the Sev receptor is essential for differentiation of the R7 photoreceptor in the compound eye [Tomlinson and Ready 1987]. Precise spatial activation of the Torso and Sev receptor tyrosine kinases does not result from the localization of the receptor or accessory proteins but, rather, from localized ligands. In the case of the Drosophila fibroblast growth factor-receptor [FGF-R], localization of the receptor may restrict signaling [Klämbt et al. 1992]. The localization of a necessary or amplifying accessory molecule such as rho represents another mechanism for achieving specificity of a signaling pathway. Thus, future studies of rho may broaden the understanding of how specificity is achieved by ligand receptor systems.

Potential mechanisms for hyperactivation of EGF-R signaling by Rho

The mechanism by which the Rho protein mediates receptor hyperactivation is unknown. Several potential mechanisms for hyperactivation of EGF-R by Rho are indicated schematically in Figure 4. In one class of models, Rho would function directly or indirectly to increase cell–cell adhesion [perhaps in combination with S] and thereby makes ligand (e.g., Spi)–receptor (e.g., EGF-R) interaction more likely. In an alternative but not mutually exclusive class of models, rho would associate as a cofactor to form part of the receptor for the ligand, for example, by increasing the affinity of EGF-R for the ligand or some other required component of the signaling pathway.

Modification of specific signaling events by augmenting cell–cell adhesion is a common mechanism for regulating the extent of cell–cell signaling. This adhesion-mediated process, referred to as cocapping [Singer 1992], increases the likelihood of receptor–ligand interaction by increasing the local concentration of the interacting molecules as an indirect consequence of the two cell membranes being brought into close register. The association between rho-expressing cells and clear adhesive domains is compelling. For example, during embryogenesis, rho is expressed in midline cells, tracheal pits, segment boundary cells, and cells at the dorsal edge of the epidermis during dorsal closure. The formation or function of all these structures is abnormal in rho mutant embryos. During wing disc development, rho is expressed along presumptive veins; the formation of these veins is strongly dependent on adhesive interactions [Brower and Jaffee 1989; Wilcox et al. 1989; Zunman et al. 1990]. Given the strong suppression of HS–rho phenotypes by S mutations, it is possible that Rho might directly mediate adhesion by binding the S product. Alternatively, Rho and S could increase adhesion by modifying the activities of other adhesion molecules.

Models in which Rho interacts directly with EGF-R or other tyrosine kinase receptors to form a more active receptor for a ligand [possibly Spi] are also plausible on the basis of current data. A version of this type of model, similar in formal terms to the adhesion model, is that Rho increases the affinity of the receptor for its ligand. Although no cofactors with molecular mass or hydrophobicity profiles similar to Rho have been found to be associated with EGF-R or other tyrosine kinase receptors in biochemical studies, the nerve growth factor-receptor [NGF] provides a precedent for interactions between two low-affinity receptors creating a high-affinity receptor [Hempstead et al. 1991; Kaplan et al. 1991]. Other possible mechanisms by which rho might hyperactivate EGF-R function include facilitation of receptor dimerization upon binding ligand, augmentation of receptor transphosphorylation, or facilitation of receptor association with all or a subset of primary effector molecules such as SH2-containing proteins.

If Rho acts to increase all functions of EGF-R equally, there must be some threshold for receptor activity above which a qualitatively different signal is elaborated. Such a threshold would explain why basal EGF-R function is required for survival of all epidermal cells during wing development and embryogenesis [Diaz-Benjumea and García-Bellido 1990b; Clifford and Schüpbach 1992; Raz and Shilo 1992], whereas hyperactivation of EGF-R signaling has specific developmental significance [Diaz-Benjumea and García-Bellido 1990b]. Distinct thresholds for EGF-R activity may also regulate the type of vulval cell produced in C. elegans [Sternberg and Horvitz 1986]. Future biochemical studies will be required to determine whether one or more of these potential mechanisms form the basis for rho amplification of EGF-R signaling.

Materials and methods

Fly stocks

All genetic markers and chromosome balancers used are described in Lindsley and Grell [1968]. Transformant HS–rho flies (kindly provided by Hannele Ruohola-Baker, University of California, San Francisco) and embryo were balanced to establish stocks. The GαK-1 and GαK-2 alleles were determined to be P-lacW insertion [Bier et al. 1989] in the GαP gene by E. Grell [University of San Francisco], B. Hay, and U. Gaul [University of California, Berkeley]. The Gα/P-1, Ras1e18, sos2/2, and dkr104 (= Drosophila GB2/Sem5) alleles were kindly provided by G. Rubin [University of California, Berkeley]. Rho alleles including Egf-R-Cif, Egf-R265, and Egf-R-F18 were kindly provided by T. Schüpbach [Princeton University, NJ]. Other stocks were obtained from the Bowling Green, Ohio and Bloomington, Indiana Drosophila Stock Centers.

Heat inductions

Late third-instar larvae or early pupae (<10 hrs AP) were placed in fresh glass vials with fly food. The vials were incubated at 38°C for 45 min [in a water bath] and were then kept at 25°C until flies emerged.

Mounting fly wings

Wings from adult flies were dissected in isopropanol and mounted in Canadian Balsam mounting medium (Gary’s Magic Mount) following the protocol of Lawrence et al. [in Roberts 1986]. Mounted wings were photographed under Nomarski optics with a 4× lens on a compound microscope.
In situ hybridization to whole-mount embryos or discs

In situ hybridization to whole-mount discs and embryos was performed using digoxigenin-labeled RNA probes (Boehringer-Mannheim, 1093 657), according to Tautz and Pfeiffle (1989), using 4 μg/ml of proteinase K instead of 40 μg/ml as required for digoxigenin-labeled DNA probes.

Double labeling of discs

Discs were fixed for 30 min in 0.1 M PIPES (pH 6.9), 2 mM MgSO4, 1 mM EGTA, 1% Triton X-100, and 4% formaldehyde. After washing five times for 10 min each in an incubation buffer containing 50 mM Tris (pH 6.8), 150 mM NaCl, and 0.5% NP-40, the tissues were then blocked for 2 hr at 4°C in incubation buffer with 5 mg/ml of BSA. Subsequent incubations and washes for antibody staining were done in the incubation buffer with 1 mg/ml of BSA. For staining neuronal precursors, discs from A101 flies (a P-lacZ reporter for neuronalized) were incubated overnight at 4°C in 200 μl of incubation buffer with 1:200 dilution of rabbit-anti-β-galactosidase (Cappel). After five 10-min washes, biotinylated goat antirabbit secondary antibodies (Vector) were added at 1:200 for 1 hr and then washed five times for 10 min each in PBS. At this point the antibody staining protocol was suspended and embryos were refixed in PBS with 50 mM EGTA and 4% formaldehyde overnight to ensure that the biotinylated secondary antibody remained in place before the subsequent whole-mount in situ protocol. The embryos were then washed and processed according to the standard whole-mount in situ protocol. Following development of the alkaline phosphatase reaction from the whole-mount in situ protocol, we resumed the antibody staining protocol at the avidin-biotinylated–HRP addition step and proceeded with the Vectastain ABC elite kit according to instructions. A more detailed description of this double-labeling method will be presented elsewhere.

Construction of the HS–rho P-element vector

As a first step in constructing the HS–rho P-element transformation vector, we trimmed a nearly full-length rho cDNA to separate the protein-coding region from 5'– and 3'–untranslated sequences. Because the pattern of rho expression during embryogenesis is highly dynamic, it is likely that these untranslated sequences lead to mRNA instability. We particularly wished to remove 3' sequences that contain 14 repeats of an RNA instability motif AUUUA. The complete Rho protein-coding open reading frame is contained on a 1060-bp HindIII fragment (Bier et al. 1990). The ends of this fragment were filled in with Klenow, and the fragment was ligated to the cDNA insert, treated with Klenow, and ligated to XbaI linkers to create a rho-coding-region insert flanked on either side by XbaI sites. The rho insert was then isolated by XbaI digestion and subcloned into the XbaI site of the P-element heat shock vector hs–CaSpeR [Bang and Posakony 1992]. We recovered both the sense (HS–rho) and antisense (HS–anti rho) versions of the vector for injection into fly embryos.

Other molecular techniques

RNA probe synthesis was performed according to Boehringer-Mannheim protocols and other cloning techniques followed standard procedures, as in Maniatis et al. (1982).

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