brother of rhomboid, a rhomboid-Related Gene Expressed during Early Drosophila Oogenesis, Promotes EGF-R/MAPK Signaling

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The Drosophila rhomboid (rho) gene participates in localized activation of EGF-receptor signaling in various developmental settings. The Rhomboid protein has been proposed to promote presentation and/or processing of the membrane-bound Spitz (mSpi) EGF-related ligand to generate an active diffusible form of the ligand. Here, we report on a new rhomboid-related gene identified by sequence similarity searching that we have named brother of rhomboid (brho). In contrast to rho, which is expressed in complex patterns during many stages of development, brho appears to be expressed only during oogenesis. brho transcripts are present in early oocytes and abut posterior follicle cells which exhibit high levels of MAPK activation. brho, like rho, collaborates with Star to promote signaling through the EGF-R/MAPK pathway, and genetic evidence indicates that Brho can activate both the mSpi and the Grk precursor EGF ligands in the wing. We propose that endogenous brho may activate the oocyte-specific Gurken ligand and thereby participate in defining posterior cell fates in the early follicular epithelium. © 2000 Academic Press

Key Words: rhomboid; brother of rhomboid; EGF-R; MAPK; spitz; gurken; Star; oogenesis.

INTRODUCTION

Receptor tyrosine kinases (RTKs) constitute a large family of proteins that function through the highly conserved intracellular RAS/MAPK signaling cascade. These receptors are activated by extracellular signals such as growth factors and control various cell fate decisions during development. Members of the EGF-receptor subfamily of RTKs have been extensively studied for their critical roles in cell survival, proliferation, and differentiation (Moghali and Sternberg, 1999). The fruit fly provides a particularly good developmental and genetic system for understanding the complex mechanisms supporting EGF-receptor activation. Only one EGF-receptor gene (Egfr) has been identified so far in Drosophila. This single Egfr gene plays key roles in many different cell fate choices throughout the Drosophila life cycle, spanning oogenesis to imaginal disc development (Bier, 1998; Perrimon and Perkins, 1997; Schweitzer and Shilo, 1997).

The participation of EGF-R signaling in a multitude of developmental processes is made possible by a combination of regulatory mechanisms controlling its localized activation and down-regulation (reviewed in Perrimon and Perkins, 1997). Several genes have been implicated in extracellular regulation of this ubiquitously expressed receptor. Among these genes, spitz, gurken, and vein encode EGF ligands that activate EGF-R in various developmental situations. The product of the vein gene (Vein) belongs to the neuregulin subfamily of EGF ligands and is directly secreted into the extracellular space (Schnepf et al., 1996). In contrast, the product of the spitz gene is initially expressed as a membrane-bound inactive precursor (mSpi) (Rutledge et al., 1992). Although the precise mechanism through which the mSpi precursor is activated remains unclear, it may involve proteolytic cleavage of mSpi to liberate a diffusible active peptide. In support of this possibility, an artificially secreted form of Spi lacking both cytoplasmic and transmembrane domains (sSpi) is much more active than the...
full-length mSpi form (Bang and Kintner, 2000; Golembo et al., 1996; Schweitzer et al., 1995). The Gurken (Grk) ligand is involved in several aspects of oogenesis (Van Buskirk and Schüpbach, 1999) and displays structural (Neuman-Silberberg and Schüpbach, 1993) and functional similarities to Spitz. For example, a truncated form of Grk lacking both transmembrane and cytoplasmic domains is highly active, in contrast to full-length Grk, which is inactive when misexpressed in the wing or in ovarian follicle cells (Queenan et al., 1999). This finding suggests that Grk may require activation through a mechanism similar to that of Spi.

The rhomboid (rho) and Star (S) genes, which encode integral membrane proteins, also play important roles in regulating EGFR signaling (Kolodkin et al., 1994; Bier et al., 1990). A variety of evidence indicates that rho and Star function in an interdependent fashion and act at least in part by promoting Spi activation. Consistent with this view, rho, S, and spi mutants have nearly identical embryonic phenotypes (Bier et al., 1990; Mayer and Nüsslein-Volhard, 1988; Rutledge et al., 1992). Analysis of gene expression and MAPK activation pattern indicates that Rho can activate EGFR/MAPK signaling in neighboring cells (Golembo et al., 1996; Guichard et al., 1999; Sapir et al., 1998). As the Rho protein is not observed to move from the cells in which it is expressed (Sturtevant et al., 1996), the non-cell-autonomous activity of rho suggests that Rho participates in the production of a diffusible EGFR signal. It has also been observed that Spi can substitute for Rho in patterning the ventral epidermis (Schweitzer et al., 1995), consistent with Rho acting to generate a sSpi-like activity. In addition, combined misexpression of mspi and Star in the developing wing leads to striking phenotypes, whereas neither of these genes has any effect when misexpressed alone (Pickup and Banerjee, 1999). Similarly, strong synergistic activation of EGFR signaling is observed in the wing when rho, Star, and mspi are coexpressed (Guichard et al., 1999). This striking synergy between misexpressed rho, Star, and mspi supports the idea that Rho and Star collaborate to convert mspi into an active EGFR ligand. More recently, Bang and Kintner used a frog heterologous assay system (Bang and Kintner, 2000) to demonstrate that Rho and Star are necessary for activating the mspi ligand. Importantly, these investigators demonstrated that this activation is followed by, but does not require, proteolytic cleavage of the Spitz precursor.

In contrast to the spi, Star, and Egfr genes, which are expressed ubiquitously in most developmentally active tissues, rho is expressed in a spatially restricted and dynamic pattern (Bier et al., 1990; Sturtevant et al., 1993). Localized expression of rho correlates with cells requiring an elevated activity of EGFR, as revealed by high levels of MAPK activation detected in situ (Gabay et al., 1997a,b; Guichard et al., 1999). In addition, as mentioned above, cells a short distance from rho-expressing cells often display elevated levels of MAPK activation. These observations suggest that the Rho protein provides the spatial and temporal clues necessary for restricting EGFR activation to appropriate cells during development.

Although models in which Rho and Star collaborate to promote spatially restricted cleavage of mSpi, and hence EGFR activation, account for many facts, several observations suggest that there may be additional missing components. For example, rho and Star are required for wing vein formation, while spi has no detectable function in the wing (Guichard et al., 1999; Nagaraj et al., 1999). One explanation for this observation is that Rho may act on a yet unknown EGF ligand during wing vein development. There are also examples in which rho does not appear to be required to activate EGF-R signaling. Thus, rho is dispensable in the female germ line, yet activation of EGFR signaling by the Gurken ligand in the oocyte plays an essential role in the germarium and in establishing both the anterior-posterior (A/P) and the dorsal-ventral (D/V) axes of the oocyte (Nilson and Schüpbach, 1999). Similarly, during eye development, Spitz and Star are involved in recruiting undifferentiated cells to the forming ommatidium (Freeman, 1996), but rho has no detectable role in this process (Freeman et al., 1992, see note added in proof). These latter observations raise the question of whether Spitz or Gurken might be activated by yet unidentified Rhomboid-like molecules.

Here, we describe the identification of a novel rhomboid-related gene, brother of rhomboid (brho), from the Drosophila genome project (Adams et al., 2000), that functions like rho to promote MAPK activation through the EGFR pathway. Also like rho, brho interacts synergistically with Star to promote vein development. However, brho expression appears to be restricted to the female germ line, in contrast to rho, which is expressed in a complex pattern during many stages of development. brho expression in the oocyte abuts posterior follicle cells that exhibit MAPK activation during stages 5–8 of oogenesis when EGFR signaling plays an essential role in determining fates of posterior follicle cells, which subsequently define the A/P axis of the embryo. The restricted pattern of brho expression suggests that it may participate in establishing the A/P axis of the egg chamber, possibly through activation of the EGF ligand Gurken in the oocyte. Consistent with this hypothesis, coexpression of brho and mgrk in the wing generates an ectopic vein phenotype much stronger than that caused by brho alone. As expression of mgrk alone cannot induce any visible effect, we propose that brho normally functions by activating mgrk during early oogenesis.

**MATERIALS AND METHODS**

**Isolation and Characterization of brho Transcripts**

RNA was extracted from flies at different developmental stages using TRIzol reagent (Life Technologies) according to manufacturer suggestions. To amplify an internal portion of the brho mRNA, primers surrounding the second intron were synthesized. The sense primer, designated RT brhoS (5′GCTGGGTTCCTTTTGGTTTA3′),
and the antisense primer, designated RT brhoAS (5’AGCATAGCGACCATAGATG3’), were used on the RNA templates extracted from various tissues and stages of development using the Superscript One Step RT-PCR system (Life Technologies). PCR was carried out in a Perkin-Elmer Thermal Cycler 480, with the following parameters: 20 min at 50°C for synthesis of cDNA followed by 45 cycles (30 s at 94°C, 45 s at 55°C, 1 min 25 s at 72°C). To confirm that the observed PCR products corresponded to brho, a second antisense primer was designed, located internally to the first PCR product (RTbrhoAS2, 5’GGCAAAGGCAAAGTC-CTGAAGG3’), and the first RT-PCR was used as template for the second PCR, with the same parameters. We determined that the full predicted open reading frame is contained in brho transcripts by using a sense primer containing the predicted ATG start codon (5’CGACTGTCTTAGATCACCTGGGATTTCACACCTGGAA3’) from the DS 02734 P1 clone DNA using Vent DNA polymerase (New England Biolabs). The resulting PCR product was checked for proper orientation and sequence. The full cDNA product was amplified from RNA extracted from adult flies with these primers in a RT-PCR (reaction parameters were the same as above) and sequenced.

In Situ Hybridization to Ovaries

A brho-specific digoxigenin-labeled probe was synthesized from a HindIII-linearized brho genomic fragment cloned into the pCR-BluntII-TOPO vector (Invitrogen), using a T7 priming site. Ovaries were dissected and processed for in situ hybridization with the brho probe as described in Suter and Steward (1991).

Activated MAPK* Staining

Ovaries were fixed for 30 min in 8% formaldehyde, 0.05 M EGTA, 1× PBS, rinsed for an hour in PBS 0.1% Tween 20 (PBST), and stored overnight in methanol. After progressive rehydration in PBST, ovaries were incubated with 1/200 anti-dp-ERK antibodies (Sigma). Remaining steps were performed according to standard procedures (e.g., Gabay et al., 1997a).

Fly Stocks and Crosses

Stocks and crosses were raised using standard methods. To determine genetic interactions between brho and known partners of the rho/Egfr pathway, UAS-brho flies were crossed to various test stocks, including GAL4 MSL1096;UAS-DN-Egfr, GAL4 MSL1096;UAS-msp1, GAL4 MSL1096;UAS-markr, GAL4 MSL1096;UAS-Star, and GAL4 MSL1096;S118 Embros.

Construction of UAS-brho Transgenic Lines

The genomic sequence of the brho gene comprising the complete open reading frame was amplified by PCR, with a sense primer located 5’ of the predicted ATG translation start codon (Drho2S, 5’GAGCATAGCGACCATAGATG3’) and an antisense primer containing the predicted STOP codon (Drho2AS, 5’CGACTGTCTTAGATCACCTGGGATTTCACACCTGGAA3’) and an antisense primer located downstream of the predicted STOP codon (5’CGACTGTTCTAGATCACCTGGGATTTCACACCTGGAA3’). A full cDNA product was amplified from RNA extracted from adult flies with these primers in a RT-PCR (reaction parameters were the same as those described above) and sequenced.

Phylogenetic Reconstruction of Rho Family Proteins

Phylogenetic relationships were inferred using neighbor joining (NJ) and maximum likelihood (ML) from an unaligned 118-amino-acid subset of the alignment derived primarily from the second through the sixth transmembrane domains. Two methods of calculating the distance matrix for use in neighbor-joining tree reconstruction were used: the simple Poisson corrected proportional difference and Lake’s paralinear distance for its robustness of inference on data sets having significant unequal rate effects (Lake, 1994). Support for NJ topologies was evaluated using bootstrap analysis with 1000 replicates. Support for maximum likelihood tree inference utilized quartet puzzling reliability values from 10,000 puzzling steps (analogous to bootstrap replicates). The NJ inference of tree topology and the subsequent bootstrap analysis were implemented in DAMBE (Xia, 2000). The quartet puzzling maximum likelihood analysis was performed with PUZZLE (Strimmer and von Haesler, 1996). The NJ tree derived from the maximum likelihood distance matrix was used to compute the parameters for the models of substitution and rate heterogeneity. The ML model of evolution allowed for among-site rate heterogeneity with 1 invariant and 8 y-distributed rate categories.

RESULTS

Identification of brother of rhomboid, a rho-Related Gene

We searched the Drosophila genome database made available by the Berkeley Drosophila Genome Project (Adams et al., 2000) with the amino acid sequence of Rhomboid using the WU-BLAST program and identified five rho-like genes (Fig. 1A). One of these rho-related genes, which we named brother of rhomboid, maps to cytological position 62A3 on the third chromosome. The complete brho transcription unit is contained within the P1 clone DS02734 and is located approximately 8 kb from rho in inverted orientation relative to rho. The Brho sequence clusters tightly with that of Rho in a cladogram of Rho-related proteins found in organisms from diverse kingdoms and phyla (Fig. 1B).

In order to confirm the computer-predicted coding sequence of brho experimentally, we amplified a brho cDNA using RT-PCR of RNA extracts from wild-type adult flies and sequenced the product. This analysis revealed that both the predicted translation start codon and the termination codons are included in the transcribed brho sequence and that the open reading frame of the cDNA exactly matches that of the predicted transcript. Sequence analysis of the cDNAs and alignment to brho genomic sequence revealed the presence of two small introns in brho, of 63 and 60 bp (Fig. 1C).

Like rho, brho is predicted to encode a protein with seven transmembrane (TM) domains, with generally very short stretches of hydrophilic amino acids between the TM domains and a longer loop between TM 1 and TM 2. Comparison of the two proteins reveals that the strongest identities fall within the transmembrane domains (see Fig.
FIG. 1. Brho is a novel Rhomboid-related protein. (A) Alignment of Rho, Brho, and a sample of other Rho-related proteins. Identities to Rho are boxed in dark blue, similarities with Rho are boxed in medium blue, and similarities among Rho-related proteins other than Rho are boxed in mauve. Transmembrane domains, indicated with heavy lines as TM1 to TM7, are those predicted by the TMHMM program, but are in close agreement with predictions made by several other structure prediction programs, Top-Pred, Tmpred, and SOSUI, all accessed through the ExPaSy Web site. For alignments of additional Rhomboid-related family members see aligned sequences online at http://dot.imgen.bcm.tmc.edu. The green stars indicate stretches of amino acids unique to \textit{Drosophila} Rho-related [31D10] of 162, 39, 85, 119, 58, and 42 amino acids (in order). (B) Cladogram of Rho-related proteins in eukaryotes and prokaryotes. The inferences of phylogenetic relationships were constructed as described under Materials and Methods. All methods recovered the same topology, although the most basal bifurcations in the animal clade were not well supported by the maximum likelihood method. Asterisks indicate proteins that are also in the alignment in A. Blue dots indicate nodes significantly supported by all methods of tree reconstruction. Coding sequences of \textit{Arabidopsis-1} (O82756), \textit{Arabidopsis-2} (O81073), and \textit{sugarcane} (\textit{Saccharum}, Q43323) rho-related genes are reported in Roundsley et al. (1998), Peters et al. (1998), and Bugos et al. (1993), respectively. Coding sequences of \textit{C. elegans-1} (Q19821), \textit{C. elegans-2} (YLTH), and \textit{C. elegans-3} (gi:5832784) rho-related genes, are reported in Wilson et al. (1994). Predicted protein sequences of \textit{Human-1} (NCBI gi:4506525) and a rat Rho (gi:888779) are reported in Pascall and Brown (1998). Rho-2, a second human rhomboid-related gene, was localized in genomic sequence mapping to chromosome 1p32.3–p34.1 (AL139260). A full-length EST for this gene has been sequenced by the NEDO human cDNA sequencing project (BAA91168). The rho-related gene of \textit{Mycobacterium} (O53632) has been reported in Cole et al. (1998) and that of \textit{Saccharomyces pombe} (YB4J) in Wood et al. (1997). The sequences of Drosophila rho (P02350) (Bier et al., 1990) and brho were determined empirically. Drosophila Rho-related [62A1] (CT2274), Drosophila Rho-related [10C6] (CT4804), Drosophila Rho-related [31D10] (CG5364), and a complete protein sequence of Drosophila Rho-related [33C1] (CT48787) are available in GadFly, the annotated Drosophila genome. For Drosophila Rho-related [62A1] and Drosophila Rho-related [10C6], a second round of HMM-based gene prediction was performed using FGENESh, available at http://genomic.sanger.ac.uk/, and for both of the genes an additional exon which encoded the putative start Met and the first transmembrane domain was found. All accession numbers provided refer to the SwissProt Database except the \textit{Human-1} and \textit{C. elegans-3} rho-related proteins, which are referred to by their NCBI numbers. The tree is rooted with the bacterial sequences. (C) The intron/exon structure of the \textit{brho} locus, as determined by comparing full-length cDNA and genomic sequences. Red arrows indicate the positions of nested primers used for RT-PCR experiments (see Fig. 3). Blue arrows indicate the positions of primers used to amplify the full \textit{brho} cDNA.
brho Promotes EGF-R Signaling in the Wing

As a first step in assessing the function of brho, we used the GAL4-UAS system (Brand and Perrimon, 1993) to misexpress a UAS-brho construct containing the full genomic sequence of brho in the wing. When this UAS-brho construct was expressed using the strong ubiquitous wing-specific GAL4 driver MS1096, we observed ectopic vein phenotypes similar to, although weaker than, those generated by misexpression of rho (Figs. 2C and 2B, respectively). This observation suggests that Brho functions like Rho by promoting EGF-R signaling. To test whether the induction of ectopic veins by brho misexpression requires EGF-R activity, we coexpressed UAS-brho with a dominant-negative EGF-R construct, UAS-DN-Egfr (Buff et al., 1998). As previously observed for rho (Fig. 2E), brho-induced ectopic veins were entirely suppressed by DN-Egfr (Fig. 2F), resulting in narrow wings with missing veins typical of DN-Egfr misexpression (Fig. 2D). We also observed strong synergism between misexpressed brho and Star, as has been shown to be the case for rho (Guichard et al., 1999), which is discussed further below. These results are consistent with brho functioning to promote EGF-R signaling.

Since EGF-R signaling results in MAPK activation, we assessed the activation state of MAPK following misexpression of brho in the wing disc. MAPK is an essential downstream component required to transduce signals from all RTKs to the nucleus. Activated MAPK (MAPK*) can be detected in situ, using an antibody directed against phosphorylated MAPK (anti dP-ERK antibodies) (Gabay et al., 1997a,b). In wild-type wing discs, MAPK activation is restricted to vein primordia, as a consequence of endogenous localized rho expression (Fig. 2G and Gabay et al., 1997a: Guichard et al., 1999). In wing discs ubiquitously misexpressing brho (Fig. 2I), we observed a strong general activation of MAPK comparable to that found in discs ectopically expressing rho (Fig. 2H) or an activated form of EGF-R (data not shown). This observation provides independent support for brho activating the EGF-R/MAPK signaling pathway.

brho Expression Is Detected Only in the Early Oocyte

We surveyed brho expression during development by in situ hybridization in embryos, third-instar imaginal discs, and the female germ line and only observed expression in early stage oocytes (Fig. 3B). We also analyzed brho expression by performing PCR on various cDNA libraries and RT-PCR on RNA extracted from various developmental stages. Consistent with the very restricted expression of brho in the female germ line determined by in situ hybridization, we could amplify a brho RNA product from adult females by RT-PCR (Fig. 3A), but not from RNA extracted from embryos, larvae, pupae, or male adults. We also did not detect brho transcripts in cDNA libraries made from embryos or imaginal discs.

Expression of brho in the female germ line is restricted to the early developing oocyte between stage 5 and stage 8 (Fig. 3B). No brho staining was observed in the surrounding follicle cells, however. EGF-R signaling has been reported to play at least three separate roles during early and late oogenesis: first to package a single oocyte and its attached nurse cells in the gerarium prior to formation of the egg chamber (Goode et al., 1996), second to establish posterior follicle cell fates (stage 1–8 egg chambers) (González-Reyes et al., 1995; Roth et al., 1995), and third to establish the D/V axis in dorsal anterior follicle cells (stage 9–12 egg chambers), which is required to pattern the egg chamber and embryo (Neuman-Silberberg and Schüpbach, 1993, 1994). The restriction of brho expression to the narrow window of oocyte development between stages 5 and 8 suggests that Brho might be involved in activating EGF-R signaling in adjacent follicle cells required to specify posterior cell fates. As a direct measurement of EGF-R activity during oogenesis, we probed wild-type ovaries with anti-dp-ERK antibodies. During early stages (5–8), MAPK activation is detected only in posterior follicle cells abutting the oocyte in...
which brho and gurken (Neuman-Silberberg and Schüpbach, 1993) are expressed (Fig. 3C). This pattern of MAPK activation is temporally correlated with brho expression and is consistent with the hypothesis that brho participates in promoting EGF-R signaling in posterior follicle cells. It is noteworthy that rho, which activates EGF-R signaling in many other developmental settings, is not expressed in the oocyte or surrounding follicle cells during this period.

During later stages of oogenesis (9–10), we observed that MAPK activation is restricted to follicle cells overlying the dorsal anterior end of the oocyte (Fig. 3D), consistent with previous reports (Peri et al., 1999). This restricted activation of MAPK is believed to be the result of the asymmetrical localization of gurken transcripts to the dorsal–anterior portion of the oocyte, which then resolves into a double peak as a consequence of rho, argos, and spitz activity in the dorsalmost anterior follicle cells at stage 11 (Fig. 3D and Wasserman and Freeman, 1998). Interestingly, a trace of posterior activation of MAPK is also observed at stage 10, suggesting that sustained posterior EGF-R activity may maintain posterior fates of the egg chamber (data not shown).

We tested whether brho could activate the EGF-R pathway in the ovary by expressing the UAS-brho construct under the control of the CY2-GAL4 driver, which is expressed only in the follicular epithelium covering the oocyte (Queenan et al., 1997). This ubiquitous follicle cell expression of brho caused dorsalization of the eggshell,
resulting in thickened dorsal appendages which were more spread apart in eggs from CY2;UAS-brho females (Fig. 3G) than in wild-type controls (Fig. 3E). In some more extreme cases, white appendage-like material filled in between the two appendages, as is typical of dorsalized eggshells (Nilson and Schönbeck, 1999). The average brho misexpression phenotype is similar to, but weaker than, that induced by ectopic rho in follicle cells using the same GAL4 driver (Fig. 3F).

**brho Interacts Synergistically with Star**

Star is expressed in the oocyte during a developmental window (stage 4 to 7) largely overlapping with brho expression (Pickup and Banerjee, 1999). As Star and rho act in concert during many stages of development (Mayer and Nüsslein-Volhard, 1988) and function in a strict interdependent fashion during wing vein development (Guichard et al., 1999), we tested whether brho might also interact...
synergistically with Star. We coexpressed UAS-brho and UAS-Star constructs during wing development using the strong ubiquitous GAL4 driver MS1096 and observed highly penetrant pupal lethality, as has been previously observed for misexpression of rho and Star (Guichard et al., 1999). Despite the pupal lethality, fully differentiated wings can be dissected from pupal cases, revealing a strong ectopic vein phenotype (Fig. 4B) which is much greater than that observed in response to ectopic brho alone (Fig. 4A). Since ectopic expression of Star alone has no detectable effect, this result reveals a potent synergism between Brho and Star in enhancing EGF-R activity during wing development. We also observed a strong effect on brho activity from reducing the dose of endogenous Star since brho-induced ectopic veins are almost completely suppressed in a Star−/− heterozygous background (Fig. 4C). These results indicate that Star can collaborate with brho, as well as with rho, to activate EGF-R signaling.

**Brho and Star Can Activate mSpi and mGrk in the Wing**

The data presented thus far suggest that the Brho protein could function early during oogenesis by activating EGF-R signaling in follicle cells adjacent to the oocyte where brho is expressed. As a possible mechanism, we propose that Brho might promote processing or activation of the Grk protein in the oocyte to stimulate EGF-R expressed in adjacent follicle cells. Consistent with the idea that mGrk, like mSpi, requires activation, the mGrk protein does not...
exhibit any activity when misexpressed in the wing (Fig. 4G and Queenan et al., 1999). In contrast, an artificially truncated version of Grk, GrkΔTM, can activate EGF-R both in the wing and in follicle cells (Queenan et al., 1999). In order to determine whether activation of mGrk involves Star, as has been observed for mSpi (Pickup and Banerjee, 1999), we coexpressed Star and Gurken during wing development. Coexpression of mgrk and Star results in a strong ectopic vein phenotype (Fig. 4H), which is greater than that caused by coexpression of mspi and Star (Fig. 4E). This finding supports the view that the Grk EGF ligand can be activated through a mechanism similar to that of mspi.

To determine whether Brho can also participate in activating Grk, we coexpressed UAS-brho and UAS-mgrk in the wing. The ectopic vein phenotype resulting from the coexpression of brho and mgrk is significantly stronger than that caused by brho alone (Figs. 4I and 4A, respectively), indicating that Brho can activate the mGrk precursor. We also observed a synergistic effect between brho and mspi, similar to that which has been previously observed between rho and mspi (Fig. 4F and Guichard et al., 1999). The phenotypes resulting from coexpressing brho + mgrk (Fig. 4I) is significantly stronger than that from coexpressing brho + mspi (Fig. 4F); however, we do not believe that this necessarily reflects a preference of Brho for activating Grk versus Spi, since coexpression of UAS-Star with these ligands also results in a much stronger phenotype with Grk than Spi. These data suggest rather that the UAS-mGrk construct may be expressed more efficiently or at higher levels than the UAS-mspi construct. Also, it was not possible in these experiments to determine whether there was a significant increase in the severity of the phenotype resulting from coexpression of UAS-rho with either UAS-mspi or UAS-mgrk in the wing since UAS-rho generates a very strong ectopic vein phenotype when misexpressed alone.

DISCUSSION

We report on the identification and characterization of a novel rhomboid-related gene, brother of rhomboid, which is expressed specifically in the female germ line. We demonstrate that brho functions like rho by collaborating with Star in activating EGF-R/MAPK signaling. Brho can potentiate the activity of both mspi and mGrk EGF ligands, consistent with the possibility that Brho may activate Grk to promote EGF-R/MAPK signaling and define posterior fates in the early follicular epithelium.

A Family of Rho-Related Proteins in Drosophila and Other Eukaryotes

Rho-related genes have been found in organisms from diverse kingdoms including the nematode Caenorhabditis elegans, rat, human, Arabidopsis, sugar cane, yeast, and bacteria. The existence of this family of rhomboid-related genes raises the question whether these genes all fulfill similar biochemical functions in these widely divergent species. In the case of Brho, which clusters tightly with Rho in evolutionary cladograms (Fig. 1B), our analysis strongly suggests that it functions like Rho to promote EGF-R signaling, possibly by activating the TGFα-like ligand Gurken. It is unclear, however, whether other rho-related genes found in Drosophila or different organisms will also activate the EGF-R pathway. No function has yet been assigned to any of the vertebrate Rho proteins. In the nematode, EGF-R plays a prominent role in vulval development (Sternberg and Han, 1998), but no mutation has been identified in either of the two nematode rho-related genes in comprehensive screens designed to identify genes involved in vulval development (Herman and Horvitz, 1997). It is possible that these two genes have redundant functions or that they are involved in regulating EGF-R activity during other developmental processes (Aroian and Sternberg, 1991). Since RTKs have not been found in plants (Satterlee and Sussman, 1998), yeast, or bacteria, the rho-related genes in these organisms presumably serve other functions. It will be interesting to determine whether the activities of these Rho-related proteins are similar to those of Rho and Brho, such as promoting the processing or activation of transmembrane polypeptides.

A striking feature of rho-related genes is that amino acid sequence conservation is most prominent in the predicted transmembrane regions. This is also true for other seven-pass transmembrane proteins such as G-protein-coupled receptors (GPCRs), although Rho proteins show no direct homology to any of the known GPCR subfamilies. Interestingly, a GPCR protein has recently been shown to be required for EGF-R signaling in a mammalian system and does so by promoting processing of an EGF ligand (Prenzel et al., 2000). Similarly, presenilin-related molecules, which form another subfamily of seven-pass transmembrane proteins, have been shown to function by activating intramembrane proteolysis of Notch (Struhl and Greenwald, 1999; Ye et al., 1999; De Strooper et al., 1999). These observations raise the question of whether these seven-pass transmembrane proteins and Rho-related proteins may be generally involved in promoting proteolysis of other integral membrane proteins.

In contrast to GPCR subfamilies, no significant homologies have been found in the N-terminal or C-terminal tails among the Rho-related proteins so far identified. Consistent with the absence of sequence conservation in the N-terminal domains of Rho-related proteins, an N-terminal truncated version of Rho is fully active in the Drosophila wing (Guichard et al., manuscript in preparation). This observation, together with the conservation of amino acid residues in the transmembrane domains, has important functional implications for the family of Rhomboid-related proteins. It may reflect the presence of a conserved active site within the plasma membrane, where Rho proteins interact with potential partner(s). The recent finding that the transmembrane domain of Spi is important for its
functional interaction with Rho and Star (Bang and Kintner, 2000) supports this view.

It is noteworthy that the Drosophila rho-related [31D10] and C. elegans rho3 genes contain an additional insert of amino acids between TM1 and TM2. This insert, which is highly conserved between these two proteins (data not shown), contains a cluster of cysteine residues typical of growth factor-like domains in extracellular proteins. This observation suggests that the loop between TM1 and TM2 lies outside of the cell and consequently that the N-terminus of Rho proteins should be cytoplasmic and the C-terminus extracellular. It will be interesting to determine whether the additional conserved domains of amino acids in the Rho-related [31D10] and C. elegans Rho3 proteins interact with other extracellular components involved in the function of these proteins.

The Gurken Ligand Can Be Activated by Star and Brho

The Spitz and Gurken proteins both have single transmembrane domains and belong to the TGFβ subfamily of EGF ligands (Neuman-Silberberg and Schüpbach, 1993; Rutledge et al., 1992). As observed for Spitz (Schwitzer et al., 1995), misexpression of the full-length Grk protein in the wing or in follicle cells has no effect. In contrast, truncated versions of Spi and Grk missing the transmembrane and cytoplasmic domains (sSpi and GrkΔTC, respectively) are potent activators of EGF-R in these same assays (Queenan et al., 1999). Curiously, GrkΔTC protein is not localized at the apical pole of the follicle cells and unlike wild-type Gurken does not get transported into adjacent follicle cells when expressed in the oocyte (Neuman-Silberberg and Schüpbach, 1996). One explanation for these results is that GrkΔTC activates EGF-R intracellularly (Queenan et al., 1999). This mechanism differs from that proposed for the Spitz ligand since a similarly truncated construct (Schwitzer et al., 1995). Also, sSpi is able to activate EGF-R a few cell diameters away from its site of expression (Golembro et al., 1996), in further support of the idea that sSpi is secreted into the extracellular space where it can diffuse or be transported to adjacent cells.

In this study, we demonstrate that membrane-bound Grk ligand can be activated by Star and Brho. This finding strongly supports the idea that endogenous mGrk is activated by a mechanism similar to that for mSpi, involving Star and a Rhomboid-related protein. This proposal is further supported by the fact that the transmembrane domain, which in Spi mediates the interaction with Star and Rho (Bang and Kintner, 2000), is highly conserved between Grk homologues of Drosophila melanogaster and of D. virilis (Peri et al., 1999). Moreover, there is a significant homology between the transmembrane domains of Grk and Spi (25% identity and 65% similarity) (Neuman-Silberberg and Schüpbach, 1993). On the other hand, no significant homology between Grk and Spi is observed in the cytoplasmic domain. This latter fact is not surprising, considering that the cytoplasmic domain of Spi can be replaced by an unrelated sequence and still have an inhibitory effect on Spi activity, suggesting that its presence but not its primary sequence is important (Bang and Kintner, 2000). The apparent uncoupling of Spi or Gurken activation from the production of a secreted cleaved product (suggested by the analysis of truncated Grk function in flies and Spi function in the heterologous Xenopus assay system) raises the possibility that the primary function of Rho and Star may not be to promote Spi or Grk cleavage, but rather to modify its presentation, which in some cases may also lead to ligand processing.

A Model for brho Function during Oogenesis

Three temporally distinct functions of EGF-R signaling can be distinguished during oogenesis (Nelson and Schüpbach, 1999; Van Buskirk and Schüpbach, 1999). First, very early (in the germarium), EGF-R activity is required for the correct packaging of a single oocyte and its attached nurse cells into a single egg chamber by follicle cells (Goode et al., 1996). Second (in stage 1–8 egg chambers), the Grk ligand is expressed in the oocyte and signals to adjacent posterior follicle cells, through activation of the EGF-R, to establish anterior–posterior polarity of the egg chamber (González-Reyes et al., 1995; Roth et al., 1995). Finally, EGF-R functions in follicle cells (in stage 9–12 egg chambers) to establish the dorsoventral polarity of the egg chamber and embryo. During stages 9–10, grk mRNA is restricted to the dorsal anterior corner of the oocyte, where it produces a localized source of Grk protein at the apical surface of the oocyte that activates the EGF receptor in adjacent follicle cells. Ultimately, dorsal EGF-R activation is refined into a pair of peaks by which it promotes differentiation of the dorsal appendages (Wasserman and Freeman, 1998). A variety of evidence indicates that rho plays a critical role in this later process (Ruohola-Baker et al., 1993; Wasserman and Freeman, 1999). In contrast, there is no evidence that rho participates in the earlier EGF-R-dependent events. Furthermore, rho expression cannot be detected in stage 1–8 egg chambers. brho, on the other hand, is expressed during stages 5–8 and may function to activate Grk-mediated EGF-R signaling during this intermediate period.

This current work indicates that Brho can activate EGF-R signaling and suggests that it may have a role in defining posterior fates of the egg chamber since MAPK is activated exclusively in the posterior follicle cells abutting the oocyte at stages 5–8. Although it has been proposed that posterior EGF-R activation occurs during stages 1–6 (González-Reyes et al., 1995), or at stage 7 (Roth et al., 1995), the data presented in this study are consistent with this event occurring any time prior to stage 8. Our direct observation of MAPK activation is most consistent with the later time window. It is possible, however, that EGF-R signaling is initiated earlier, but that the levels of MAPK activation are
not easily detectable during these stages. Since the Grk ligand requires activation in order to signal to neighboring cells, we propose that endogenous Brho participates in activating Grk during midoogenesis, which then activates EGF-R signaling to define posterior cell fates in the follicular epithelium. Confirmation of this hypothesis awaits the isolation of brho loss-of-function mutants or antisense analysis.

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Note added in proof. After this manuscript was accepted for publication another study has been published which reports that the rho-related gene roughoid (designated rho-related 62A6 in our study) functions in collaboration with rho to promote EGF-R signaling during eye development (Wasserman et al., 2000). It is noteworthy that rho, brho, and roughoid cluster within the same evolution clade (see Fig. 1B).

REFERENCES


