INTRODUCTION

Overactivation of receptor tyrosine kinases (RTKs), caused by either oversupply of ligands or mutations that result in ligand-independent constitutive activation, has been linked to many cancers and other human diseases (reviewed by Robertson et al., 2000). An important question is why such overactivation, which has been generally presumed to be quantitative in nature, could lead to qualitative changes of cellular properties. It has been proposed that a constitutively activated RTK hyperactivates a canonical downstream signal transduction pathway, such as the Ras-MAPK signaling cassette, and that the qualitative changes in gene expression are determined by the variation in signaling duration and/or intensity (Ghiglione et al., 1999; Greenwood and Struhl, 1997; Marshall, 1995; Sewing et al., 1997; Woods et al., 1997). Alternatively, however, overactivated RTK could signal via alternative pathways that are not essential for or engaged by wild-type RTK under physiological conditions, thus augmenting the signaling capacity of this overactivated RTK.

To investigate how overactivation of an RTK results in aberrant gene expression, we chose to study the Torso (Tor) pathway in the early Drosophila embryo. Tor is a fly RTK most homologous to the mammalian PDGF receptor. During Drosophila development, Tor specifies cell fates in the terminal regions of the embryo (Duffy and Perrimon, 1994). Tor mRNA is synthesized during oogenesis, deposited into the unfertilized egg, and translated following fertilization. Tor proteins are uniformly distributed on the cell membrane of the early embryo, but are activated at the terminal regions by a ligand that diffuses from the egg poles (Casanova and Struhl, 1993; Sprenger and Nusslein-Volhard, 1992). Previous studies have documented that Tor activates the evolutionarily conserved Ras1/Draf/MEK/MAPK signaling cassette (Duffy and Perrimon, 1994) to induce the expression of target genes such as tailless (tll) (Pignoni et al., 1990; Pignoni et al., 1992), which is essential for specifying cell fates in the terminal regions (Steingrimsson et al., 1991). The current model is that the tll promoter is repressed in the early embryo. The MAPK pathway abrogates tll repression, thereby enabling tll activation by an unknown ubiquitous transcription factor(s) (Liaw et al., 1995; Paroush et al., 1997).

tll expression at the posterior end is precisely restricted in a domain from 0 to 15% of the egg length (EL) in wild-type embryos (Fig. 1A). The extent of this domain is a sensitive readout of the strength of Tor activation (Hou et al., 1995; Li et al., 1998; Li et al., 1997). Thus, a decrease in Tor signaling, such as caused by tor or Draf loss-of-function mutations, results in reduction or elimination of tll expression in the posterior domain in a manner reflecting the severity of the mutation
(<15%EL; not shown). Conversely, in tor gain-of-function (torGOF) mutations associated with an increase in Tor signaling, expansion of the posterior tll expression domain towards the middle region of the embryo is observed (>15%EL; Fig. 1C). The signal generated by either wild-type Tor or TorGOF, as visualized by the tll expression readout, can be completely blocked by null mutations in Draf (also known as pole hole; phi) (Ambrosio et al., 1989). Thus, it has been proposed that the major output of Tor signaling is the activation of MAPK.

In a recent genetic screen intended to isolate additional components of RTK signaling, we identified the Drosophila STAT, encoded by marelle (mrl; DStat92E), as an essential mediator of TorGOF (W. L., unpublished data). This suggests that STAT might be required for signaling by the Tor RTK.

The JAK/STAT pathway was first elucidated by studying the mechanisms of interferon signaling. In the canonical model, STAT is activated by the cytoplasmic tyrosine kinase JAK, which itself is activated by a non-tyrosine kinase receptor in response to ligand binding (Darnell et al., 1994). It is now well established that activation of STAT is associated with many cancers and other human diseases (Sahni et al., 1999; Su et al., 1997), and indeed, activated STAT3 behaves as an oncogene in causing cellular transformation and tumor formation (Bromberg et al., 1999).

JAK and STAT proteins are conserved between flies and humans (Binari and Perrimon, 1994; Hou et al., 1996; Yan et al., 1996). The hop and mrl genes were isolated in genetic screens for determining the maternal effects of zygotic lethal genes (reviewed by Hou and Perrimon, 1997). Embryos lacking the maternal product of either hop or mrl exhibit identical morphological defects when their cuticles are examined at the end of embryogenesis. They are missing the fourth and fifth ventral abdominal denticle belts, A4 and A5, respectively (see Fig. 2B). In the early embryo, Hop and Mrl are essential for the correct expression of a number of segmentation genes including even-skipped (eve) and runt that are normally expressed in alternating parasegments, forming seven stripes along the anteroposterior axis (Hou and Perrimon, 1997).

To understand the mechanism by which STAT is involved in RTK signaling, we investigated the requirement of the JAK/STAT pathway downstream of the Drosophila RTK Torso (Tor). Our results show that the effects of TorGOF require not only the Ras/Raf/MEK/MAPK pathway, but also the Drosophila STAT protein Mrl. Mutations in mrl suppress the ectopically expressed tll and embryonic defects caused by TorGOF. In contrast, Mrl is not essential for the normal tll expression patterns controlled by wild-type Tor. These results indicate that the biological effects of wild-type Tor and TorGOF require distinct downstream signaling components.

MATERIALS AND METHODS

Genetics and examination of embryos
The torGOF alleles used in this study are torV9 and torPL3 (Klingler et al., 1988). The Ras1BECD (Hou et al., 1995), mrl6346 (Hou et al., 1996) and hopC111 allele (Binari and Perrimon, 1994) used in this study are strong or null alleles. The Sevenmaker (SEM) allele of the rI locus is a gain-of-function allele (Brummer et al., 1994). Drafc110 and Drafb826 are weak and intermediate alleles, respectively (Melnick et al., 1993). We used the dominant female sterile (DFS) technique (Chou and Perrimon, 1992) to generate homozygous germline clone (GLC) embryos for null alleles, such as mrl6346, hopC111, to test genetic interactions. Since hop and Draf, mrl and Ras1, are located on the same chromosome arm, respectively, we generated recombinant chromosomes to test the double mutant GLC phenotypes. To generate mrl GLC embryos from torV9/+ females, we crossed w; torV9/Cyo; FRT52B [ovod1, w1]/TM3 males to w hs-Flp/w hs-Flp; +/+; FRT52B mrl6346/TM3 females to produce y w hs-Flp/w hs-Flp; torV9/+; FRT52B mrl6346/FRT52B [ovod1, w1] females. To remove the maternal mrl gene product from the embryos produced by rIFEM/+ females, we crossed y w hs-Flp; rIFEM/Cyo; [ovod1, w1]/FEM males to y w hs-Flp/w hs-Flp; +/+; FRT52B mrl6346/TM3 females and generated y w hs-Flp/w hs-Flp; rIFEM/+; FRT52B mrl6346/FRT52B [ovod1, w1] females.

Co-immunoprecipitation
To extract embryonic proteins, embryos of 0 to 4 hours after egg-laying were collected and homogenized in Buffer A [10 mM Tris-HCl pH 8.0; 150 mM NaCl; 1 mM EDTA; 0.1% Triton X-100; Protease Inhibitor cocktail (Sigma), and 1 mM PMSF final concentration]. To treat embryos with vanadate, a protein tyrosine phosphatase inhibitor, sodium orthovanadate (Sigma) was added to Buffer A prior to homogenization at 1 mM final concentration. To immunoprecipitate Tor from embryo extracts, we incubated anti-Tor antibody (Cleghon et al., 1996) with wild-type and torGOF embryo extracts (200 µl), respectively, at 4°C overnight at 1:200 dilution. The immunoprecipitates were resolved by 8% SDS-PAGE and blotted with anti-Tor antibody at 1:5000 dilution (Cleghon et al., 1996) to reveal the presence of Tor. The blot was then stripped of antibodies and reprobed with an anti-Mrl antibody (raised by immunizing rat with bacterially expressed Mrl) at 1:500 dilution to detect whether Mrl was bound to Tor in the embryo extracts.

Plasmids and fly transformants
A PCR based mutagenesis was performed on a 5.9 kb tll upstream regulatory fragment (Liau et al., 1995) to introduce nucleotide changes in the two Mrl-binding sites. As a result, site 1 was changed from ATTCTGAGGAT to ATGCCGCGCT to create a NotI site (underlined), and site 2 from ATTCCTCGAAGAC to ATTCCTCGGTACC to create a KpnI site (underlined). A lacZ reporter transgene was generated by replacing the wild-type tll regulatory region with this mutant 5.9 kb fragment in a tll-lacZ fusion gene (Liau et al., 1995) and used to transform Drosophila by P element-mediated transformation.

RESULTS
Mrl mediates the effects of TorGOF on embryos
To determine whether Mrl plays a role in TorGOF signaling, we examined the phenotype of embryos derived from female germ cells that carry a torGOF mutation and lack mrl activity (see Materials and Methods). Strikingly, these embryos exhibited the characteristic mrl mutant phenotype, while the torGOF segmentation phenotype was mostly suppressed (Fig. 1). Consistent with the cuticle phenotype, the domain of tll expression in these embryos was nearly wild type (Fig. 1E). This suppression is not allele-specific, as a second torGOF mutant allele was also suppressed by lack of Mrl in embryos (data not shown). Both alleles of torGOF are due to point mutations in the extracellular, ligand-binding domain, presumably causing ligand-independent dimerization of the receptor (Sprenger and Nusslein-Volhard, 1992). These results demonstrate that removal of mrl suppresses the effects of torGOF mutation on tll expression and larval cuticles, suggesting that Mrl mediates the effects of TorGOF.
Mrl and Hop are not essential for wild-type Tor signaling

To determine whether mutations in the JAK/STAT pathway show genetic interactions with members of the Ras1/Draf pathway, we generated embryos doubly mutant for various combinations of alleles. We used two Draf mutations with reduced activities, \( \text{Draf}^{C110} \) and \( \text{Draf}^{PB26} \), as well as a null \( \text{Ras1} \) mutation, \( \text{Ras1}^{AC40B} \). Unlike Draf null GLC embryos, which exhibit no posterior \( tll \) and cuticle structures, \( \text{Draf}^{C110} \) GLC embryos have a wild-type cuticle and show a near wild-type \( tll \) expression (not shown) (see also Melnick et al., 1993). \( \text{Draf}^{PB26} \) GLC embryos have reduced posterior \( tll \) expression domains to 6-10% EL, and defects in the posterior cuticle structures that include frequent deletions of A8 (not shown) (see also Melnick et al., 1993). While most of the \( \text{Ras1}^{AC40B} \) GLC embryos are identical to \( \text{tor} \) or \( \text{Draf} \) null embryos and exhibit no posterior \( tll \) expression and cuticle structures, about 20% of these embryos have residual posterior \( tll \) expression as well as posterior cuticle structures due to a Ras1-independent activation of Draf (see also Hou et al., 1995).

Since the phenotypes associated with \( \text{tor}^{GOF} \) are suppressed by a null \( \text{mrl} \) mutation, we investigated whether Mrl or Hop activities are essential for the expression of \( tll \) in wild-type embryos. We found that in either \( \text{mrl} \) or \( \text{hop} \) mutant embryos, the posterior domain of \( tll \) expression, which is invariably reduced in mutations that affect Tor signaling, appears wild type (about 15% EL; Fig. 2A), indicating that the Hop/Mrl pathway is not essential for the wild-type patterns of \( tll \) expression.

These results, however, do not fully exclude the possibility that Hop and Mrl constitute a branch of the Tor signaling pathway that acts in parallel and redundant to the Ras1-MAPK branch, and that the inability to detect any influence of the JAK/STAT pathway on wild-type \( tll \) expression could result from a compensatory up-regulation of the Ras1/Draf/MEK/MAPK pathway. We therefore examined the role of the JAK/STAT pathway in a number of sensitized genetic backgrounds wherein the efficiency of Tor signaling had been suppressed by null \( \text{mrl} \) and \( \text{hop} \) mutants.
compromised. First, we examined \textit{tll} expression and cuticle phenotype in embryos that were doubly mutant for a \textit{hop} null allele and weak alleles of \textit{Draf}. Elimination of \textit{hop} did not increase the severity of the \textit{Draf} mutations in these assays (Fig. 2C-F). Second, we examined the phenotype of embryos doubly mutant for \textit{mrl} and \textit{Ras1}. A fraction (about 20\%) of \textit{Ras1} null mutant embryos exhibits residual \textit{tll} expression due to activation of \textit{Draf} by a Ras1-independent mechanism (Hou et al., 1995; Li et al., 1998; Li et al., 1997). Removal of \textit{mrl} activity did not enhance the \textit{Ras1} phenotypes (Fig. 2G,H). Thus, neither Hop nor Mrl appear to be required for \textit{tll} expression patterns in wild-type embryos, therefore they are unlikely to be integral components of the Tor pathway. This conclusion, however, does not apply to \textit{TorGOF} since we find that Mrl activity is required for the full activity of \textit{TorGOF}.

\textbf{TorGOF is capable of activating Mrl}

The above results are consistent with the possibility that \textit{TorGOF} causes Mrl activation to exert its biological functions. To test whether \textit{TorGOF} can cause Mrl activation, we examined Mrl activity in \textit{Drosophila Schneider (S2)} cells transfected with DNA encoding different Tor molecules. As reported previously (Yan et al., 1996), transfection of Hop into S2 cells increased Mrl DNA-binding activity in these cells (Fig. 3A, lane 4). This increase in DNA binding was specific to Mrl, as addition of an anti-Mrl antibody causes the bound complex to be supershifted (Fig. 3A, lane 6). Interestingly, transfection of Tor or \textit{TorGOF} also resulted in activation of endogenous Mrl in S2 cells (Fig. 3A, lane 2 and 3). Based on the intensity of the gel shift bands, Tor and \textit{TorGOF} activate Mrl to levels similar to those observed after Hop transfection (Fig. 3A, lane 4). In these transfection experiments, Tor and \textit{TorGOF} similarly activated Mrl, presumably because when overexpressed in transfection experiments wild-type Tor can dimerize, mimicking the effect of \textit{TorGOF} mutations. These results are consistent with our hypothesis that \textit{TorGOF} causes Mrl activation in vivo.

\textbf{TorGOF activates Mrl independently of MAPK and JAK and is capable of associating with Mrl}

How does \textit{TorGOF} RTK activate Mrl? There are at least three possible mechanisms through which STAT activation by RTK can occur. RTK could directly bind and activate STAT proteins (Fu and Zhang, 1993). Alternatively, STAT could be indirectly activated by the RTK, either via JAK or MAPK (Wen et al., 1995). Genetic evidence allows us to rule out the possibilities that \textit{TorGOF} activates Mrl via JAK or MAPK. First, we examined whether removal of Hop activity modifies the \textit{torGOF} phenotype. Surprisingly, a \textit{hop} null mutation did not suppress \textit{torGOF} (Table 1), indicating that unlike Mrl, Hop is not required for ectopic \textit{tll} expression. Second, removal of \textit{mrl} did not suppress \textit{rSevenmaker (rSevenmaker)} (Table 1), which encodes a GOF mutant form of \textit{Drosophila MAPK} (Brunner et al., 1994), suggesting that Mrl is not essential for the effects of GOF mutation in MAPK. To test for a physical interaction between Mrl and \textit{TorGOF}, we immunoprecipitated Tor from wild-type and \textit{torGOF} embryos, respectively, with anti-Tor antibody (Cleghon et al., 1996), and examined the presence of Mrl in the immune complexes. As shown in Fig. 3B, we detected a specific band corresponding to Mrl in the immunoprecipitates. The Tor-Mrl association, however, is only observed in the presence of vanadate (a general tyrosine phosphatase...
Table 1. hop or mrl mutations do not suppress the phenotypes associated with torGOF or rGOF, respectively

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>Percentage of embryos with &lt;4 denticle belts</th>
<th>Percentage of embryos with ≥4 denticle belts</th>
<th>Total number of embryos scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>torY9/+; +/+</td>
<td>94.0 (n=3576)</td>
<td>6.0 (n=24)</td>
<td>400</td>
</tr>
<tr>
<td>torY9/+; hopC111 GLC</td>
<td>91.3 (n=364)</td>
<td>8.7 (n=29)</td>
<td>103</td>
</tr>
<tr>
<td>rSEM/+; +/+</td>
<td>10.7 (n=123)</td>
<td>89.3 (n=1157)</td>
<td>400</td>
</tr>
<tr>
<td>rSEM/+; mrlL346 GLC</td>
<td>15.1 (n=113)</td>
<td>84.9 (n=73)</td>
<td>86</td>
</tr>
</tbody>
</table>

GOF mutations in tor and, to a lesser extent, in r often result in deleterions and disruption of the ventral denticle belts in the embryo. Strong alleles are associated with deletions of most or all denticle belts (see Fig. 1D); weaker alleles exhibit partial deletions or disruptions of ventral denticle belts (not shown) (Klingler et al., 1988). We categorized the embryonic phenotypes of GOF mutations into strong (≥4 denticle belts) and weak (≥4 denticle belts) classes. Removal of maternal hop activity did not cause significant changes in the cuticle phenotypes associated with torY9. Similarly, removal of maternal mrl did not significantly suppress rSEM.

Mrl-binding sites in tll promoter are essential only for TorGOF-induced ectopic tll expression

Since Mrl activation is required for ectopic tll expression induced by TorGOF, we examined whether Mrl-binding sites (TTCNNNGAA) were present in the regulatory region of the tll gene. A search in the tll regulatory region revealed two putative Mrl-binding sites with the consensus TTCNNNGAA located at -2357 (site 1) and -2462 (site 2) upstream of the tll transcription start site (Fig. 4B). These two sites were able to bind Mrl, although site 2 showed a much lower affinity (Fig. 4A). Interestingly, the two Mrl sites are located 105 bp apart in the tll regulatory region. This configuration is reminiscent of that existing in the eve stripe 3 enhancer, where cooperative binding of two Mrl homodimers was demonstrated (Yan et al., 1996). To assess the functional relevance of the two Mrl sites in tll expression, transgenes containing the 5.9 kb regulatory fragment upstream of the tll transcription start site fused to the lacZ gene were introduced into flies. This 5.9 kb fragment had been shown previously to drive lacZ expression in a pattern almost identical to that of the endogenous tll gene (Fig. 4C) (see also Rudolph et al., 1997). Accordingly, lacZ expression is greatly expanded in a torGOF background (Fig. 4D). A 5.9 kb fragment with the two Mrl binding sites mutated, showed wild-type activity for lacZ expression in wild-type embryos (Fig. 4E), suggesting that these Mrl-binding sites are dispensable for tll expression under normal Tor signaling. However, in a torGOF background, the mutant 5.9 kb fragment shows greatly diminished ability to drive lacZ expression in an expanded domain compared to the situation when the Mrl binding sites are wild type (compare Fig. 4D and F). These results are consistent with the genetic results that Mrl is required for the full activity of gain-of-function, but not wild-type Tor.

DISCUSSION

A general assumption regarding the pathophysiology inherent to gain-of-function RTKs has been that more activity of the receptor translates into a higher level of activation of the downstream signaling pathway, in our case the Ras1/Draf/MEK/MAPK pathway. The requirement of STAT in RTK signaling has been controversial. Contrary to general expectations that higher MAPK activation accounts for the effects of RTK overactivation, we provide genetic evidence that wild-type and gain-of-function mutant RTKs require distinct downstream signaling components to exert their effects. Signal transduction by TorGOF requires Drosophila STAT (Mrl). In contrast, Mrl is not essential for the Tor RTK to promote...
In this manuscript we demonstrate that TorGOF requires Mrl but not Hop for its ability to induce ectopic target gene expression and causing deleterious effects on embryos. In addition, we show that TorGOF can associate with and cause Mrl activation in embryos and transfected cells. These results are most consistent with a model in which TorGOF directly phosphorylates Mrl, which in turn binds to the tll promoter to exacerbate its expression levels. Activation of STAT by RTKs has previously been suggested following studies in cultured mammalian cells. For example, transfected EGF or PDGF receptors can directly interact with and activate STAT by phosphorylation (Fu and Zhang, 1993; Paukku et al., 2000). Taken together with these studies, our results seem to suggest that the intracellular kinase domain of several RTK proteins may have an intrinsic ability to activate STAT proteins.

To account for the involvement of Mrl in tll regulation we propose that a hyperactivated RTK requires a downstream pathway that is not essential for wild-type RTK under normal physiological situations. In wild-type embryos, Tor is activated only in the two terminal regions and defines the spatial limits of tll expression domains by relieving the transcriptional repressors bound to the tll promoter. Mrl is not an essential factor for tll activation in the terminal regions, although it remains to be determined whether Mrl contributes to the activation of tll expression redundantly with other yet unidentified factors. In torGOF mutant embryos, TorGOF is constitutively active in all regions of the embryo and causes ectopic tll expression. In this case, Mrl activation is indispensable for the ectopic tll expression in the central regions of the embryo. The differential requirement for Mrl in central and terminal regions might be due to the lack of other activators of tll and/or the presence of additional repressors in the central region of the embryo. Consistent with this idea, we and others have previously shown that, in the absence of Tor signaling (such as in tor mutant embryos), tll can be induced by uniformly expressing activated forms of downstream signaling components (such as RasV12 or 14-3-3). The resulting induction of tll expression happens preferentially in the terminal regions (Greenwood and Struhl, 1997; Li et al., 1998; Li et al., 1997). Thus tll expression could be determined by the balance between repressors and activators that can bind to the tll promoter (Fig. 5).

Our findings may explain some of the conflicting observations on the role of STAT in RTK signaling in mammals. For example, thanatophoric dysplasia type II (TD II) dwarfism in humans is caused by mutations that lead to constitutive activation of a human RTK FGF receptor 3 (FGFR3). Similar to TorGOF activating Mrl, it has recently been shown that an activated mutant FGFR3 specifically activates STAT1 in both human patient tissues and mouse models. The activated STAT1 in this case induces expression of the cell-cycle inhibitor p21^{WAF1/CIP1}, resulting in growth inhibition of bone tissues (Sahni et al., 1999; Su et al., 1997). However, STAT1 is not known to be required for bone development. STAT1 knockout mice have perfect bones, although they exhibit defective immune systems (Durbin et al., 1996; Meraz et al., 1996). This might be explained by a redundancy among different STAT proteins. Alternatively, STAT1 may not be required for normal FGFR3 signaling in bone development. The presence of several STAT genes in mammals makes it technically difficult to distinguish between the above two possibilities using the mouse as a genetic model. In contrast, the presence of a single JAK and a single STAT gene in Drosophila allows us to examine the relationship between RTK and JAK/STAT signaling, without being limited by gene redundancy. Our observations in Drosophila suggest that the TD II syndrome in humans could be explained if STAT1 is not normally required for FGFR3 signaling, but it becomes essential only for the activating mutant FGFR3.
Altered gene expression is commonly found in cancerous growth. The initiation and maintenance of the changes in gene expression often require the activation of multiple signaling molecules. STAT activation is found in many human cancers or transformed cells (Bromberg et al., 1999; Campbell et al., 2001; Catlett-Falcone et al., 1999; Garcia et al., 1997). In light of our finding in Drosophila, STAT activation might play essential roles for the activation of genes that are required for malignant growth and other pathological conditions. More importantly, we found that STAT activation is insignificant for the normal patterns of gene expression that are controlled by an RTK. It would be interesting to investigate if it is generally true that STAT activation is an important factor only in aberrant RTK signaling. If so, a broad implication of our results is that STAT rather than Ras, should be viewed as premier target for drug interference in the treatment of human diseases and cancers associated with hyperactivation of receptor tyrosine kinases.

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